FASTKD1 as a diagnostic and prognostic biomarker for STAD: Insights into m6A modification and immune infiltration

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Abstract. Fas-activated serine/threonine kinase domain 1 (FASTKD1), a known modulator of mitochondrial-mediated cell death and survival processes, has garnered attention for its potential role in various biological contexts. However, its involvement in gastric cancer remains unclear. Thus, the present study aimed to investigate the relationship between FASTKD1 expression and key factors, including clinicopathological characteristics, immune infiltration and m6A modification in stomach adenocarcinoma (STAD). The expression of FASTKD1 was analyzed in STAD and normal adjacent tissues to assess its association with clinicopathological characteristics and survival prognosis. Data from The Cancer Genome Atlas (TCGA) and Gene Expression Omnibus (GEO) databases were used in this study. Additionally, the findings were validated through immunohistochemical staining. Co-expression analysis of FASTKD1 was performed using Gene Ontology and Kyoto Encyclopedia of Genes and Genomes (GO/KEGG) enrichment analysis, Gene Set Enrichment Analysis (GSEA) and LinkedOmics database analysis. An in-depth analysis was conducted using databases, such as Tumor Immune Estimation Resource (TIMER), Gene Expression Profiling Interactive Analysis (GEPIA), GEO and TCGA to explore the potential correlation between FASTKD1 expression and immune infiltration and m6A modification in STAD. The results revealed that FASTKD1 was significantly upregulated across different tumor types, including STAD. Notably, FASTKD1 was able to distinguish between tumor and normal tissue samples with accuracy. Furthermore, the expression levels of FASTKD1 were significantly associated with clinical stage and survival. Through GO/KEGG enrichment analysis and GSEA, it was revealed that the genes co-expressed with FASTKD1 were active in a variety of biological processes. Within the TIMER, GEPIA and TCGA databases, a notable inverse correlation was observed between FASTKD1 expression and the abundance of immune cell subsets. Notably, significant correlations were established between FASTKD1 and m6A modification genes, YTHDF1 and LRPPRC, in both TCGA and GEO datasets. In conclusion, FASTKD1 may serve a significant role in m6A modification and immune infiltration processes, making it a potentially valuable diagnostic and prognostic biomarker in STAD.

Introduction

Gastric cancer is a malignant tumor of the digestive system, and is the fifth most common type of cancer and the fourth leading cause of cancer mortality worldwide (1). In East Asia, China is one of the countries with the highest incidence of gastric cancer (1). Notably, gastric cancer displays heterogeneity with regard to phenotypes and genotypes (2). Stomach adenocarcinoma (STAD), the main pathological subtype of gastric cancer, arises from the malignant transformation of somatic cells in the gastric glands, accounting for ~95% of all cases (3). The primary treatment approach for gastric cancer is surgical resection. Despite advancements in endoscopic, surgical and systemic therapies, as well as an increased focus on multidisciplinary evaluation and treatment, the 5-year survival rates remain unsatisfactory (4). Therefore, it is necessary to urgently explore new and specific molecular biomarkers for the diagnosis and prognosis of STAD. These biomarkers may facilitate the development of targeted diagnostic and therapeutic strategies.

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Fas-activated serine/threonine kinase domain 1 (FASTKD1) belongs to the FASTK family, which comprises six members, including FASTK and its homologs, FASTKD1-5. These proteins have exclusive expression in the mitochondrial matrix and extensively regulate diverse mitochondrial functions (5-7). Each member has distinct functions in governing various aspects of mitochondrial RNA biology, such as mRNA processing, maturation, ribosome assembly and translation (5-7). All six proteins are found solely in vertebrates and share a conserved arrangement of three homology domains: FAST_1, FAST_2 and RAP (8). FASTKD1 specifically regulates the mitochondrial ND3 domain and is thought to serve a role in RNA stability (8). It has previously been suggested that FASTKD1 may function as a sensitive RNA biomarker for endometrial carcinoma when detected in uterine aspirates (9). Additionally, upregulation of FASTKD1 has been associated with negative prognoses in pediatric and adult patients with acute lymphoblastic leukemia (10). However, to the best of our knowledge, there is currently no available evidence supporting the involvement of FASTKD1 in STAD.

The present study aimed to assess the possible importance of FASTKD1 in STAD by examining The Cancer Genome Atlas (TCGA) STAD dataset and Gene Expression Omnibus (GEO) datasets. Using R software and GENT2 online database (http://gent2.appex.kr), the variation in FASTKD1 expression was examined across different tumor types. Additionally, immunohistochemical staining was performed to verify the differential expression of FASTKD1 in STAD tumors in comparison to adjacent tissues. Furthermore, a comprehensive analysis of the enriched pathways of FASTKD1 was performed, exploring their biological functions and mechanisms of signal transduction. Additionally, the correlation between FASTKD1 expression levels, and immune cell infiltration and m6A modification we investigated. The present study emphasizes the significant involvement of FASTKD1 in STAD, and indicates its potential as a biomarker for the diagnosis and prognosis of patients with STAD.

Materials and methods

Expression of FASTKD1 in STAD. The RNA-sequencing (RNA-seq) data utilized in the present study were sourced from the XENA-TCGA database (https://xena.ucsc.edu) and consist of transcript per million values. The dataset comprises 10,534 samples, which were uniformly processed by UCSC XENA using the Toil process (11). To analyze the differences in FASTKD1 expression across various tumors, the GPL570 platform data from the GENT2 database was used, which includes microarray expression data from 72 tumor tissues or cell lines from the GEO database (12). Furthermore, the RNA-Seq data from 407 patients diagnosed with STAD were obtained from TCGA website (https://portal.gdc.cancer.gov); the dataset used consists of 32 normal adjacent samples and 375 tumor samples from patients with STAD. The dataset also provides relevant clinical characteristics for further analysis (13). To explore the differences in FASTKD1 expression between STAD and normal tissue, GSE27342, GSE29272, GSE33335 and GSE63089 datasets were collected from the GEO database (www.ncbi.nlm.nih.gov/geo) (14-17). The datasets chosen for the present study were obtained from patients diagnosed with STAD and the number of tumor samples and normal adjacent samples analyzed in the study were matched accordingly.

Receiver operating characteristic (ROC) and Kaplan Meier (KM) curves served as tools to objectively evaluate the diagnostic and prognostic significance of FASTKD1 in patients with STAD. The TCGA STAD dataset was utilized to evaluate the ROC predictive efficacy of FASTKD1. To assess the prognostic significance of FASTKD1 mRNA expression on overall survival (OS), the Kaplan Meier Plotter (https://kmplot.com/analysis/) was used, using data from various resources within the GEO, including GSE14210, GSE15459, GSE22377, GSE29272 and GSE51105 (18-22). GSE62254 was excluded from the analysis because it exhibited significantly distinct clinical and genomic data (longer survival and shifted expression) upon examination with the Kaplan Meier Plotter. The resulting KM plots displayed hazard ratios (HR), 95% confidence intervals (CI) and log-rank P-values. $P<0.05$ was considered to indicate a statistically significant difference in the prognostic outcomes. Furthermore, TCGA STAD dataset was utilized to investigate the correlation between the expression levels of FASTKD1 and the clinicopathological characteristics of patients diagnosed with STAD.

STAD tissue samples. Tumor tissues were collected from 48 patients (the subjects were between the ages of 28-78, with a median age of 56.5 years and a male to female ratio of 1.82:1) with STAD who underwent surgical resection at the Taihe Hospital (Shiyan, China) between January 2019 and April 2022. The research protocol was authorized by the Ethics Committee of Taihe Hospital affiliated with Hubei University of Medicine (approval no. 2022KS010). The research was conducted following the principles stated in the Declaration of Helsinki and its succeeding amendments.

IHC staining. To prepare for IHC staining, the collected tumor tissues were fixed in 10% neutral buffered formalin for 24 h at room temperature, then the tissues were dehydrated using disposable tissue embedding cassettes and then embedded after being dipped in wax. The 5-micron pathology sections underwent deparaffinization with xylene, followed by dehydration with alcohol. Endogenous peroxidase activity was blocked by applying a 3% H_2O_2 solution for 5 min at room temperature. Antigen retrieval was achieved by incubating the samples in a pressure cooker for 3 min with sodium citrate buffer (10 mM Sodium Citrate; 0.05% tween-20; pH 6.0). After blocking with 5% goat serum (Guangzhou Dingguo Biotechnology Co., Ltd.) at room temperature for 30 min, the sections were incubated overnight at 4˚C with a rabbit monoclonal FASTKD1 antibody (1:50; cat. no. D122349-0100; Sangon Biotech). Subsequently, a goat anti-mouse IgG-HRP secondary antibody (1:200; cat. no. ab6802; Abcam) was applied to the sections for 1 h at room temperature. Finally, the sections were stained using DAB (A:B; 1:50) reagent for 3-5 min at room temperature, followed by counterstaining with hematoxylin for 4 min. After sealing the slices with neutral gum, the results were analyzed using a light microscope.

Enrichment analysis of the gene co-expression network for FASTKD1 in STAD. LinkedOmics (https://linkedomics.org/) was used to investigate the co-expressed genes of FASTKD1 in TCGA STAD. The results were visually represented by

volcano plots and heatmaps (23). In order to further illuminate the functional characteristics of the co-expressed genes, Gene Ontology (GO) functional analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis were conducted using the clusterProfiler package (version 3.14.3) (24) in R software (version 3.6.3) (25). The resulting bubble chart displays the top five significant findings based on count scores, meeting the criteria of the corrected p-value obtained by the p-value correction method (P.adj) <0.05 and q-value <0.2. The resulting data were then graphically visualized using the ggplot2 software package (version 3.1.0; https://ggplot2.tidyverse.org).

Gene set enrichment analysis (GSEA). To gain deeper insights into the underlying mechanisms of FASTKD1, TCGA STAD dataset was stratified into high and low expression groups based on the median expression levels of FASTKD1. Subsequently, single-gene differential analysis was conducted using the DESeq2 package (version 1.26.0) (26) to assess variations between the two groups. Furthermore, all genes displaying differential expression were subjected to GSEA using the clusterProfiler package (version 3.14.3) (24) to explore associated biological enrichment processes. The dataset utilized for GSEA originated from the MSigDB database, accessible at https://www.gsea-msigdb.org/gsea/msigdb/index.jsp (27). For GSEA, c2.cp.all.v2022.1.Hs.symbols.gmt [ALL Canonical Pathways] was employed as the reference gene set. Enriched pathways meeting the criteria of FDR (q-value) <0.25 and P<0.05 were visualized using the ggplot2 package (version 3.1.0).

Tumor-infiltrating immune cells. To investigate the possible role of FASTKD1 in the regulation of tumor-infiltrating immune cells, the Tumor Immune Estimation Resource (TIMER) database (https://cistrome.shinyapps.io/timer/) (28) was used. Using this tool, the correlation between FASTKD1 expression and the presence of immune-infiltrating cells in TCGA STAD samples was evaluated. The study focused on B cells, neutrophils, $CD4^+$ T cells, macrophages, $CD8^+$ T cells and dendritic cells (DCs). Furthermore, the association between FASTKD1 copy number variation (CNV) and the infiltration of immune cells was explored utilizing the SCNA module presented in the TIMER database. The present study employed the survival module in the TIMER database to illustrate the correlation between clinical outcomes, immune cell infiltration, and FASTKD1 gene expression. The GSVA software package (version 1.34.0) (29) was used to analyze the expression differences of 24 immune cells in STAD samples between the groups with high and low expression of FASTKD1.

FASTKD1 expression and m6A modification in STAD. The present study utilized the R software (version 3.6.3) to investigate the correlation between FASTKD1 expression and the expression of 21 m6A-related genes in TCGA STAD dataset. These genes include eight writers (METTL3, METTL14, RBM15, RBM15B, WTAP, VIRMA/KIAA1429, CBLL1 and ZC3H13), two erasers (ALKBH5 and FTO) and 11 readers (YTHDC1, YTHDC2, YTHDF1, YTHDF2, YTHDF3, IGF2BP1, HNRNPA2B1, HNRNPC, FMR1, LRPPRC and ELAVL1) (30). The data obtained from this analysis were subsequently visualized and assessed using the ggplot2 software package (version 3.1.0). Furthermore, the Kaplan-Meier Plotter (31) was utilized to evaluate the prognostic value of these 21 m6A-associated genes in STAD samples.

Statistical analysis. All statistical analyses in the present study were performed using R software (version 3.6.3). P<0.05 was considered to indicate a statistically significant difference in all analyses. When comparing categorical variables between groups, either the χ^2 test, the χ^2 test with Yates correction, or the Fisher's exact test was used. The statistical methods used to analyze differences between subgroups of clinical variables were the independent samples t-test, and one-way ANOVA or Welch one-way ANOVA followed by the Tukey HSD or Games Howell post-hoc tests. A comparison was made between changes in 24 immune cell subtypes in STAD tumor samples using the Wilcoxon rank-sum test.

Results

Pan-cancer analysis of FASTKD1 expression. FASTKD1 mRNA expression was analyzed across diverse tumor types using the GENT2 and XENA-TCGA datasets. Fig. 1 displays the observed differences in FASTKD1 expression levels between various tumor types and some normal adjacent tissues. The findings revealed that FASTKD1 expression was elevated in several cancer types compared with in normal tissues (Fig. 1A). Further analysis revealed a significant increase in the mRNA expression levels of FASTKD1 in various types of tumors, such as bladder cancer, breast cancer, cervical cancer, bile duct cancer, esophageal cancer, head and neck cancer, kidney chromophobe, liver cancer, lung adenocarcinoma, lung squamous cell carcinoma, pheochromocytoma and paraganglioma, prostate cancer, STAD and endometrioid cancer (Fig. 1B).

Expression levels of FASTKD1 in patients with STAD. A comparison of FASTKD1 expression between STAD and some normal adjacent tissues was conducted by analyzing the STAD datasets from TCGA and GEO. In TCGA dataset, there was a significant increase in FASTKD1 mRNA expression levels observed in STAD samples compared with those in control normal samples (Fig. 2A). Moreover, analysis of the GSE63089, GSE29272, GSE33335 and GSE27342 datasets demonstrated a marked increase in the expression levels of FASTKD1 in STAD samples compared with in control samples (Fig. 2B-E). The ROC analysis revealed an area under the ROC curve of 0.87 (95% CI, 0.801-0.942; Fig. 2F). This finding indicated that FASTKD1 expression may accurately differentiate between STAD and normal samples. Additionally, the survival analysis showed that high expression of FASTKD1 in STAD could significantly predict poor survival (HR, 1.64; 95% CI, 1.32-2.02; $P=4.2x10^{-6}$; Fig. 2G). In addition, IHC analysis indicated a marked increase in FASTKD1 protein levels in STAD tumor tissue compared with in adjacent normal tissue (Fig. 2H). Taken together, the increased expression levels of FASTKD1 mRNA and protein in STAD tissues suggest its potential as a diagnostic marker.

Further analysis was conducted on clinical data obtained from TCGA STAD cohort to investigate the relationship between FASTKD1 expression and various clinical

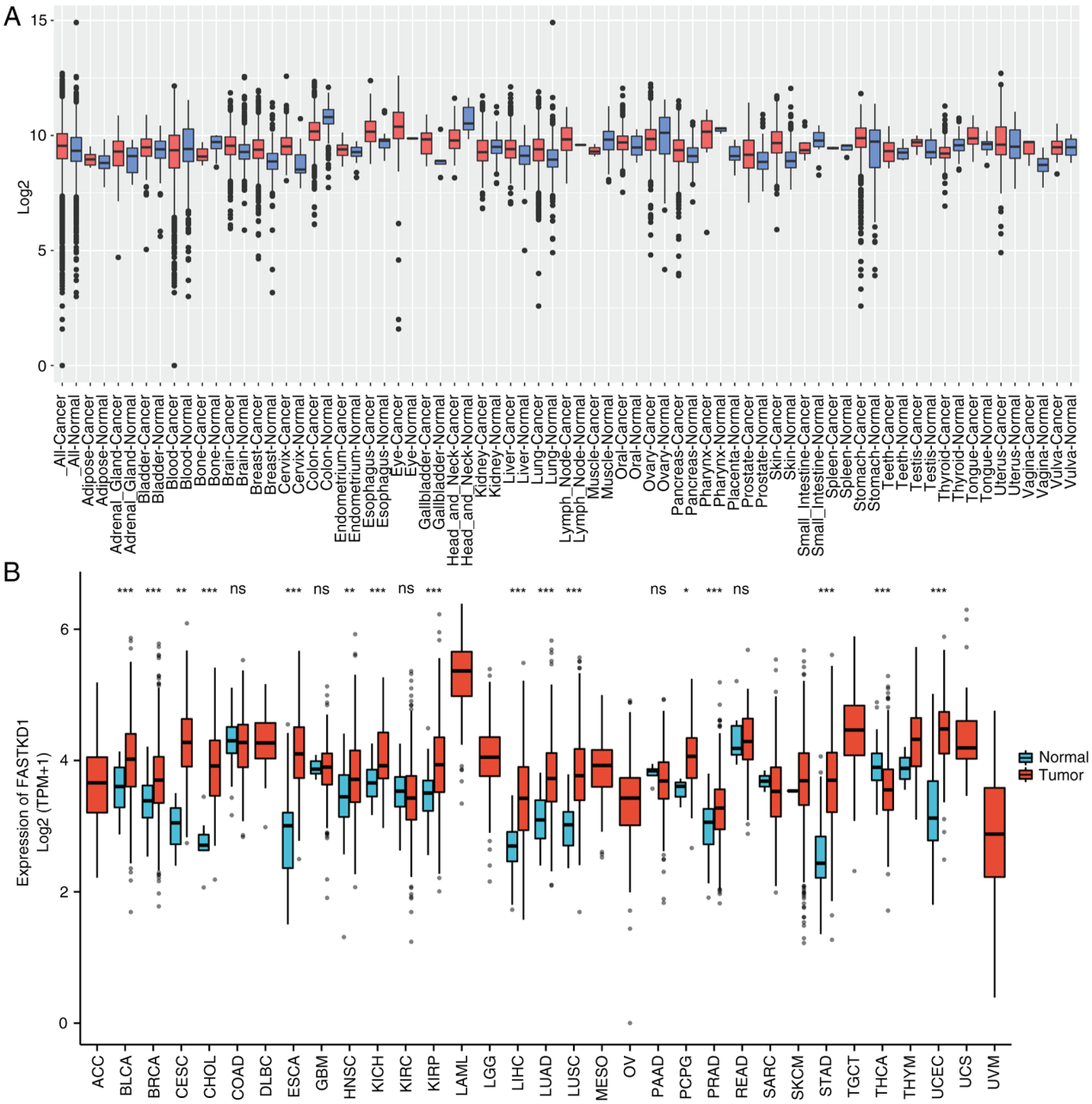

Figure 1. Pan-cancer analysis of the expression of FASTKD1. (A) FASTKD1 mRNA expression across several types of cancer from the GENT2 database. (B) Analysis of FASTKD1 mRNA expression across multiple cancer types using the XENA-TCGA datasets. *P<0.05, **P<0.01, ***P<0.001. ns, not significant; FASTKD1, Fas-activated serine/threonine kinase domain 1.

characteristics. A total of 407 clinical samples were included in the investigation. The data revealed that the expression levels of FASTKD1 were higher in patients aged >65 years in comparison to those aged ≤65 years (Fig. 3B). However, there were no significant differences in FASTKD1 expression based on sex or ethnicity (Fig. 3A and C). In addition, the results indicated that FASTKD1 expression levels were higher in patients with T1 stage STAD compared with T2 and T3. In addition, higher expression levels of FASTKD1 were observed in patients with T4 stage STAD compared with T2 (Fig. 3E). However, no significant differences were identified among the analyzed clinical samples in terms of lymph node involvement, metastasis and pathological stage (Fig. 3D, F and G). Subsequently, it was revealed that clinical patients who did not undergo anti-reflux therapy exhibited considerably higher FASTKD1 expression levels compared with the treated individuals (Fig. 3I). However, no notable distinction was observed in the presence or absence of *Helicobacter pylori* infection (Fig. 3H). The FASTKD1 expression levels were also not significantly different between deceased and surviving patients in relation to OS, disease-specific survival and progression-free interval events (Fig. 3J-L). The clinical features of FASTKD1 in STAD are summarized in detail in Table I.

STAD co-expression network analysis of FASTKD1. To analyze the co-expressed genes of FASTKD1 in STAD, LinkedOmics was used. The results showed a positive correlation between FASTKD1 and 10,229 genes, whereas 9,994 genes were

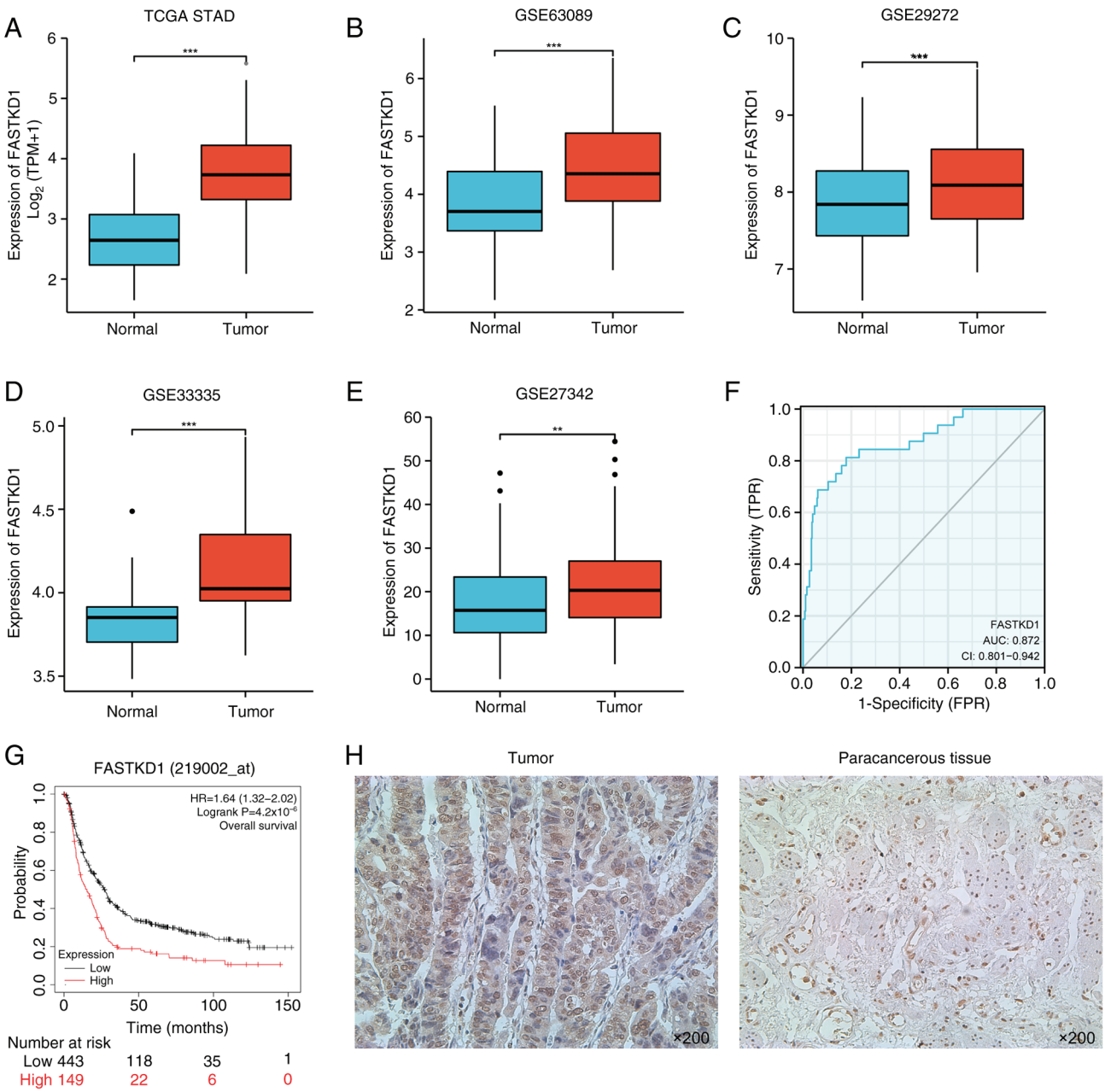

Figure 2. FASTKD1 expression analysis in STAD and normal tissues. (A) Comparison of FASTKD1 expression between STAD and normal tissues using TCGA dataset. Differential expression analysis of FASTKD1 between STAD and normal tissues using (B) GSE63089, (C) GSE29272, (D) GSE33335 and (E) GSE27342 datasets. (F) Receiver operating characteristic curve analysis for the diagnosis of FASTKD1. (G) Survival curve analysis of FASTKD1. (H) Immunohistochemical staining of FASTKD1 in STAD tissues and adjacent normal tissues. **P<0.01, ***P<0.001. ns not significant; FASTKD1, Fas-activated serine/threonine kinase domain 1; STAD, stomach adenocarcinoma; TCGA, The Cancer Genome Atlas.

negatively correlated with FASTKD1 (Fig. 4A). Heatmaps were generated to visually represent the top 50 genes that exhibited a positive or negative correlation with FASTKD1 expression. Fig. 4B displays the heatmap for genes with a positive correlation to FASTKD1 expression, whereas Fig. 4C presents the heatmap for genes with a negative correlation to FASTKD1 expression. The top 200 co-expressed genes that showed a positive correlation with FASTKD1 expression were subjected to GO/KEGG functional enrichment analysis. When P.adj <0.05 and q-value <0.2, there were 183 biological process (BP) terms, 46 cellular component (CC) terms, 31 molecular function (MF) terms and four KEGG pathways identified. The bubble charts present the five most significantly enriched GO BP, CC and MF terms, and KEGG pathways. The GO bubble map analysis revealed that FASTKD1 co-expression was mainly linked to 'nuclear chromosome segregation', 'chromosomal region', 'catalytic activity, acting on RNA' and 'ATPase activity' (Fig. 4D-F). The KEGG pathway bubble map analysis demonstrated that FASTKD1 co-expression was predominantly related to 'Cell cycle' and 'Spliceosome' (Fig. 4G).

GSEA. To examine the potential role of FASTKD1 in STAD, GSEA was performed using the single gene differential analysis associated with FASTKD1. Out of a total of 249 genesets, the top six results were obtained according to the normalized enrichment score. These results included 'WP

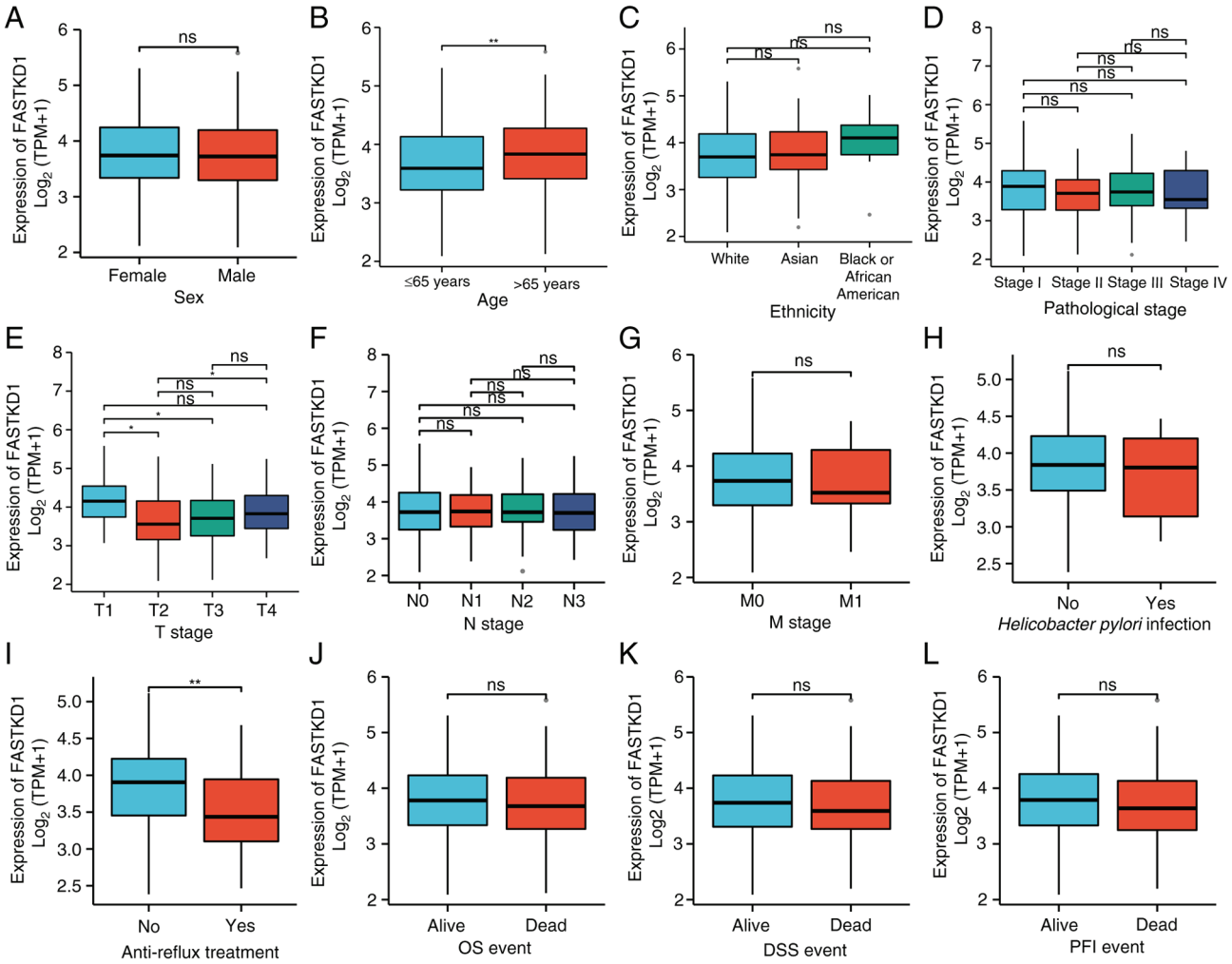

Figure 3. Association of FASTKD1 mRNA expression levels with clinicopathological characteristics in patients with stomach adenocarcinoma. (A) Sex, (B) age, (C) ethnicity, (D) pathological stage, (E) T stage, (F) N stage, (G) M stage, (H) *Helicobacter pylori* infection, (I) anti-reflux treatment, (J) OS event, (K) DSS event, (L) PFI event. *P<0.05, **P<0.01, ns not significant; FASTKD1, Fas-activated serine/threonine kinase domain 1; OS, overall survival; DSS, disease-specific survival; PFI, progression-free interval.

RETINOBLASTOMA GENE IN CANCER' (FDR=0.027, P.adj=0.032), 'REACTOME ACTIVATION OF ATR IN RESPONSE TO REPLICATION STRESS' (FDR=0.013, P.adj=0.015), 'REACTOME RESOLUTION OF D LOOP STRUCTURES' (FDR=0.013, P.adj=0.015), 'PID PLK1 PATHWAY' (FDR=0.014, P.adj=0.017), 'REACTOME HOMOLOGOUS DNA PAIRING AND STRAND EXCHANGE' (FDR=0.013, P.adj=0.016) and 'REACTOME NUCLEAR PORE COMPLEX NPC DISASSEMBLY' (FDR=0.013, P.adj=0.015) (Fig. 5).

Associations between FASTKD1 and tumor-infiltrating immune cells. To investigate the possible role of FASTKD1 in tumor immunity, the present study examined the relationship between FASTKD1 expression and the presence of immune-infiltrating cells in STAD. The analysis used the TIMER database and TCGA STAD dataset to evaluate correlations between FASTKD1 expression and immune cell infiltration (Fig. 6A) illustrates a positive correlation between the expression of FASTKD1 and B cells (ρ=0.139; P=7.34x10^{-3}), and a negative correlation between the expression of FASTKD1 and CD8^{+} T cells (ρ=-0.217; P=2.58x10^{-5}), CD4^{+} T cells (ρ=-0.094; P=7.32x10^{-2}), macrophages (ρ=-0.239; P=3.22x10^{-6}), neutrophils (ρ=-0.192; P=1.93x10^{-4}) and dendritic cells (ρ=-0.229; P=8.36x10^{-6}). (Fig. 6B) illustrates that varying copy numbers of FASTKD1 may affect the degree of immune infiltration in STAD. The results demonstrated that the generalized change in FASTKD1 copy number significantly influenced the level of immune infiltration in STAD, mainly including deep deletion, arm-level deletion, diploid/normal, arm-level gain. Based on R package (GSVA package, version 1.34.0) analysis, it was observed that the expression levels of FASTKD1 were associated with tumor-infiltrating immune cells, such as B cells (P<0.05), CD8 T cells (P<0.001), cytotoxic cells (P<0.001), DCs (P<0.001), immature DCs (P<0.01), macrophages (P<0.05), mast cells (P<0.001), natural killer (NK) cells (P<0.001), plasmacytoid DCs (P<0.001), T helper (Th) cells (P<0.001), central memory T cells (P<0.05), T follicular helper cells (P<0.001), γδ T cells (P<0.05), Th17 cells (P<0.05) and Th2 cells (P<0.001) (Fig. 6C). Kaplan-Meier plots of immune infiltration and the FASTKD1 gene were generated using the TIMER online database to visualize survival differences, the use of KM curves demonstrated that the prognosis of STAD was associated with macrophages (P=0.004; Fig. 6D).

Table I. Clinical characteristics of FASTKD1 in stomach adenocarcinoma.

Characteristics	Low expression of FASTKD1 (n=41) (%)	High expression of FASTKD1 (n=54) (%)	P-value
Sex, n (%)			0.639
Female	14 (14.7)	16 (16.8)	
Male	27 (28.4)	38 (40.0)	
Age, n (%)			0.361
≤65	19 (20.0)	20 (21.1)	
>65	22 (23.2)	34 (35.8)	
Ethnicity, n (%)			0.247
Asian	3 (3.2)	5 (5.3)	
Black or African American	1 (1.1)	6 (6.3)	
White	37 (38.9)	43 (45.3)	
Pathological T stage, n (%)			0.020
T1	1 (1.1)	0 (0.0)	
T2	14 (14.7)	6 (6.3)	
T3	18 (18.9)	33 (34.7)	
T4	8 (8.4)	15 (15.8)	
Pathological N stage, n (%)			0.923
N0	9 (9.5)	11 (11.6)	
N1	9 (9.5)	14 (14.7)	
N2	11 (11.6)	16 (16.8)	
N3	12 (12.6)	13 (13.7)	
Pathological M stage, n (%)			>0.999
M0	38 (40.0)	51 (53.7)	
M1	3 (3.2)	3 (3.2)	
Pathological stage, n (%)			0.067
Stage I	8 (8.4)	3 (3.2)	
Stage II	5 (5.3)	13 (13.7)	
Stage III	23 (24.2)	35 (36.8)	
Stage IV	5 (5.3)	3 (3.2)	
Helicobacter pylori infection, n (%)			0.901
No	36 (37.9)	49 (51.6)	
Yes	5 (5.3)	5 (5.3)	
Reflux history, n (%)			0.075
No	33 (34.7)	51 (53.7)	
Yes	8 (8.4)	3 (3.2)	
Anti-reflux treatment, n (%)			0.023
No	30 (31.6)	49 (51.6)	
Yes	11 (11.6)	5 (5.3)	
OS event, n (%)			0.854
Alive	22 (23.2)	30 (31.6)	
Dead	19 (20)	24 (25.3)	
DSS event, n (%)			0.964
No	26 (27.4)	34 (35.8)	
Yes	15 (15.8)	20 (21.1)	
PFI event, n (%)			0.309
No	20 (21.1)	32 (33.7)	
Yes	21 (22.1)	22 (23.2)	

FASTKD1, Fas-activated serine/threonine kinase domain 1; OS, overall survival; DSS, disease-specific survival; PFI, progression-free interval.

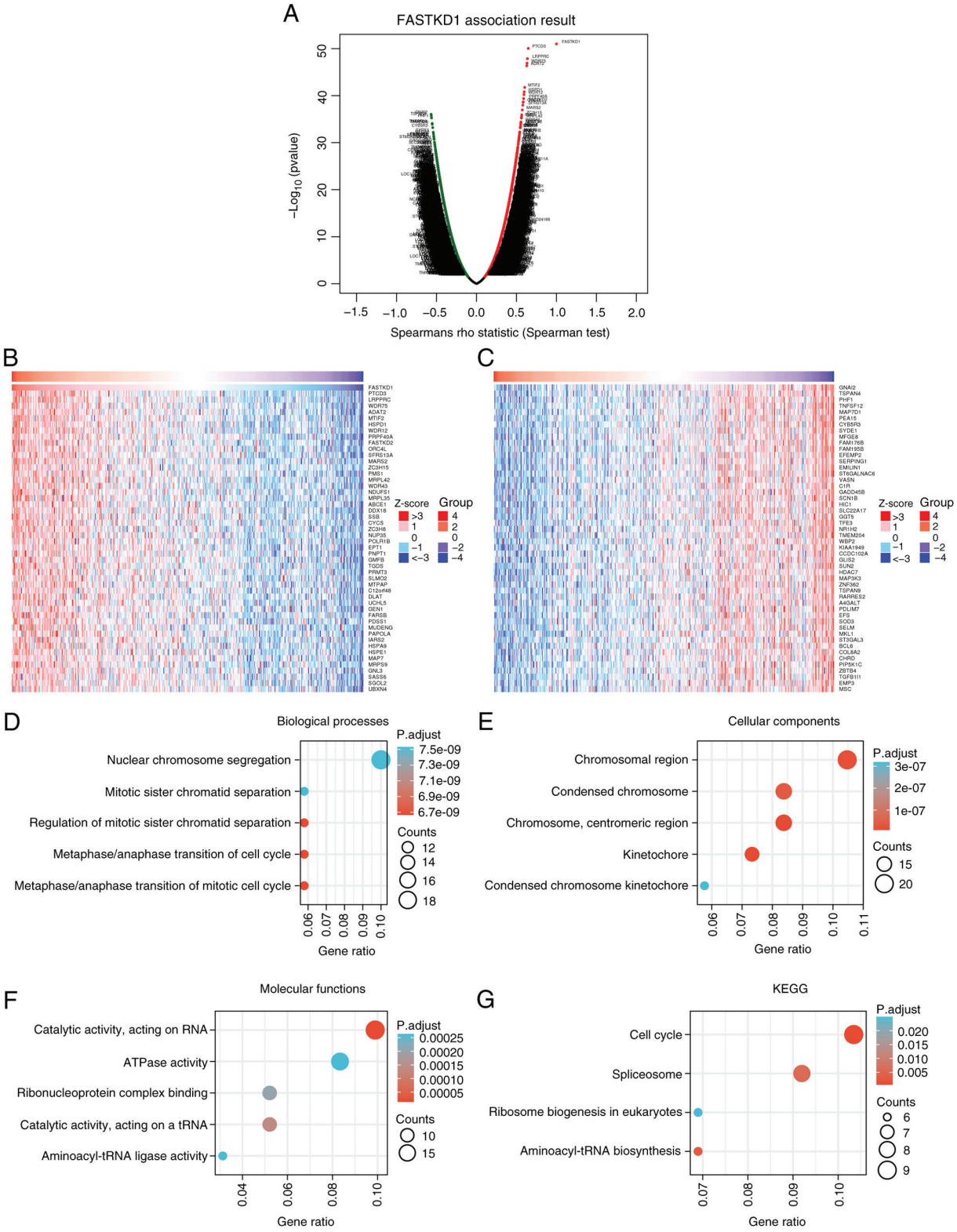

Figure 4. Enrichment analysis of the FASTKD1 co-expression network in STAD. (A) Volcano plot of genes co-expressed with FASTKD1 in The Cancer Genome Atlas STAD dataset. (B) Heatmap of the top 50 positively correlated co-expressed genes with FASTKD1 in STAD. (C) Heatmap of the top 50 negatively correlated co-expressed genes with FASTKD1 in STAD. (D) Biological processes in Gene Ontology enrichment analysis of FASTKD1 co-expressed genes. (E) Cellular components in Gene Ontology enrichment analysis of FASTKD1 co-expressed genes. (F) Molecular functions in Gene Ontology enrichment analysis of FASTKD1 co-expressed genes. (G) KEGG pathway enrichment analysis of FASTKD1 co-expression genes. FASTKD1, Fas-activated serine/threonine kinase domain 1; STAD, stomach adenocarcinoma; KEGG, Kyoto Encyclopedia of Genes and Genomes.

Further exploration is required to investigate the role of FASTKD1 in tumor immunity. Therefore, the present study analyzed the correlation between the expression levels of FASTKD1 in STAD and various immune infiltration markers, using the TIMER and GEPIA databases. The expression of FASTKD1 was weakly negatively correlated with T-cell

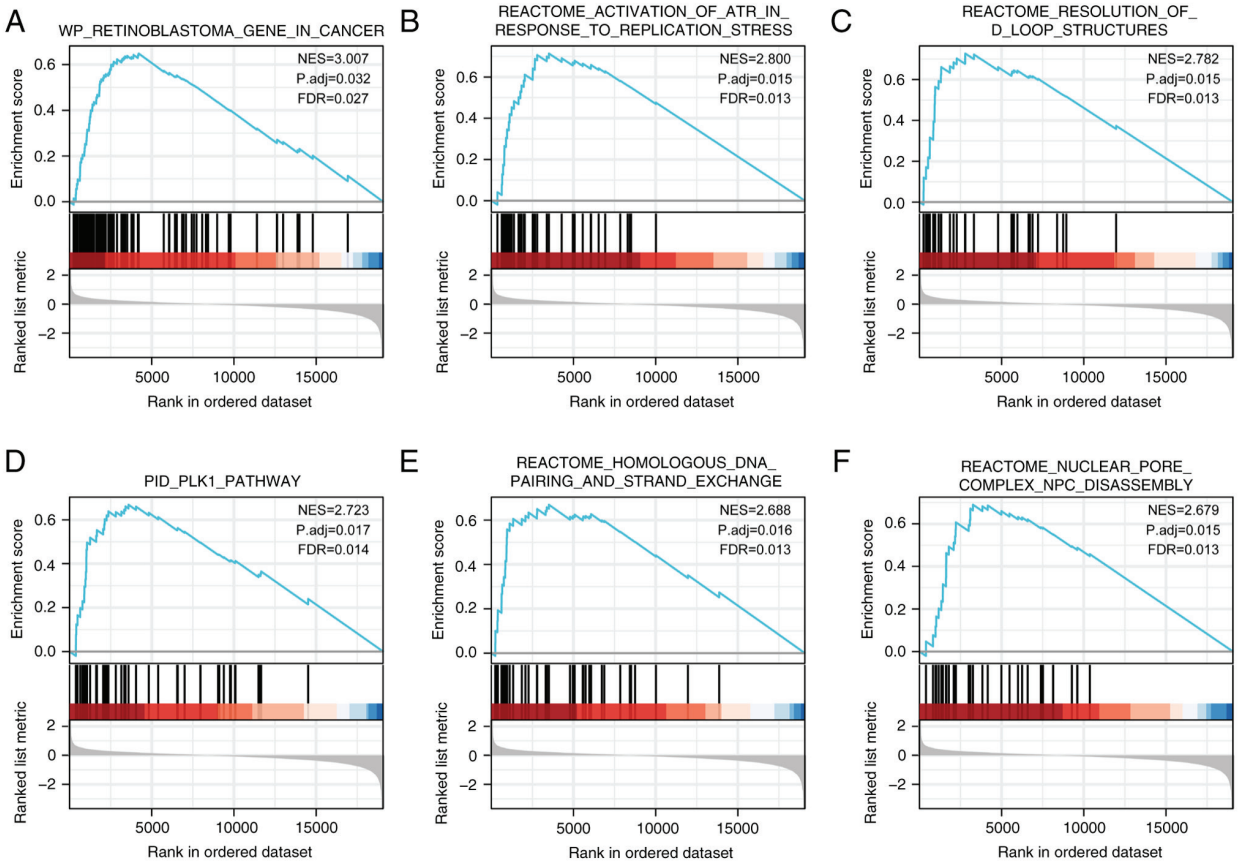

Figure 5. Gene Set Enrichment Analysis for Fas-activated serine/threonine kinase domain 1 in stomach adenocarcinoma. (A) Enrichment in the retinoblastoma gene in cancer pathway. (B) Enrichment in the ATR activation in response to replication stress pathway. (C) Enrichment in the resolution of D-loop structures pathway. (D) Enrichment in the PLK1 pathway. (E) Enrichment in the homologous DNA pairing and strand exchange pathway. (F) Enrichment in the nuclear pore complex NPC disassembly pathway.

biomarkers (CD4, CD3D and CD3E), B-cell biomarkers (CD79A and CD19), DC biomarkers (CD1C, HLA-DPB1 and HLA-DQB1), NK cell biomarkers (NCAM1 and KLRK1), neutrophil biomarkers (MPO, ITGAM and ITGAX) and macrophage biomarkers (CD86 and MRC1) (Fig. 7).

Correlations between the expression levels of FASTKD1 and m6A modification in STAD. Accumulating evidence has supported the crucial involvement of m6A modifications in processes such as inflammation, innate immunity and antitumor responses. These processes are mediated by interactions with various m6A regulatory factors (32-34). The present study analyzed both TCGA STAD cohort and the GSE15459 cohort to investigate the correlation between the expression levels of FASTKD1 and 21 m6A-related genes in STAD (Fig. 8A) shows TCGA STAD and GSE15459 datasets. In TCGA STAD dataset, significant positive correlations were detected between FASTKD1 and the expression levels of METTL3, METTL14, RBM15, RBM15B, WTAP, VIRMA/KIAA1429, CBLL1, ZC3H13, ALKBH5, YTHDC1, YTHDC2, YTHDF3, HNRNPA2B1, HNRNPC, FMR1, LRPPRC and ELAVL1. In the GSE15459 dataset, significant positive correlations were detected between FASTKD1 and the expression levels of METTL14, YTHDF1, YTHDF2 and LRPPRC. In the GSE15459 dataset, there were also negative correlations detected between FASTKD1 and the expression levels of WTAP, ZC3H13, ALKBH5, FTO, YTDHDC1 and HNRNPA2B1. Scatter plots (Fig. 8B) were created to illustrate the correlation between FASTKD1 and m6A modification-related genes. Furthermore, a forest plot (Fig. 8C) was employed to present the prognostic importance of the 21 m6A-related genes in STAD. A Venn plot (Fig. 8D) was also generated to display the intersection of m6A expression-related genes and prognostic genes. For the overlapping genes, KM survival analysis was performed. The KM-plotter indicated that high expression levels of YTHDF1 (HR=1.38; log-rank P=0.0028) and LRPPRC (HR=1.54; log-rank P=$8.1x10^{-5}$) were associated with a worse prognosis in STAD (Fig. 8E). The present study identified an association between FASTKD1 and m6A modifications in STAD, especially through its interaction with the LRPPRC and YTHDF1 genes. This interaction may affect the progression and prognosis of STAD.

Discussion

STAD is a type of cancer resulting from the malignant transformation of somatic cells in the gastric glands. It is one of the most prevalent types of gastrointestinal cancer, with an estimated annual incidence of >1 million cases worldwide (2). Due to its frequently advanced stage upon diagnosis, the mortality rates associated with gastric cancer remain high. In addition, although the cancer incidence rate is decreasing

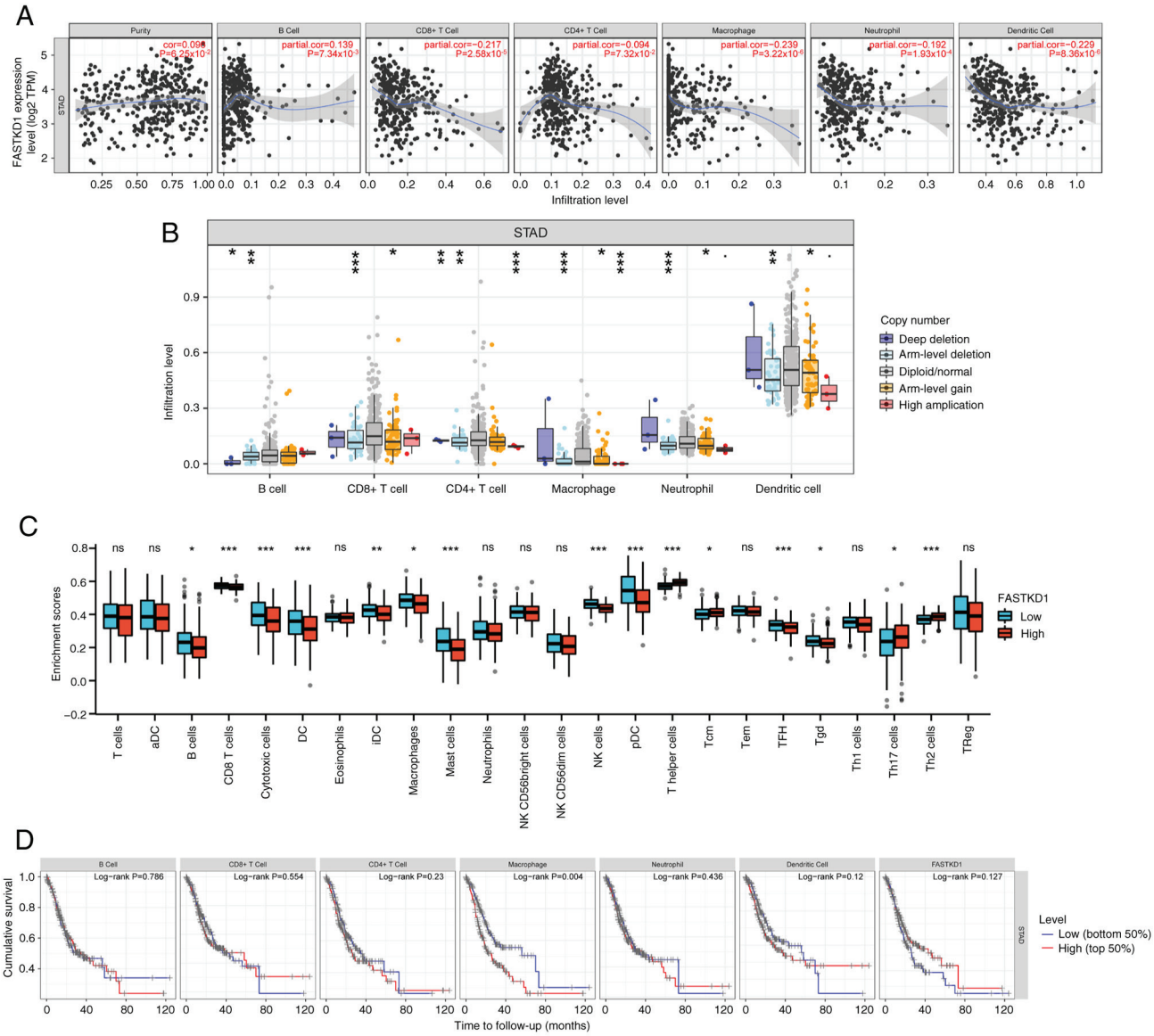

Figure 6. Correlation between FASTKD1 and tumor-infiltrating immune cells in STAD. (A) Spearman correlation analysis shows the correlation between FASTKD1 expression and infiltrating immune cells in STAD. (B) Effect of FASTKD1 copy number variation on the levels of infiltrating B cells, $CD8^+$ T cells, $CD4^+$ T cells, macrophages, neutrophils and dendritic cells in STAD. (C) Comparison of changes in 24 immune cell subtypes between high and low FASTKD1 expression groups in STAD tumor samples using the Wilcoxon rank-sum test. (D) Kaplan-Meier plots to assess the relationship between immune infiltration and FASTKD1 expression levels in STAD. *P<0.05, **P<0.01, ***P<0.001. ns, not significant; FASTKD1, Fas-activated serine/threonine kinase domain 1; STAD, stomach adenocarcinoma.

in most countries, clinicians predict an increase in cancer cases in the future due to the aging population. Therefore, researching new molecular targets and pathways is crucial for providing innovative insights into the diagnosis, treatment and prognosis of STAD.

FASTKD1 is a member of the FASTK family, which includes six members: FASTK, the original member, and its homologs, FASTKD1-5. These FASTK family proteins are found exclusively in vertebrates and have widespread expression in mitochondria across multiple body tissues. They serve a vital role in preserving and stabilizing mitochondria, emerging as critical regulators of post-transcriptional gene expression within these organelles (6). FASTKD1 is situated on chromosome 2q31.1 and is expressed in the mitochondrial matrix. It features an amino-terminal mitochondrial targeting signal and a C-terminal region with three conserved domains, FAST1, FAST2 and RAP (35). FASTKD1 controls the ND3 domain within mitochondria and may participate in processes associated with RNA stability. Furthermore, FASTKD1 operates as a protective factor against reactive oxygen species-induced oxidative stress and cell death, although the exact mechanism of this protection is currently unknown (5). Additionally, FASTKD1 alters mitochondrial dynamics in a CypD-independent manner and impacts processes, such as autophagy/mitophagy and caspase-3 activation (35). However, limited research has examined the role of FASTKD1 in carcinogenesis (36).

The present study analyzed XENA-TCGA and GEO datasets to observe the expression levels of FASTKD1 in various tumors, including STAD. The elevated expression of FASTKD1 in STAD tissue was subsequently confirmed through IHC

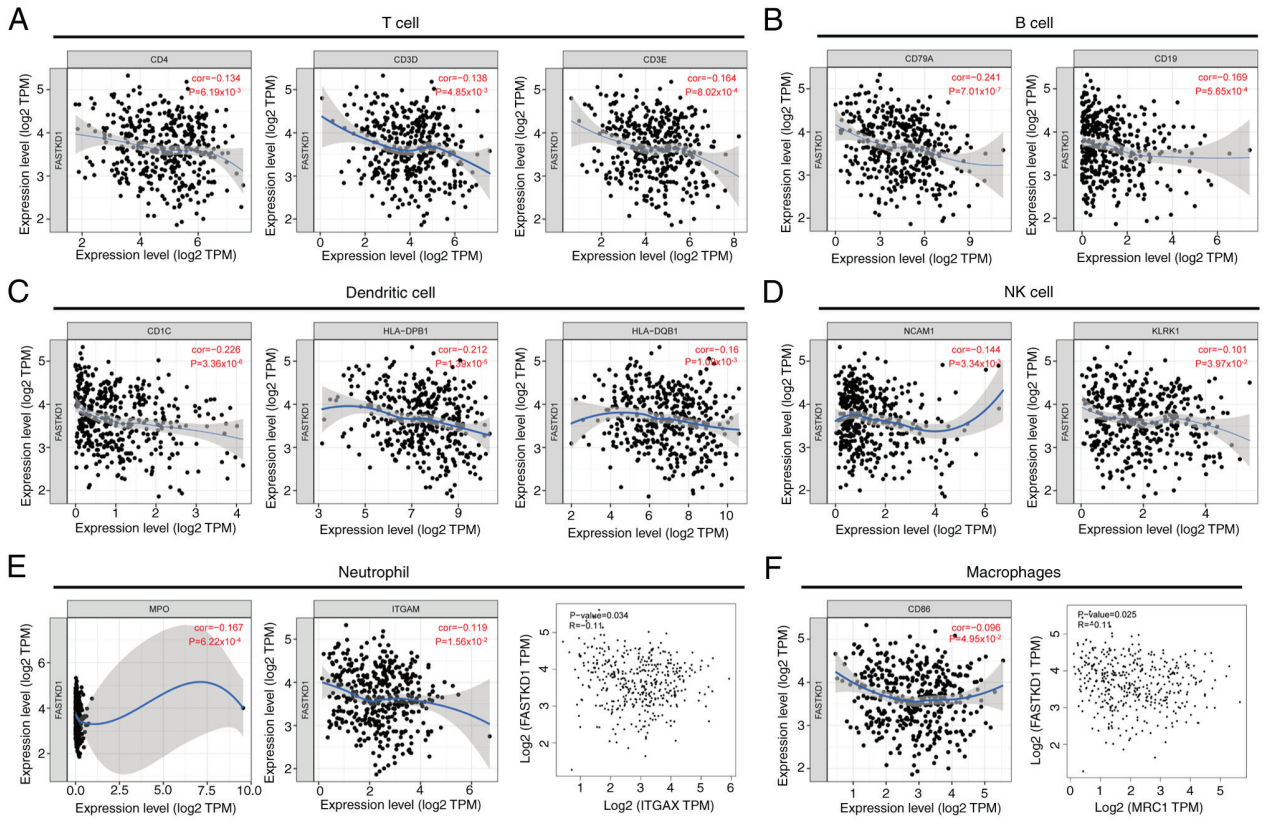

Figure 7. Analysis of the relationship between Fas-activated serine/threonine kinase domain 1 expression and marker genes of immune cells in stomach adenocarcinoma using Tumor Immune Estimation Resource and Gene Expression Profiling Interactive Analysis databases. (A) T cell; (B) B cell; (C) dendritic cell; (D) NK cell; (E) Neutrophil; (F) Macrophage.

staining. In addition, the present study revealed that increased levels of FASTKD1 expression may aid the diagnosis of STAD, and could be strongly linked to unfavorable prognosis and clinical features in patients with STAD. For example, administering anti-reflux therapy was shown to significantly reduce the expression levels of FASTKD1 in patients with STAD.

The GO/KEGG enrichment analysis revealed that the co-expressed genes of FASTKD1 were active in a variety of terms and pathways, such as 'nuclear chromosome segregation', 'chromosomal region', 'catalytic activity, acting on RNA', 'ATPase activity', 'Cell cycle' and 'Spliceosome'. Several studies have demonstrated the significant involvement of the aforementioned biological functions in the initiation and progression of tumors (37-40). Proper segregation of nuclear chromosomes is crucial to maintaining genomic stability during cell division and errors in this process can lead to aneuploidy, a condition in which cells have an abnormal number of chromosomes, which is often observed in cancer cells. Aneuploidy contributes to genomic instability, promotes tumorigenesis and enables the acquisition of genetic alterations that propel tumor progression (41). Structural variations, amplifications, deletions and rearrangements in particular chromosomal regions are associated with different types of cancer, and these changes bring about alterations in gene dosage, disruption of regulatory elements, and activation or inactivation of oncogenes and tumor suppressor genes found within these regions. These alterations can lead to uncontrolled growth and survival of cells, and promote the development and progression of tumors (42). Catalytic activity, specifically acting on RNA, includes RNAs involved in processing, modification and degradation, and dysregulation of these RNA activities can affect critical cellular processes, including the stability, translation and splicing of mRNA. Aberrant RNA metabolism is frequently observed in cancer cells and can lead to altered gene expression patterns, which promote tumor cell proliferation and survival (43). ATPase activity is crucial to multiple cellular processes, such as DNA repair, chromatin remodeling and protein folding, whereas dysregulated ATPase activity in cancer is associated with defects in DNA damage response and repair mechanisms, compromised chromatin structure and altered protein homeostasis; these disruptions can contribute to genomic instability, a hallmark of cancer, and promote tumor progression (44). The cell cycle is regulated to guarantee precise DNA replication and cell division, whereas excessive cell proliferation and accumulation of genetic abnormalities can be caused by the dysregulation of checkpoints. Notably, dysregulation of the cell cycle is a hallmark of cancer, contributing to uncontrolled cell proliferation, genomic instability and tumor progression (45). The spliceosome is a sophisticated molecular apparatus responsible for RNA splicing, which is a key process in the regulation of gene expression; notably, dysregulation of splicing events can lead to the production of abnormal isoforms with oncogenic properties. In addition, disruptions in spliceosome components or splicing factors have been reported in numerous types of cancer, and markedly affect essential cellular processes, including cell proliferation, survival and metastasis, thereby contributing to tumor progression (46).

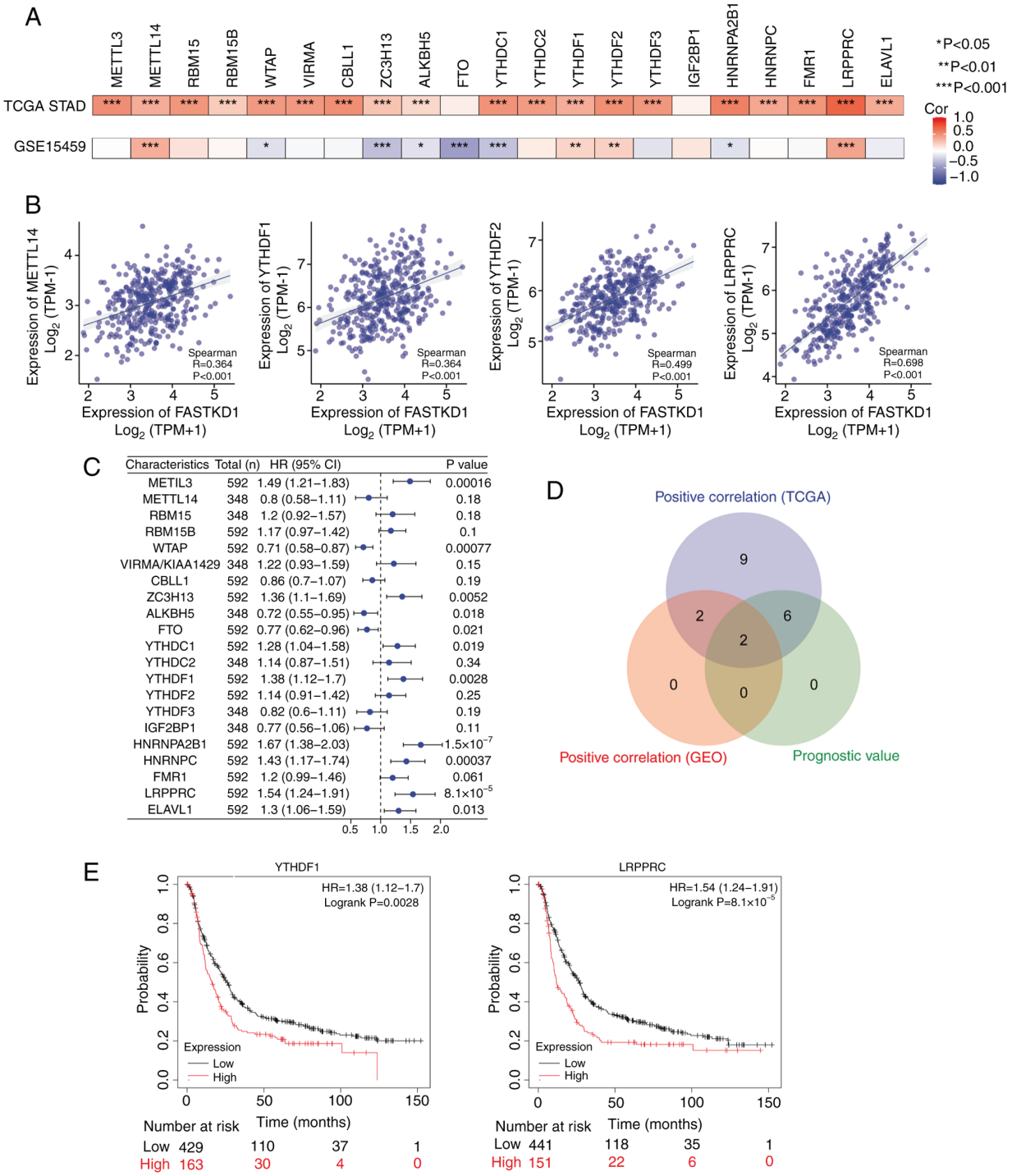

Figure 8. Correlation analysis between FASTKD1 expression and m6A-related genes in STAD. (A) Comparative analysis (correlation) of FASTKD1 and m6A-associated gene expression using the GSE15459 and TCGA STAD datasets. (B) Scatterplot visualization of the relationship between FASTKD1 and m6A-associated gene expression. (C) Forest plot showing survival outcomes based on m6A-associated gene expression. (D) Venn diagram illustrating the overlap of m6A-associated genes based on expression patterns and survival data. (E) Kaplan-Meier survival analysis focusing on YTHDF1 and LRPPRC. *P<0.05, **P<0.01, ***P<0.001. FASTKD1, Fas-activated serine/threonine kinase domain 1; STAD, stomach adenocarcinoma; TCGA, The Cancer Genome Atlas; GEO, Gene Expression Omnibus.

Single gene differential GSEA analysis revealed that the differentially expressed genes associated with FASTKD1 were enriched in the following pathways: 'Wp Retinoblastoma Gene in Cancer', 'Reactome Activation Of Atr in Response to Replication Stress', 'Reactome Resolution of D Loop Structures', 'Pid Plk1 Pathway', 'Reactome Homologous Dna Pairing and Strand Exchange' and 'Reactome Nuclear Pore Complex Npc Disassembly'. These cellular processes and pathways serve a critical role in the initiation and progression of cancer. A complete understanding of their dysregulation and functional implications may provide valuable insights into the underlying mechanisms of STAD. The retinoblastoma pathway in cancer involves the central role of the RB1 gene in regulating cell cycle progression and inhibiting tumorigenesis; notably, RB1 disruptions, caused by mutations or inactivation, can significantly contribute to the development of various

types of cancer (47). Activation of ATR in response to replication stress is a pathway related to the role of ATR proteins in response to DNA replication stress; ATR serves a critical role in maintaining genomic integrity by initiating signaling pathways involved in the DNA damage response, leading to cell cycle arrest and DNA repair (48). The association between D-loop resolution and homologous recombination suggests a DNA repair mechanism responsible for the accurate repair of DNA double-strand breaks; accurate D-loop resolution has a critical role in maintaining genomic stability and preventing the accumulation of genetic abnormalities (49). PLK1 is a critical regulator of cell division and exerts its influence at multiple stages of mitosis, including mitotic entry, spindle assembly, chromosome segregation and cytokinesis; by contrast, disruption of PLK1 activity has been reported in several cancer types, and is associated with aberrant cell division, chromosomal instability and tumor progression (50). Homologous DNA pairing and strand exchange is a pathway associated with the critical steps in DNA repair by homologous recombination; specifically, this pathway highlights the importance of homologous DNA pairing and subsequent strand exchange events, since dysregulation of these complex processes can lead to genomic instability and increase the risk of tumorigenesis (51). The NPC disassembly pathway refers to the process of NPC disassembly during mitosis to facilitate accurate chromosome segregation; dysregulation of NPC disassembly disrupts normal cell division and contributes to chromosomal instability, a common feature observed in cancer cells (52). The intricate relationship between these processes and pathways suggests their potential involvement in the biological functions and mechanisms associated with FASTKD1 in STAD. Further investigation of these pathways may provide valuable insights into the role of FASTKD1 in the development and progression of STAD.

The assessment of immune infiltration of tumor cells has become increasingly important in cancer research and clinical practice. Understanding the composition and functional properties of the immune infiltrate can assist in identifying potential therapeutic targets and developing immunotherapies. The present study provided valuable insights into the interplay between FASTKD1 and the immune microenvironment in the context of STAD. The results indicated a negative correlation between the expression levels of FASTKD1 in STAD and the presence of several immune cell types. Specifically, a negative correlation was observed between FASTKD1 expression and the infiltration of $CD8^+$ T cells, $CD4^+$ T cells, macrophages, neutrophils and DCs. These results suggested that FASTKD1 may serve a role in modulating the tumor immune microenvironment in STAD by potentially affecting the abundance or activity of these immune cell populations (53).

The present study also revealed that CNVs in FASTKD1 may have an impact on the levels of infiltrating immune cells in STAD. Changes in FASTKD1 CNV were shown to be associated with changes in the abundance of B cells, $CD4^+$ T cells, macrophages, neutrophils and DCs within the tumor microenvironment. In the present study, it was demonstrated that the prevalent copy number alterations associated with FASTKD1 in STAD were characterized by two patterns: Arm-level deletion and arm-level gain. Arm-level deletion is a genomic alteration commonly observed in various tumor types, including STAD. It is characterized by the loss of genetic material spanning an entire chromosomal arm. The loss of genetic material from an entire chromosomal arm can result in the inactivation or loss of multiple genes located within that region. These genes may include tumor suppressor genes, which normally regulate cell proliferation and prevent tumor formation (54). By contrast, arm-level gain is often associated with amplification of oncogenes, which are genes that promote tumor growth when abnormally activated or amplified. The increased copy number of these oncogenes leads to increased expression or functional activity, which drives tumor proliferation and survival (55). These findings suggested a potential role for FASTKD1 CNV in modulating the composition of immune cells and potentially influencing the immune response in STAD. In the context of disease, CNVs have been implicated in a variety of conditions, including developmental disorders, neurodegenerative diseases (56,57). In cancer, CNVs may contribute to tumorigenesis by affecting oncogenes or tumor suppressor genes, disrupting key signaling pathways or altering the genomic stability of cancer cells (58,59).

By comparing groups with high and low FASTKD1 expression, the differential immune cell composition between the two groups can be examined. This analysis aims to uncover potential differences in the abundance of various immune cell subtypes, including, but not limited, to T cells (including $CD8^+$ T cells and $CD4^+$ T cells), B cells, NK cells, macrophages, neutrophils, DCs and other identifiable immune cell populations. Notably, a significant association between macrophage infiltration and survival was detected in patients with STAD. This finding suggested that the presence or abundance of macrophages in the tumor microenvironment may have a role in determining patient outcome (60). In addition. FASTKD1 expression was revealed to be weakly negatively correlated with most immune infiltration markers in the TIMER and GEPIA databases. This finding suggested that, as FASTKD1 expression increases, the expression or abundance of immune cell infiltration markers tends to decrease. It is possible that FASTKD1 directly or indirectly affects the release of immunosuppressive factors or alters the expression of chemokines that drive immune cell migration. A comprehensive analysis to investigate the relationship between FASTKD1 expression and changes in the immune cell landscape within STAD tumors may provide valuable insights into the potential role of FASTKD1 in influencing the immune microenvironment.

m6A modification is a common and reversible RNA modification that has an important role in post-transcriptional gene regulation. In recent years, extensive research has been conducted to understand the functional significance of m6A modification and its impact on various biological processes (61-63). In the present study, a significant correlation was observed between the expression levels of FASTKD1 and two genes, namely YTHDF1 and LRPPRC. YTHDF1 is a member of the YTH domain family of RNA-binding proteins that specifically recognizes and binds to m6A-modified mRNA. As an m6A reader, YTHDF1 has a role in the regulation of RNA metabolism and translation. It promotes translation efficiency by interacting with the translation initiation machinery and recruiting ribosomes to m6A-modified transcripts. YTHDF1 also influences mRNA decay processes, where it can either stabilize or promote degradation of m6A-modified

transcripts, depending on the context (64). LRPPRC is a versatile protein involved in several cellular processes, including mitochondrial function and RNA metabolism, which has been implicated in the stabilization and maintenance of mitochondrial transcripts, as well as the post-transcriptional regulation of nuclear-encoded mRNAs. It interacts with specific RNA targets, including those involved in oxidative phosphorylation and mitochondrial biogenesis, to regulate their processing, stability or translation (65,66). The present study detected a significant correlation between FASTKD1 expression and both YTHDF1 and LRPPRC. This correlation suggested a possible relationship between FASTKD1 and these two genes in the context of post-transcriptional gene regulation. Further investigation is required to determine the nature and functional implications of these correlations, such as whether FASTKD1 directly interacts with YTHDF1 and LRPPRC, or whether they share a common regulatory pathway or mechanism.

The present study has some limitations. Firstly, the association between high FASTKD1 expression and poor prognosis in patients with STAD lacked validation from clinical trials. Secondly, despite combining metabolic and genomic signatures to explore potential biomarkers and underlying mechanisms, the lack of molecular biology validation represents another limitation of this study. Future studies should focus on addressing this limitation to provide more robust evidence for the identified biomarkers and mechanisms.

In conclusion, the present study demonstrated that FASTKD1 may be upregulated in STAD, and identified its associations with clinical characteristics and survival in patients with STAD. Furthermore, the interaction between FASTKD1, immune infiltration and m6A modification was investigated. The results indicated that FASTKD1 is a promising independent diagnostic and prognostic marker for STAD, and may be a potential target for future molecularly targeted therapies.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

YY performed the experiments, analyzed the results and wrote the manuscript. YG assisted in completing immunohistochemical staining and scoring based on a points system. ZMH, XSL, YZ, YHZ, ZYL and YXC interpreted the results. YY collected the clinical samples. ZJP designed and supervised the project, and revised and finalized the manuscript. YY and YG confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The studies involving human participants were reviewed and approved by the Ethics Committee of Taihe Hospital affiliated with Hubei University of Medicine (approval no. 2022KS010). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements. The Institutional Review Board waived the need for informed consent due to the retrospective nature of the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Use of artificial intelligence tools

During the preparation of this work, AI tools were used to improve the readability and language of the manuscript or to generate images, and subsequently, the authors revised and edited the content produced by the AI tools as necessary, taking full responsibility for the ultimate content of the present manuscript.

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