Multi-omics Analysis of Prognostic Significance and Immune Infiltration of FASTK Family Members in Kidney Renal Clear Cell Carcinoma

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ABSTRACT

OBJECTIVE: The Fas-activated serine/threonine kinase (FASTK) family of proteins has been recently found to be able to regulate mitochondrial gene expression post-transcriptionally. Nonetheless, there is a paucity of study about the role of the FASTK family in kidney renal clear cell carcinoma (KIRC). This study was conducted to explore the correlation of FASTK family genes with expression, prognosis, and immune infiltration in KIRC.

METHODS: We collected the data from the UALCAN, GeneMANIA, STRING, CancerSEA, cBioPortal, Kaplan-Meier plotter, GEPIA, TISIDB and TIMER databases to evaluate the genetic alterations, differential expression, prognostic significance, and immune cell infiltration of FASTKs in patients with KIRC.

RESULTS: In tumor tissues of KIRC, the mRNA expression level of FASTK and TBRG4 was elevated, whereas that of FASTKD1, FASTKD2, and FASTKD5 was lowered compared with normal tissues ($P < .05$). Patients with KIRC and high FASTK and Transforming growth factor β regulator 4 (TBRG4) expression had worse overall survival (OS) and disease specific survival (DFS), while those with lower expression of FASTKD2/3/5 had worse outcomes. FASTK was positively correlated with DNA damage. FASTKD1 was positively related to differentiation. FASTKD2 was inversely related to proliferation and FASTKD5 was inversely related to invasion and EMT in KIRC cells. FASTK expression in KIRC was inversely linked to the presence of several immune cells including Tgd, macrophages, Tcm, and Mast cells ($P < .05$).

CONCLUSIONS: Our research provided fresh insight and in-depth analysis to the selection of prognostic biological markers of FASTK family members in KIRC.

KEYWORDS: Multi-omics analysis, FASTK family, biomarker, prognosis, kidney renal clear cell carcinoma

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Introduction

Renal cell carcinoma (RCC) is a malignancy that has witnessed an increase in incidence over the past few decades worldwide. Specifically, RCC is a urological tumor that has the highest annual death rate.[1] Approximately 80% of all RCC cases in adults are diagnosed as clear cell renal cell carcinoma (ccRCC).[2] Notably, ccRCC is typified by the deletion of the von Hippel Lindau (VHL) gene or a mutation in that gene in which the oxygen-sensing mediator protein VHL (pVHL) is encoded in most malignant tumors.[3,4] It is estimated that 30% of RCC patients are already in a metastatic state when they are first diagnosed.[5] Nearly 30% of individuals who have had the primary tumor completely removed will develop recurrence.[6] The 5-year survival rate of RCC is as dismal as 11.7% if diagnosed at an advanced stage.[7] Presently, those who have metastatic RCC (mRCC) are often treated with immunotherapy, specifically Interferon alfa (IFN-α) or high-dose Interleukin-2 (IL-2). Combinations of immune checkpoint inhibitors and anti-VEGF (Vascular Endothelial Growth Factor, VEGF) treatments are now under ongoing exploration, which significantly alters the mRCC therapeutic landscape.[8] Immunotherapy response rates, although encouraging, are largely dismal, spanning between 15% and 25%,[9] therefore the need to find better treatment targets for ccRCC cannot be neglected. Moreover, it is crucial to discover new biomarkers that can help with earlier detection and prognosis improvement.

FASTK family comprises 6 members of FASTK together with its homologs FAST Kinase Domains 1-5 (FASTKD1-5) and the founder member. In recent years, study discovered important roles of members of this family,[10] which also encouraged us to further probe into the important role of FASTK family in post-transcriptional regulation of mitochondrial gene expression. FASTK becomes activate during the Fas-mediated apoptosis via phosphorylation of translational repressor T cell intracellular Ag-1 (TIA1), which precede the onset of DNA fragmentation. TIA1 is an RNA-binding protein, involved in

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translational control pathway.[11] Gene expression studies using microarrays have shown that many members of the FASTK family are overexpressed in a wide range of cancers. In a variety of cystic pancreatic tumors with the propensity to progress into malignancy as well as in pancreatic ductal adenocarcinoma (PDAC), upregulation of FASTK has been detected.[12] Overexpression of this protein has also been detected in the most prevalent form of cutaneous T-cell lymphoma, mycosis fungoides. In mycosis fungoides, upregulated expression of FASTK was shown to be linked to a chromosomal change consisted of a gain of 7q36, which is the location of the FASTK gene.[13]

In uterine aspirate samples, the FASTKD1 gene (which is part of the FASTK family) is a highly sensitive RNA-based biological marker for endometrial carcinoma.[14] Furthermore, a meta-analysis on freely accessible gene expression datasets has shown that the overexpression of FASTKD1 is a poor predictive marker for both pediatric and adult patients suffering from acute lymphoblastic leukemia (ALL).[15] Similar to FASTK, the expression of FASTKD2 is elevated in pancreatic cancer samples and is linked to a dismal prognosis.[16] On the other hand, the upregulation of FASTKD3 is indicative of a higher survival rates in patients with bladder cancer.[17] No studies have shown alterations in FASTKD4 (TBRG4) or FASTKD5 expression in cancer so far. Nevertheless, the roles of various FASTK family members in the prognostic outcomes of KIRC patients is still unclear.

In this research, we searched openly available datasets including the Kaplan-Meier plotter, Gene Expression Profiling Interactive Analysis (GEPIA), and University of Alabama at Birmingham Cancer Data Analysis Portal (UALCAN) to study the expression and prognostic relevance of the FASTK family in KIRC. CancerSEA was used for the purpose of assessing the role of the FASTK in KIRC. We employed Tumor-Immune System Interactions Database (TISIDB) and Tumor Immune Estimation Resource (TIMER) to examine how FASTK expression was linked to immune inhibitors and infiltrating immune cells. This was the first study of how the FASTK family gene expression correlated with specific features of KIRC across clinical, molecular, and immune dimensions. Referring to a previous study,[18] the present study workflow is presented in Figure 1. Our findings may contribute to the development of more effective immunotherapies for patients with KIRC.

Materials and Methods

Data source

The Cancer Genome Atlas (TCGA) (https://portal.gdc.cancer.gov) is a large-scale cancer genome data bank containing the clinical and pathological data on 33 different types of cancer.[19] From the TCGA database, we collected patient-level clinical data on ccRCC diagnoses and high-throughput RNA sequencing (RNA-seq) data. The fragments per kilobase per million fragments mapped (FPKM) approach available in

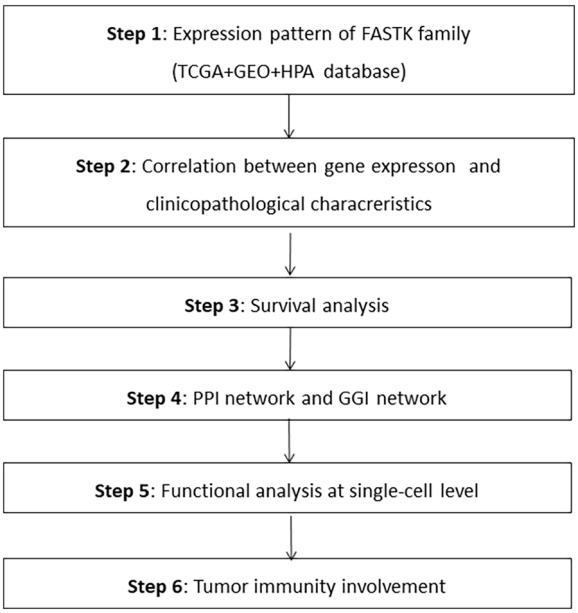

Figure 1. The workflow of this study.

HTSeq were applied to compute transcript expression levels. Additionally, transcripts per million (TPM) readings were generated using the clinical information and level 3 HTSeq-FPKM values of RNA-Seq gene expression obtained from 539 individuals with KIRC. The Gene Expression Omnibus (GEO) software was used to download the gene expression profile data from the GEO datasets (GSE66271), and the limma package was used to examine the differences between the 2 groups.

FASTK family expression level analysis

We analyzed the FASTK expressions in cancerous and normal samples with the help of UALCAN (http://ualcan.path.uab.edu/index.html), an online tool for conducting a thorough analysis of TCGA gene expression profiles.[20] Moreover, UALCAN was utilized to determine the expression of the FASTK family of proteins in KIRC patients of varying racial/ethnic backgrounds, tumor grades, ages, and several other clinical and pathological variables.

The HPA databases

The Human Protein Atlas (HPA) now stores information on the cell-specific locations for 44 normal tissues and 20 of the most common malignancies.[21] HPA covers tissue, cell, and pathology Atlas, provides extensive information on the transcriptome and proteome of numerous human specimens. The database furthermore offers information on protein immunohistochemistry in normal human tissues and cancerous tissues. The antibodies of FASTK, TBRG4, FASTKD5, FASTKD3, and FASTKD1 protein were HPA031621. HPA020582, HPA043833, HPA068525, and HPA043719, respectively.

Clinical statistical analysis of prognosis, model development, and assessment

Using the patient data from TCGA, the clinical meaning module of the Xiantao platform (https://www.xiantao.love/)[22] was adopted to evaluate prognostic metrics, notably, overall survival (OS), disease-specific survival (DSS), and progression-free interval (PFI). Both the Cox regression and Kaplan-Meier were applied to perform these analyses. The dividing line between subgroups with high- and low-FASTK expression was established on the premise of the median value. FASTK expression in relation to clinical-pathological variables was examined using Wilcoxon signed-rank sum test together with log regression. Based on a multi-factor Cox regression model, we evaluated the impact of FASTK expression on survival and several other clinical outcomes. The significance level was set at a Probability value of $<.05$. The survival odds over 1, 3, and 5 years were predicted as per the results of the Cox regression model and the independent prognostic markers derived from the multi-factor analysis. The predicted rates were matched to the observed rates using calibration curves. The line at 45° indicated a high accuracy of the predicted value.

FASTK family gene alterations and correlation analysis

To assess the FASTK family mutations in the TCGA KIRC sample, we used the cBio Cancer Genomics Portal (http://cbioportal.org), a free-to-use platform that enables the interactive investigation of multiple-dimensional cancer genomic datasets.[23] Additionally, it was employed for analyzing the relationships among members of the FASTK family. This combined study contains samples from 7 studies including 1814 samples/1744 patients.

Gene-gene interaction and protein-protein interaction (PPI) networks

GeneMANIA (http://genemania.org/) is a convenient method for the development of gene networks and the predictions of their functions in Cytoscape,[24] and Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) (https://string-preview.org/) was utilized for the functional enrichment analysis of PPI networks.[25] Both of these tools were utilized to analyze the FASTK family gene and protein network. For GeneMANIA, in this study, we showed the top 20 related genes. The PPI network data showed the predicted high number of edges (65) and nodes (16).

Single-cell analysis

To examine the function of the FASTK family expression, we employed the CancerSEA (http://biocc.hrbmu.edu.cn/CancerSEA/home.jsp), the first specially-created database decoding the functional statuses of cancerous cells at the single-cell level.[26]

Diagnostic value analysis

The effectiveness of the FASTK family members in the ccRCC diagnosis was evaluated using the "pROC" R program. *P*-values under .05 were regarded as significant. The area under the curve (AUC) of receiver operating characteristic (ROC) curve reflected the diagnostic impact.

DNA methylation analysis

Between ccRCC and normal tissues, the promoter methylation of FASTK family was compared using the UALCAN database. Beta value in the range between 0 (unmethylated) and 1 (fully methylated) was used to show DNA methylation level. A *P*-value$<.05$ was defined as significant.

Immune cell infiltration and immunoinhibitor analysis

The marker genes were acquired following the methods proposed by Bindea et al.[27] The Single-sample Gene Set Enrichment Analysis (ssGSEA) approach studies tumor cell infiltration using the 24 distinct types of immune cells. On the basis of the findings from immune infiltration, Xiantao tool, Wilcoxon signed-rank sum, Spearman correlation, the correlations of immune infiltration with the expression of FASTK family genes and with the values of various gene expression subgroups of FASTK family were comprehensively examined in the module of the "Xiantao tool." The TISIDB (http://cis.hku.hk/TISIDB/index.php), which is a tumor-immune system interaction applications that stores heterogeneous data sources,[28] was introduced here to further study the correlation between immune inhibitors and FASTK family members.

Correlation analysis of genes

GEPIA (http://gepia.cancer-pku.cn/index.html) provides data on 9736 distinctive tumors and 8587 normal tissues from GTEx and TCGA.[29] Specifically, GEPIA can interpret the results of RNA-seq studies. There are a total of 60 498 different types of genes and 198 619 different types of isoforms, both of which are specified by the Gene and the Isoform Classes, respectively. Research on the link between FASTK expression and several immune cell biomarkers was performed based using the GEPIA database. The *x*-axis depicted the FASTK expression levels, whereas the *y*-axis showed expression levels for the other examined genes. Based upon the information from TIMER (http://cistrome.org/TIMER/), we further analyzed the expression of genes strongly correlated with FASTK expression in GEPIA.

Results

Differential expression of FASTKs in KIRC patients

Expression analyses on the FASTK family mRNA between 2 types of tissues (normal and tumorous) in pan-cancers using the TIMER database revealed a higher expression of FASTK,

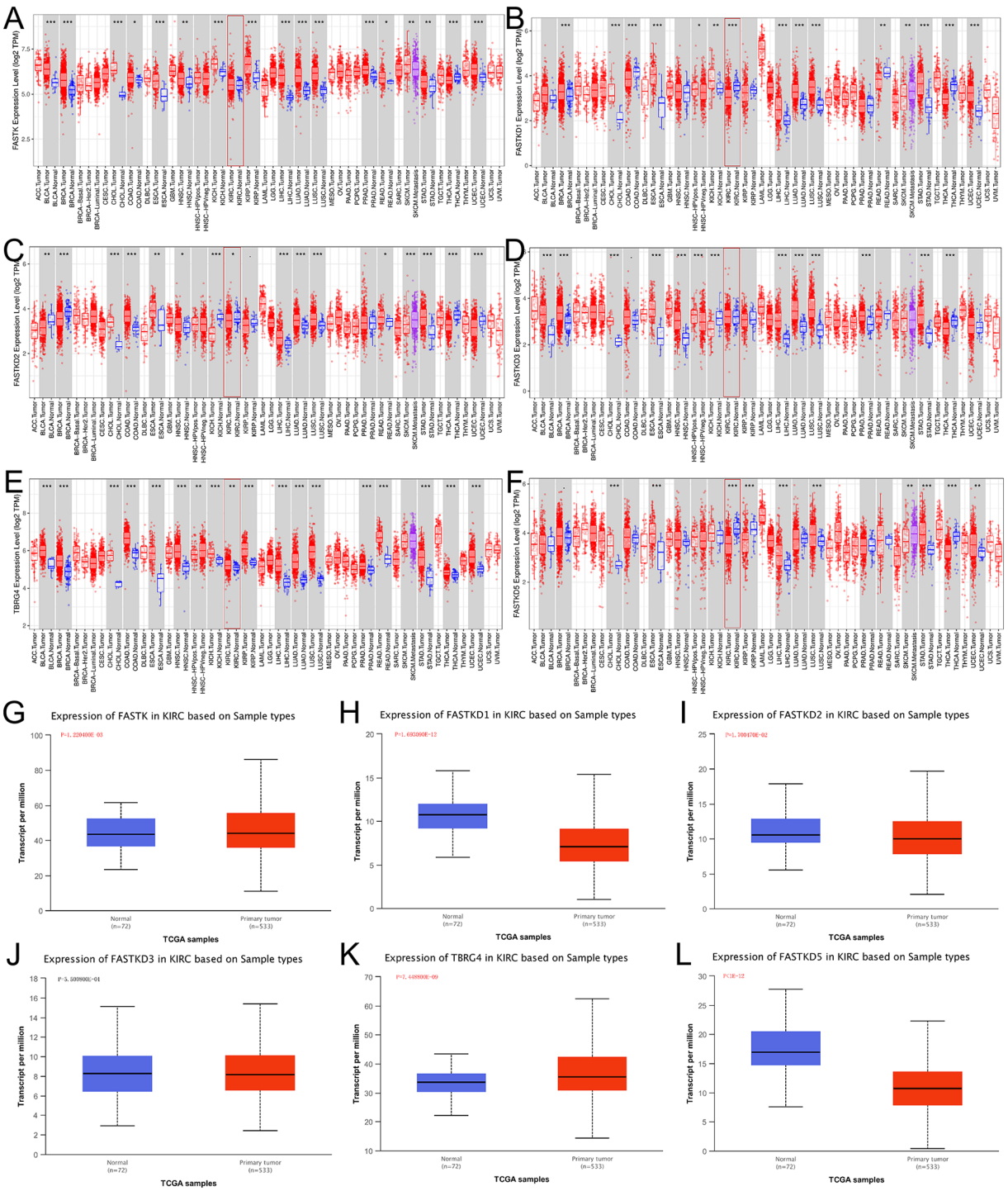

Figure 2. FASTK family mRNA expression in KIRC and normal tissues. In different tumor: (A) mRNA expression of FASTK. (B) mRNA expression of FASTKD1. (C) mRNA expression of FASTKD2. In pan-cancer: (D) mRNA expression of FASTKD3. (E) mRNA expression of TBRG4. (F) mRNA expression of FASTKD5. (G-L)Box plot illustrating elevated expression level of FASTK and TBRG4 and lowered expression level of FASTKD1, FASTKD2, and FASTKD5 in KIRC tissues in contrast with the level in normal tissues from UALCAN (*$P < .05$, **$P < .01$, ***$P < .001$ and ****$P < .0001$ and ns means no significance.).

FASTKD1, FASTKD2, FASTKD3, TBRG4, and FASTKD5 in many cancers, including CHOL, LIHC, LUSC (Figure 2A-F). Following that, we evaluated the mRNA expression level of FASTKs in KIRC and normal kidney samples based on the UALCAN dataset. The findings demonstrated that FASTK and TBRG4 were expressed at a higher level, whereas FASTKD1, FASTKD2, and FASTKD5 were expressed at a lower level in KIRC tissues in comparison to normal samples (Figure 2G-L). Additionally, the expression of the FASTK was linked to T stage, gender, Histologic grade, M stage, N stage, and Pathological stage (Table 1). Additionally, the gene expression of FASTKs in the GSE66271 were analyzed (Supplemental Figure S1). Downregulated FASTKD1, FASTKD3, TBRG4, and FASTKD5 protein in ccRCC tissue opposite to that in

Table 1. Association between the Clinical and pathological features of patients with KIRC and FASTK expression.

CHARACTERISTIC	LOW EXPRESSION OF FASTK	HIGH EXPRESSION OF FASTK	*P*
N	269	270	
T stage, n (%)			0.008
T1	157 (29.1%)	121 (22.4%)	
T2	28 (5.2%)	43 (8%)	
T3	81 (15%)	98 (18.2%)	
T4	3 (0.6%)	8 (1.5%)	
N stage, n (%)			0.040
N0	117 (45.5%)	124 (48.2%)	
N1	3 (1.2%)	13 (5.1%)	
M stage, n (%)			0.004
M0	239 (47.2%)	189 (37.4%)	
M1	29 (5.7%)	49 (9.7%)	
Pathologic stage, n (%)			0.005
Stage I	154 (28.7%)	118 (22%)	
Stage II	24 (4.5%)	35 (6.5%)	
Stage III	60 (11.2%)	63 (11.8%)	
Stage IV	30 (5.6%)	52 (9.7%)	
Histologic grade, n (%)			0.004
G1	5 (0.9%)	9 (1.7%)	
G2	133 (25%)	102 (19.2%)	
G3	104 (19.6%)	103 (19.4%)	
G4	25 (4.7%)	50 (9.4%)	
Serum calcium, n (%)			0.060
Elevated	3 (0.8%)	7 (1.9%)	
Low	111 (30.3%)	92 (25.1%)	
Normal	67 (18.3%)	86 (23.5%)	
Age, n (%)			0.763
⩽60	132 (24.5%)	137 (25.4%)	
>60	137 (25.4%)	133 (24.7%)	
Primary therapy outcome, n (%)			0.317
PD	5 (3.4%)	6 (4.1%)	
SD	5 (3.4%)	1 (0.7%)	
PR	1 (0.7%)	1 (0.7%)	
CR	57 (38.8%)	71 (48.3%)	
Gender, n (%)			0.005
Female	77 (14.3%)	109 (20.2%)	
Male	192 (35.6%)	161 (29.9%)	
Age, median (IQR)	61 (53, 72)	60 (51, 69)	0.421

normal tissue were observed on the basis of HPA and UALCAN data. For example, In KIRC cells, FASTKD1 protein cannot be detected, but the staining of FASTKD1 protein is medium in normal glomeruli cells and high in normal tubules cells (Antibody:HPA043719) (Figure 3A and B).

Relationship between FASTK family expression and clinical variables

Next, we compared the KIRC patients' clinical variables such as histologic grade, pathological stage, M stage, T stage, N stage, and OS event with the differentially expressed FASTKs. In KIRC, a positive correlation of lymph node metastasis and distant metastasis with a high FASTK mRNA expression was observed. Lower FASTKD1 mRNA expression in KIRC showed a negative correlation with T stage, pathological stage, histologic grade and distant metastasis. Higher TBRG4 mRNA expression in KIRC showed a positive relation to histologic grade, pathological stage, and distant metastasis. Lower FASTKD5 mRNA expression in KIRC was negatively correlated with T stage, pathological stage, and histologic grade (Figure 4A-F). As supported by these findings, FASTKs played a crucial function in KIRC carcinogenesis and development.

In addition, the outcomes of the univariate analysis showed a remarkable correlation between FASTK expression and clinical indicators, especially pathological grade [odds ratio (OR) = 1.487 (1.048-2.113), $P = .027$], histological grade [OR = 1.475 (1.048-2.079), $P = .026$], N stage [OR = 4.089 (1.279-18.161), $P = .031$], M stage [OR = 2.137 (1.308-3.547), $P = .003$], and gender [OR = 0.592 (0.413-0.847), $P = .004$] (Table 2). Our results did, illustrate a significant variation in terms of the T stage [OR = 1.423 (0.999-2.034), $P = .051$], age [OR = 0.935 (0.667-1.311), $P = .698$] (Table 2). These results manifested a relationship between FASTK expression and KIRC-related clinical variables.

Prognosis value of mRNA expression of FASTK in KIRC

After analyzing the data from the TCGA database, associations between FASTK mRNA expression and prognostic marker (OS) was shown in Figure 5A. Elevated FASTK mRNA expression level was related to worse OS outcomes [hazards ratio (HR), $P = .0054$]. Several clinical variables including FASTK mRNA expression, N stage, histological grade, M stage, age, T stage, pathological grade were all introduced to develop a clinically applicable risk score for KIRC prognosis (Figure 5B). The data indicated that the high-risk patients with KIRC exhibited elevated FASTK expression levels in contrast to the low-risk patients (Figure 5C). In addition, this study analyzed how the different groups were correlated with FASTK mRNA expression. Notably, a higher FASTK mRNA expression was observed in the T3-T4 stage [HR = 1.62 (1.10-2.40), $P = .016$], pathological grade III-IV [HR = 1.69

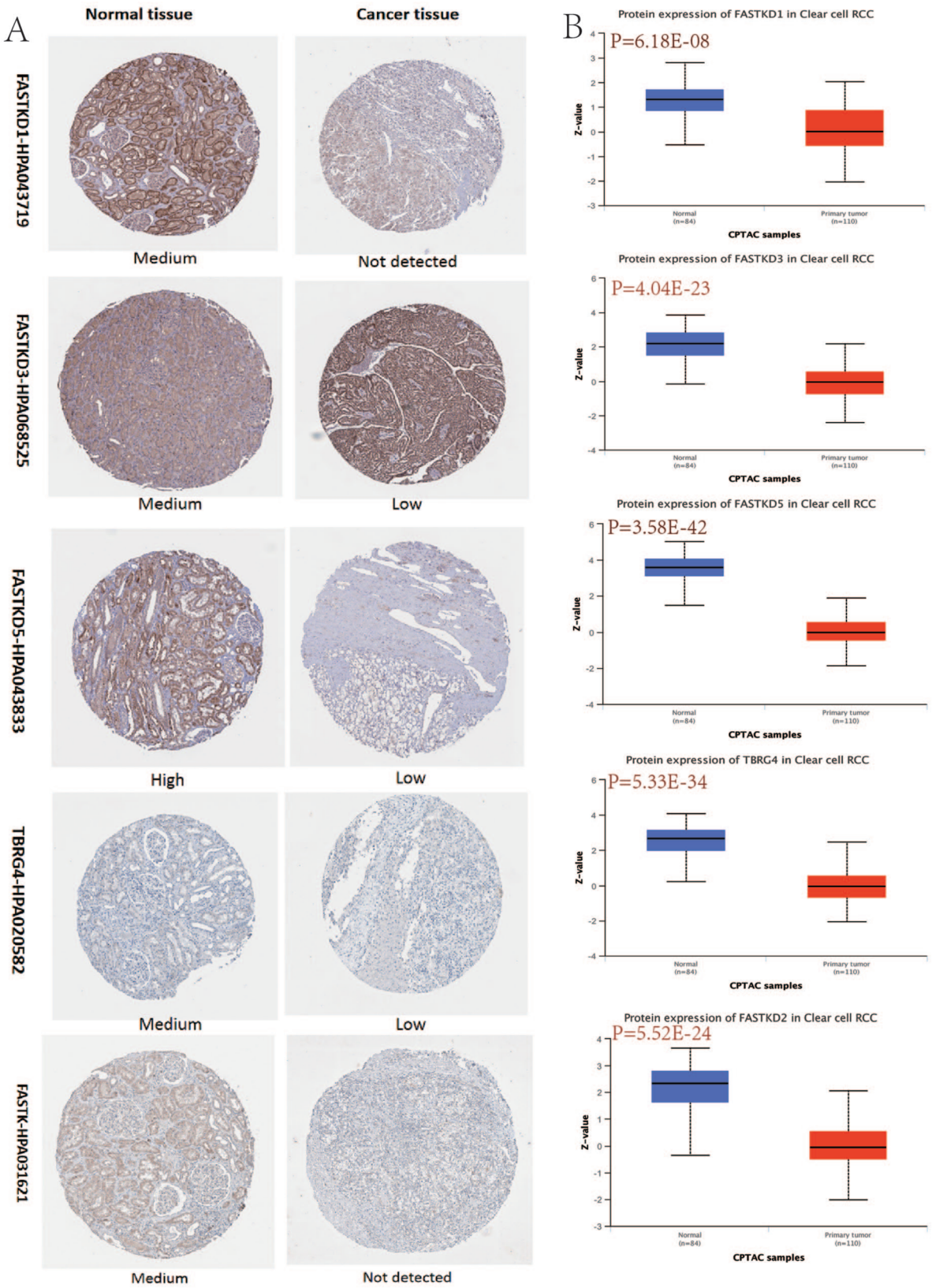

Figure 3. FASTK family protein expression in KIRC and normal tissues. (A) Comparison of the expression of FASTK family in KIRC tissues with those in non-cancerous tissue (100×) using the data from the Human Protein Atlas database. (B) Box plot illustrating lowered expression level of FASTKD1, FASTKD2, FASTKD3, TBRG4 and FASTKD5 in KIRC tissues in contrast with the level in normal tissues from UALCAN ($P < .05$.).

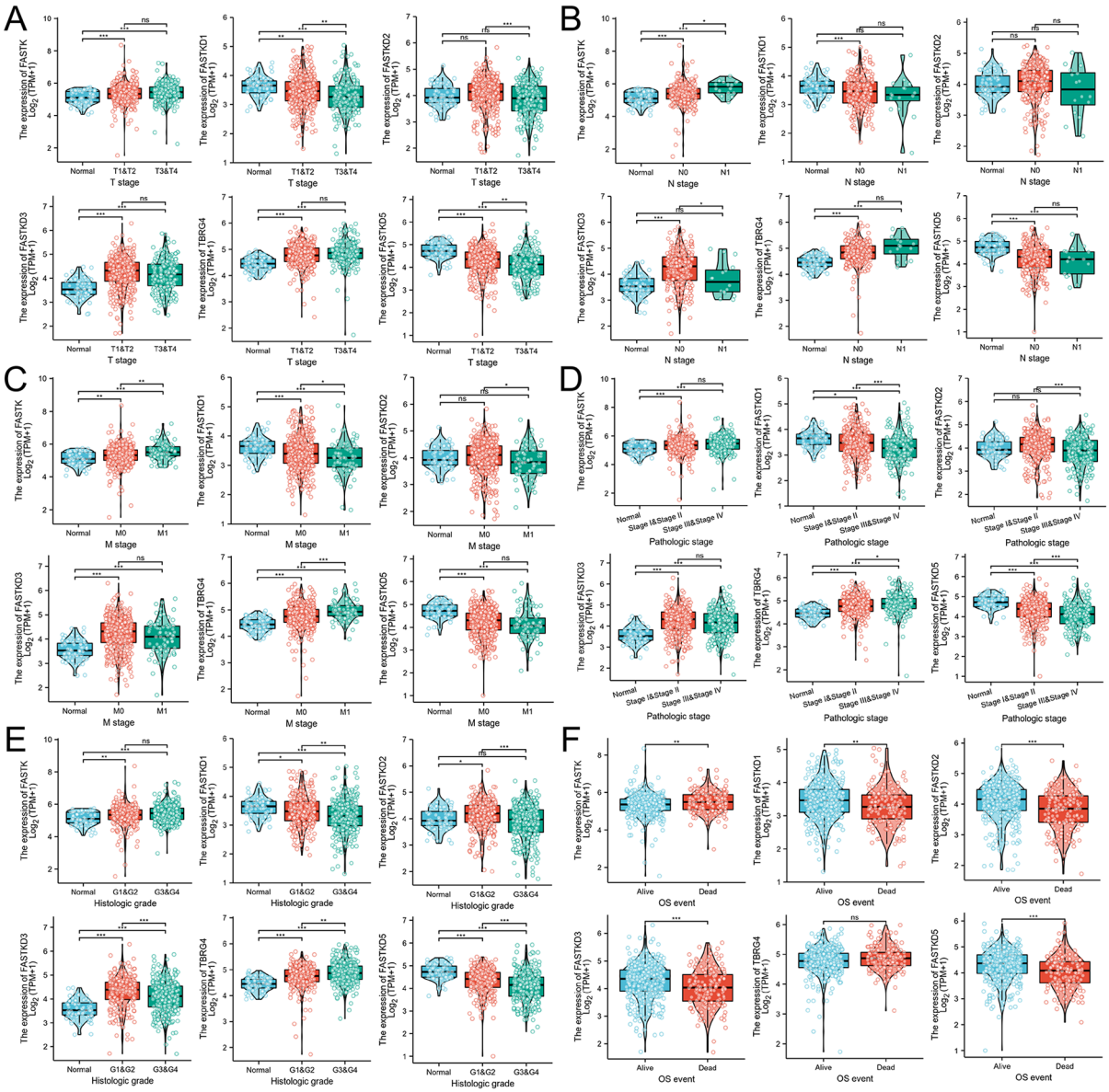

Figure 4. Differential expression of FASTK family members was correlated with histologic grade, pathologic stage, T stage, N stage, M stage, and OS event in KIRC patients. (A) The advanced T stage in KIRC patients was correlated with lower FASTKD1 and FASTKD5 expression. (B) Lymph node metastasis was correlated with higher FASTK expression. (C) Distant metastasis was correlated with higher FASTK and TBRG4 expression. (D) Advanced pathologic stage in KIRC patients was correlated with lower FASTKD1 and FASTKD5 expression. (E) Higher histologic grade was correlated with higher TBRG4 expression and lower FASTKD1, FASTKD5 expression. (F) Lower FASTKD1, FASTKD2, FASTKD3, FASTKD5 expression were correlated with Dead. (A) (*$P < .05$, **$P < .01$, ***$P < .001$ and ****$P < .0001$ and ns means no significance.).

Table 2. Analyzing FASTK expression using a logistic regression model.

CHARACTERISTICS	TOTAL (N)	ODDS RATIO (OR)	*P* VALUE
T stage (T4&T3 vs T1&T2)	539	1.423 (0.999-2.034)	.051
N stage (N1 vs N0)	257	4.089 (1.279-18.161)	.031
M stage (M1 vs M0)	506	2.137 (1.308-3.547)	.003
Histologic grade (G3&G4 vs G1&G2)	531	1.475 (1.048-2.079)	.026
Age (>60vs ⩽60)	539	0.935 (0.667-1.311)	.698
Pathologic stage (Stage III & Stage IV vs Stage I & Stage II)	536	1.487 (1.048-2.113)	.027
Gender (male vs female)	539	0.592 (0.413-0.847)	.004

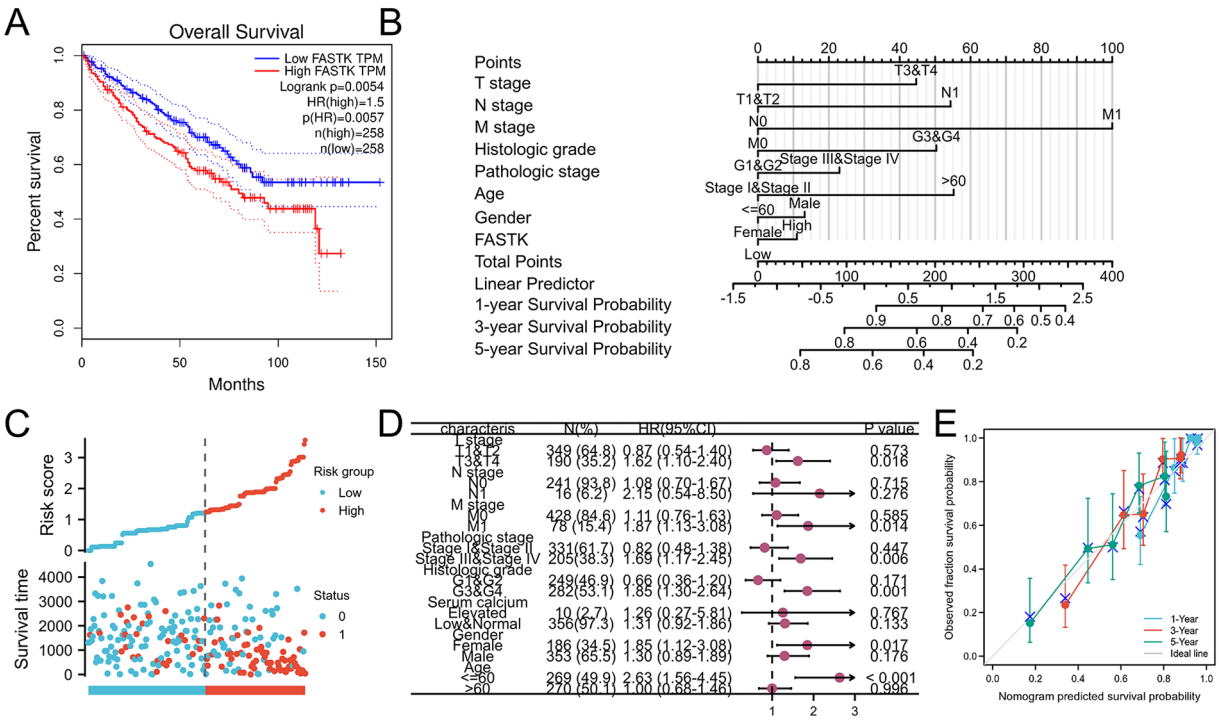

Figure 5. Prognostic analysis of FASTK mRNA expression. (A) When comparing patients with high- and low-FASTK expression, the former group had a worse prognosis, including shorter overall survival (OS). (B) Multivariate analysis nomogram premised on clinicopathological factors associated with FASTK expression. (C) Risk scores and survival status of FASTK gene in KIRC patients. (D) Prognostic prediction using FASTK expression in several clinical characteristic types (OS). (E) Multi-factor Cox regression analysis was conducted to examine the accuracy of the model's predictions, and the results are depicted in the calibration curve.

(1.17-2.45), $P = .006$], and histological grade G3-G4 [HR = 1.85 (1.30-2.64), $P = .001$] (Figure 5D). We simultaneously used a calibration curve to examine the predictive effectiveness of the model (Figure 5E). The accuracy in predicting the survival rates of patients over 3 and 5 years could be improved based on FASTK mRNA expression. The predicted results showed a high consistency with the observed results, indicating a strong link between KIRC prognostic outcomes with mRNA expression of FASTK, as supported by the calibration curve.

Prognostic significance of the mRNA expression of FASTKs in KIRC patients

Kaplan-Meier analysis was plotted to examine the link between the various FASTKs and clinical outcomes, hoping to provide novel insight into the role of differentially expressed FASTKs in the onset and progression of KIRC. Figure 6 displays DFS, OS, and PFI curves. Patients with KIRC who had high-expressed FASTK had a considerably shorter DFS [HR = 1.75 (1.19-2.58), $P = .005$], short OS [HR = 1.45 (1.07-1.95), $P = .016$] (Figure 6A). Similarly, elevated expression level of TBRG4 in patients with KIRC was significantly related to shorter DFS duration [HR = 1.85 (1.25-2.73), $P = .002$], shorter OS [HR = 1.35 (1.00-1.83), $P = .048$] and shorter PFI [HR = 1.49 (1.09-2.04), $P = .013$] (Figure 6E). Other FASTK family members, including low mRNA expression of FASTKD1, FASTKD2, FASTKD3, and FASTKD5, were closely correlated with the dismal OS and DSS rates in KIRC patients (Figure 6B-D and F). We also used GEPIA 2.0 database to visualize the survival heatmap of FASTK family genes in multiple pan-cancers (Supplemental Figure S2).

Gene alterations, expressions, and interaction analyses of FASTKs in KIRC patients

We applied the web-based resource cBioPortal to analyze FASTK gene mutations in KIRC patients. Mutation modifications were widely present in KIRC samples, and 2 or multiple alterations were found in several analyses of KIRC. In total, 61 out of 1744 KIRC patient samples (3%) showed mutated FASTKs. Also, FASTK, FASTKD1, FASTKD2, FASTKD3, TBRG4 and FASTKD5 were altered in 0.7-, 0.7-, 0.7-, 0.9-, 0-, and 0.8% of the KIRC samples, respectively (Figure 7A). We also displayed the 3D protein structure of each FASKT gene (Supplemental Figure S3). Meanwhile, the correlation between FASTKs mRNA and copy number alteration (CNA) was analyzed (Supplemental Figure S4).

Additionally, STRING was applied to analyze the PPI network of the differentially expressed FASTKs for the purpose of discovering possible links between them (Figure 7B). Some of the biological processes related to these FASTKs

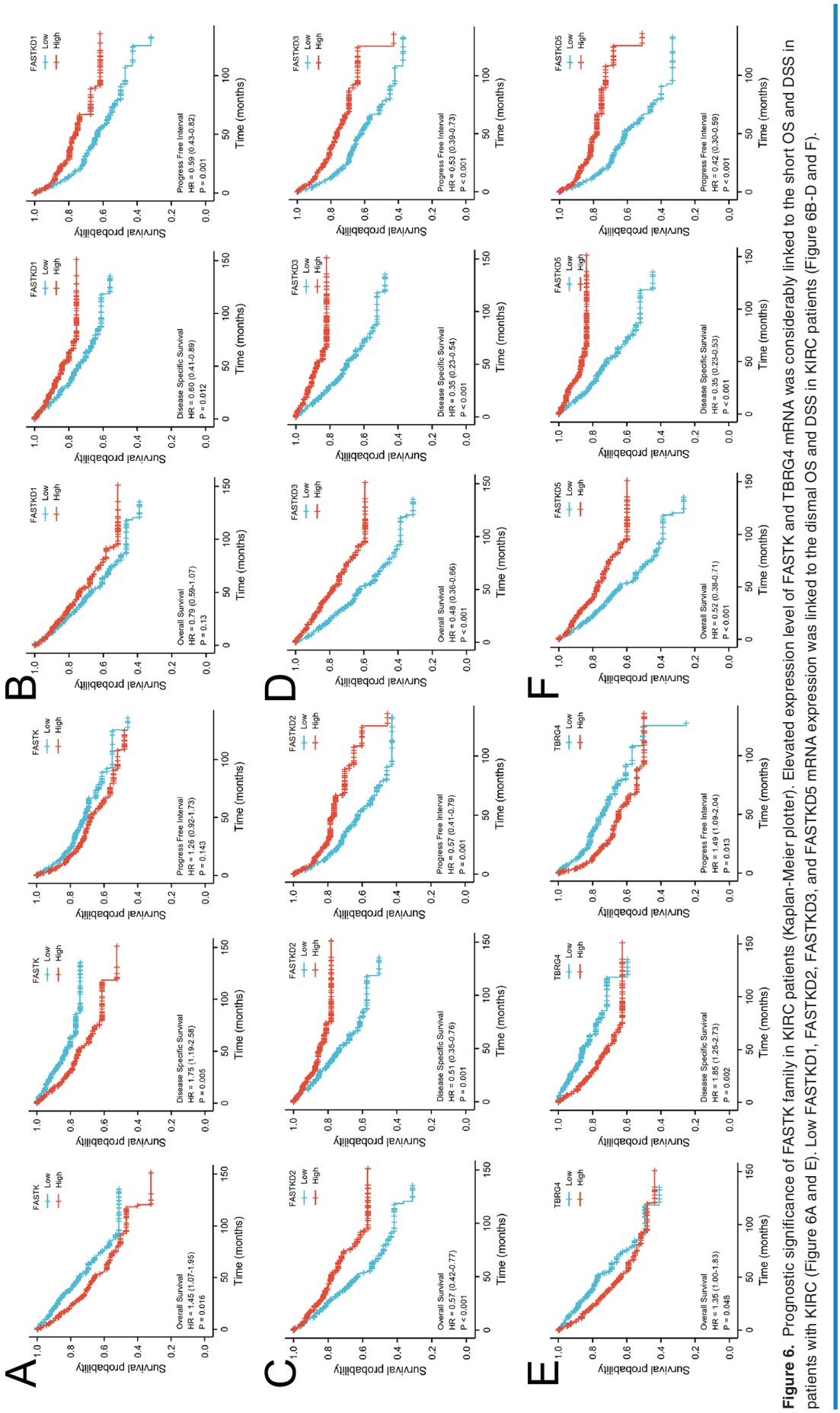

Figure 6. Prognostic significance of FASTK family in KIRC patients (Kaplan-Meier plotter). Elevated expression level of FASTK and TBRG4 mRNA was considerably linked to the short OS and DSS in patients with KIRC (Figure 6A and E). Low FASTKD1, FASTKD2, FASTKD3, and FASTKD5 mRNA expression was linked to the dismal OS and DSS in KIRC patients (Figure 6B-D and F).

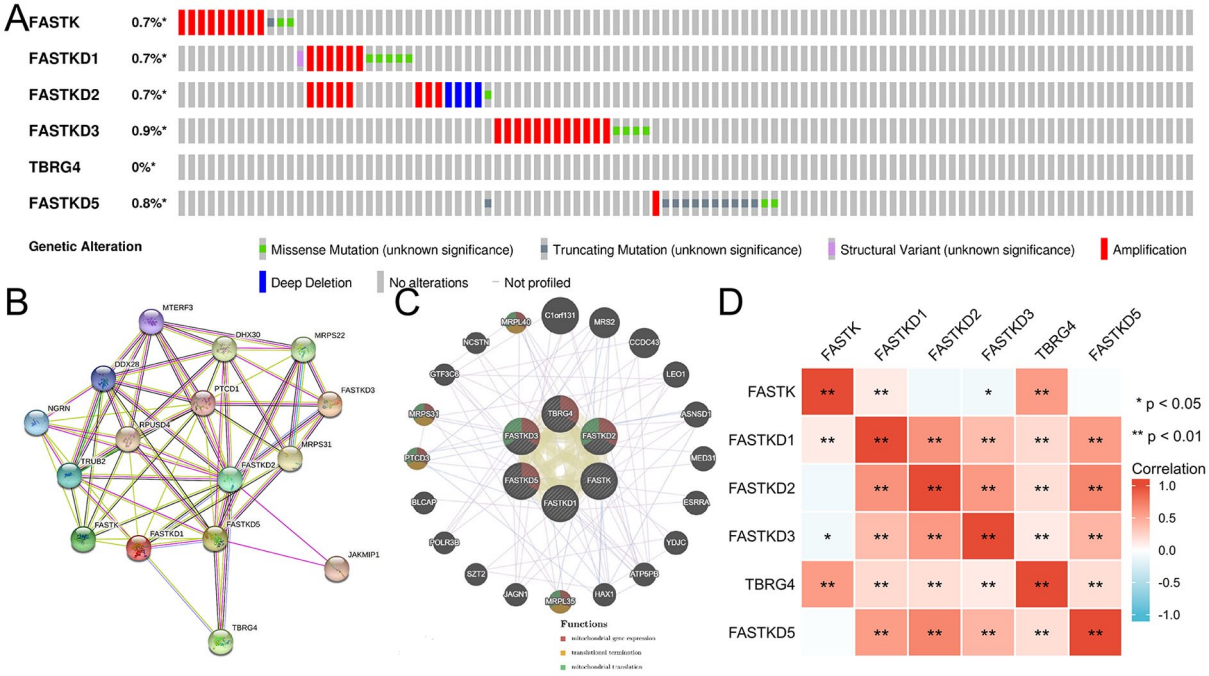

Figure 7. Gene modifications of FASTK family members and networks of gene-gene and protein-protein interactions among FASTK family genes. (A) The cBioPortal diagram offers a summary of the genomic changes that were found in the FASTK family in KIRC data derived from TCGA. (B) The STRING program was employed to generate a network graphic depicting the interplay between proteins that are encoded by members of the FASTK gene family. (C) GeneMANIA was used to design the gene network that is related to the FASTK gene family. (D) The correlation analysis among FASTK family members. *$P < .05$; **$P < .01$.

with differential expression levels included the assembly of mitochondrial large ribosomal subunits, the modulation of mitochondrial mRNA stability, and the metabolism of mitochondrial RNA. The findings from GeneMANIA showed that the functions of FASTKs with differential expression and their corresponding molecules (including MRS2, CCDC43, LEO1, ASNSD1, MED31, ESRRA, YDJC, ATP5PB, and HAX1) were primarily associated with mitochondrial gene expression, translational termination, and mitochondrial translation (Figure 7C). Subsequently, correlation analysis on the mRNA levels of FASTK family using TCGA data revealed that FASTK showed a negative correlation with FASTKD3, whereas most FASTK family members were significantly positively correlated with each other (Figure 7D).

Genes overlapped by the correlated genes of each FASTK gene were identified. As shown in Supplemental Figure S5, LRPPRC and PNPT1 were correlated with each FASTK family gene.

Functions of the FASTKs family in KIRC

CancerSEA was introduced to perform a single-cell analysis to probe into the roles of FASTKs family in KIRC. The findings showed that FASTK was positively correlated with DNA damage in KIRC cells (correlation coefficient (r) = .35, $P < .01$, Figure 8A). FASTKD1 was positively related to differentiation in KIRC cells ($r = .72$, $P < .01$, Figure 8B). FASTKD2 was inversely related to proliferation in KIRC cells ($r = -.42$, $P < .01$, Figure 8C). FASTKD5 was inversely related to invasion and EMT in KIRC cells ($r = -.37$, $P < .01$, Figure 8D).

Association of the expression of FASTKs family members with immune infiltration

Applying the findings of immune infiltration, the expression of members belonging to the FASTKs family and the presence of Tumor-infiltrating immune cells (TIICs) in KIRC were examined in the module of the "Xiantao tool." It was shown that Tgd, macrophages, Tcm, and Mast cells infiltration levels were inversely associated with FASTK expression. Furthermore, TReg, Cytotoxic cells, NK CD56dim cells, and Th1 cells infiltration level were inversely linked to FASTKD1. Infiltration levels of NK CD56dim cells, Cytotoxic cells, TReg, CD8+ T cells were inversely correlated with FASTKD2 expression. On the contrary, infiltration of NK CD56dim cells, Cytotoxic cells, B cells, and TReg was inversely related to FASTKD3 expression. The infiltration of pDC, NK cells, Tgd and Mast cells was negatively correlated to TBRG4 expression. FASTKD5 was negatively related to the infiltration of pDC, NK CD56dim cells, Cytotoxic cells, and TReg (Figure 9A-F).

The TIMER database was employed to examine the associations of FASTK with multiple immune marker sets for investigating the potential function of FASTK in the infiltration status of distinct immunocytes in KIRC. These immune cells comprised natural killer (NK) cells, CD8+T lymphocytes, monocytes, neutrophils, M1/M2 macrophages, and

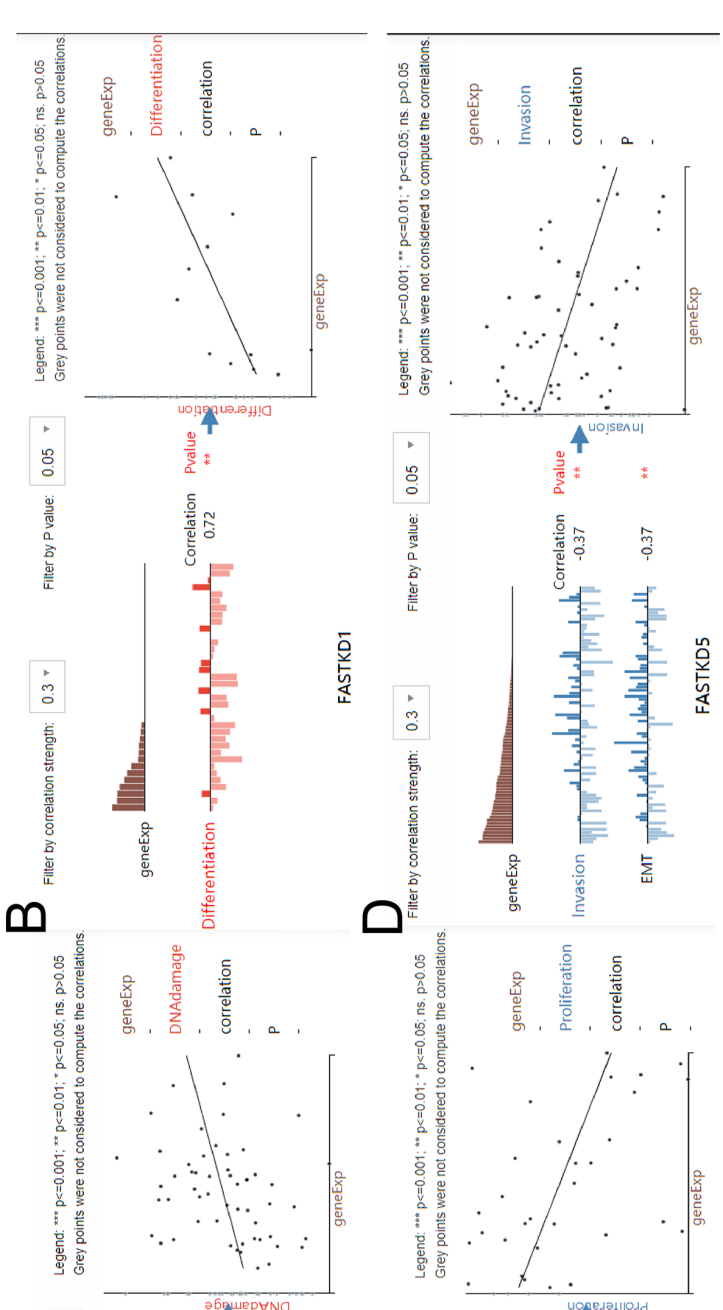

Figure 8. The functions of the FASTK family in KIRC. (A) CancerSEA data showed a positive link between FASTK and the regulation of DNA damage. (B) CancerSEA results showed that FASTKD1 has a positive link to the modulation of differentiation. (C) CancerSEA data showed that FASTKD2 has a negative link to the modulation of differentiation. (D) FASTKD5 was shown to have a negative association with Invasion and EMT regulation, according to data gathered from CancerSEA.

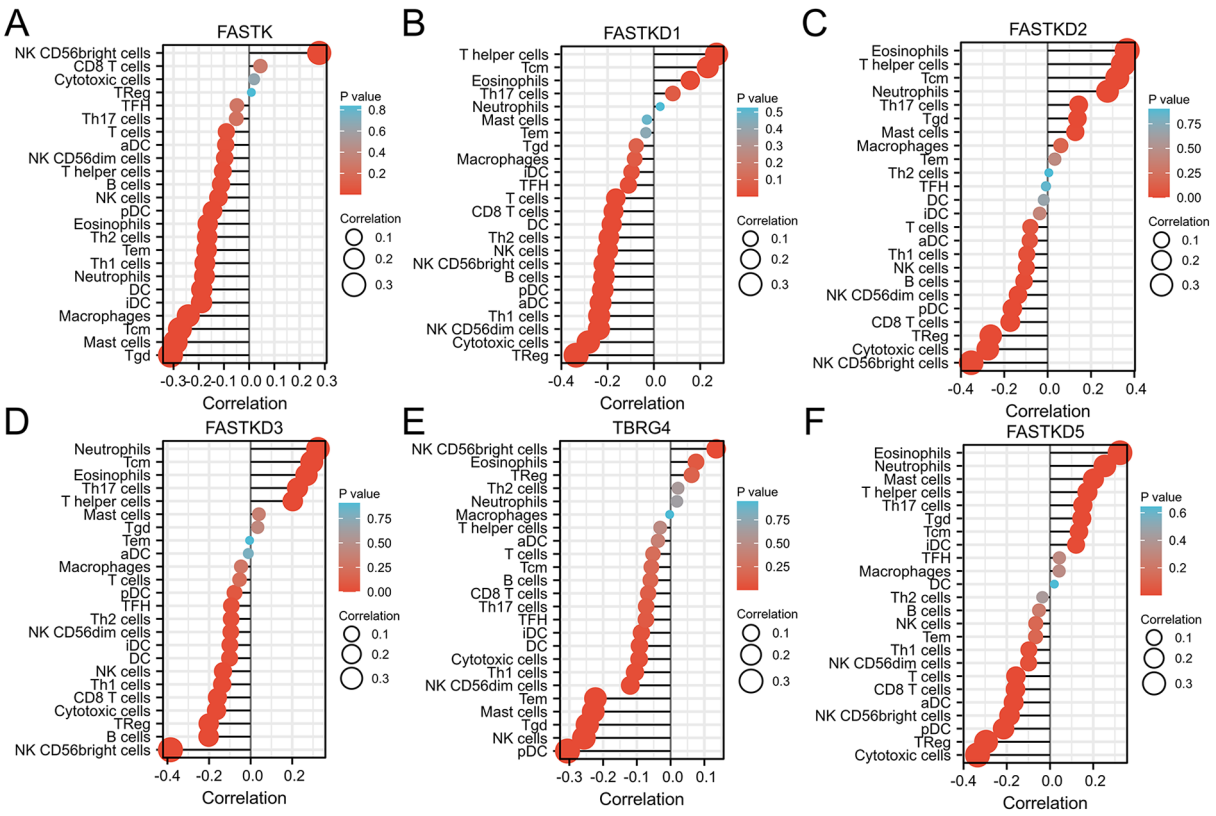

Figure 9. Relationship between FASTK family expression in KIRC and immune cell infiltration. (A) Infiltration of Tgd, macrophages, Tcm, and Mast cells were inversely linked to FASTK expression. (B) Infiltration of TReg, Cytotoxic cells, NK CD_{56}dim cells, and Th1 cells infiltration as inversely linked to FASTKD1. (C) Infiltration of CD8+ T cells, NK CD_{56}dim cells, Cytotoxic cells, TReg were inversely linked to FASTKD2. (D) Infiltration of NK CD_{56}dim cells, B cells, Cytotoxic cells, and TReg were inversely linked to FASTKD3 expression. (E) Infiltration of pDC, NK cells, Tgd and Mast cells were negatively linked to TBRG4 expression. (F) There was a negative correlation between FASTKD5 and the infiltration levels of pDC, NK CD_{56}dim cells, Cytotoxic cells, and TReg.

T cells (general), tumor-associated macrophages (TAMs), B cells, dendritic cells (DCs), (Table 3). In addition, many types of functional T cells, such as T helper type 1 (Th1), Th2, Th9, Th17, and Th22, T-regulatory cells (Treg), T follicular helper cells (Tfh), exhausted T cells, were investigated. A correlation was found between FASTK expression in KIRC and the levels of most immune sets such as various T cells, DCs, M1/M2 macrophages, and TAMs. We also evaluated the association of FASTK expression with the above mentioned markers of T-cell exhaustion, monocytes, M2 macrophages, and TAMs using the GEPIA database, and found that the findings were consistent with those in TIMER (Supplemental Table S1).

Diagnosis significance of FASTK family in KIRC

Receiver operating characteristic (ROC) curve was analyzed to further assess the diagnostic significance of FASTK family members. As can be observed from the area under curve (AUC) of FASTK, FASTKD1, FASTKD2, FASTKD3, TBRG4 and FASTKD5 was 0.660, 0.646, 0.540, 0.794, 0.795 and 0.775, respectively (Figure 10A-F). FASTKD3, TBRG4 and FASTKD5 might be potential diagnostic biomarkers for KIRC patients due to the high performance discriminating KIRC from the control samples (AUC > 0.7).

DNA methylation analysis of FASTK family in KIRC

FASTK Family methylation was examined based on UALCAN database. As shown in Figure 11A, the DNA methylation of FASTK showed a dramatically lower level in KIRC tissues compared with normal samples, and that of TBRG4 and FASTKD5 were similarly lower in KIRC tissues (Figure 11E and F).In contrast, the DNA methylation of FASTKD2 and FASTKD3 in KIRC tissues was significantly higher (Figure 11C and D).

Relationship between FASTKs family expression and immune check inhibitors

In the last 20 years, innovative ICIs have made significant achievements to advance our knowledge of how the human immune system works. Therefore, the association of FASTKs family expression with ICIs was examined. The TISIDB was selected to study the relation between FASTK expression and immune-suppressive properties. The findings demonstrated that CD274 was positively related to FASTKD1, FASTKD2, FASTKD3, and FASTKD5. PDCD1 was positively related to FASTK and TBRG4. LAG3 was inversely correlated with FASTKD5 but positively correlated with FASTK and TBRG4. PDCD1LG2 was inversely associated with FASTK and

Table 3. Correlation analysis between FASTK and related genes and markers of immune cells in Tumor Immune Estimation Resource (TIMER).

DESCRIPTION	GENE MARKERS	KIRC			
		NONE		PURITY	
		COR	*P*	COR	*P*
CD8+ T cell	CD8A	−.009	.828	.005	.918
	CD8B	.023	.598	.043	.359
T cell (general)	CD3D	−.002	.957	.007	.877
	CD3E	−.019	.669	−.013	.788
	CD2	−.042	.330	−.038	.417
B cell	CD19	.028	.524	.020	.673
	CD79A	−.059	.171	−.057	.224
Monocyte	CD86	−.161	***	−.175	***
	CD115 (CSF1R)	−.089	*	−.091	*
TAM	CCL2	−.048	.269	−.037	.430
	CD68	−.191	***	−.189	***
	IL10	−.181	***	−.191	***
M1 macrophage	INOS (NOS2)	−.245	***	−.221	***
	IRF5	.388	***	.417	***
	COX2(PTGS2)	−.270	***	−.312	***
M2 macrophage	CD163	−.311	***	−.322	***
	VSIG4	−.152	***	−.144	**
	MS4A4A	−.284	***	−.298	***
Neutrophils	CD66b	−.040	.363	−.036	.439
	CD11b (ITGAM)	−.037	.398	−.024	.614
	CCR7	−.172	***	−.171	***
Natural killer cell	KIR2DL1	−.110	*	−.108	*
	KIR2DL3	−.067	.121	−.041	.381
	KIR2DL4	−.015	.730	−.007	.883
	KIR3DL1	−.134	**	−.100	*
	KIR3DL2	−.035	.415	−.020	.665
	KIR3DL3	.003	.945	.010	.838
	KIR2DS4	−.052	.234	−.054	.246
Dendritic cell	HLA-DPB1	−.102	*	−.092	*
	HLA-DQB1	−.042	.332	−.023	.617
	HLA-DRA	−.191	***	−.188	***
	HLA-DPA1	−.184	***	−.188	***
	BDCA-1(CD1C)	−.128	**	−.111	*
	BDCA-4(NRP1)	−.414	***	−.402	***
	CD11c (ITGAX)	.186	***	.178	***

(Continued)

Table 3. (Continued)

DESCRIPTION	GENE MARKERS	KIRC			
		NONE		PURITY	
		COR	*P*	COR	*P*
Th1	T -bet (TBX21)	.005	.903	.023	.625
	STAT4	−.063	.147	−.061	.194
	STAT1	−.161	***	−.152	**
	IFN-γ (IFNG)	.064	.139	.085	.0687
	TNF-α (TNF)	.044	.311	.051	.277
Th2	GATA3	.060	.164	.089	.0576
	STAT6	.036	.403	.071	.129
	STAT5A	.024	.583	.044	.341
	IL13	.109	*	.105	*
Tfh	BCL6	−.140	**	−.117	*
	IL21	−.057	.190	−.063	.175
Th17	STAT3	−.296	***	−.267	***
	IL17A	−.034	.435	−.062	.182
Treg	FOXP3	.005	.914	.008	.858
	CCR8	−.125	**	−.122	**
	STAT5B	−.215	***	−.185	***
	TGFβ (TGFB1)	−.202	***	−.184	***
T cell exhaustion	PD-1 (PDCD1)	.158	***	.172	***
	PDL1 (PDCD1LG2)	−.266	***	−.283	***
	CTLA4	.086	*	.103	*
	LAG3	.139	**	.143	**
	TIM-3 (HAVCR2)	−.045	.304	−.009	.851
	GZMB	.029	.509	.047	.318

Abbreviations:Cor, R value of Spearman's correlation; None, correlation without adjustment; Purity, correlation adjusted by purity; TAM, tumor-associated macrophage; Tfh, Follicular helper T cell; Th, T helper cell; Treg, regulatory T cell.
*$P < .05$; **$P < .01$; ***$P < .001$.

positively associated with FASTKD1, FASTKD2, FASTKD3, and FASTKD5. In addition, FASTK was also positively linked to CD160, CD244, and HAVCR2 (Figure 12A-F).

Discussion

Recent studies have given considerable attention to FASTK family members in cancers, while the biological significance and prognosis value of FASTK family members, the role of FASTKs in KIRC remained to be carefully studied. In terms of expression, mutation, connections with immune cell infiltration and immunological checkpoints, diagnostic and prognostic use, and DNA methylation, this study performed a thorough analysis of the FASTK family in KIRC for the first time. In KIRC tumor tissue, UALCAN revealed elevated levels of the 2 FASTK members, namely FASTK and TBRG4. The expression levels of 3 FASTK family members, FASTKD1, FASTKD2, and FASTKD5, were considerably downregulated in KIRC tumor tissue than healthy tissue. Furthermore, the expression of 2 FASTK family including FASTKD2 and FASTKD5 were linked to all the 4 KIRC stages and was associated with sex, histologic grade, M stage, and nodal metastatic status. To validate the function of FASTK family members as a viable KIRC biological marker, additional research is required. Kaplan-Meier analysis revealed that upregulated FASTK and TBRG4 as well as downregulated FASTKD1, FASTKD2, FASTKD3, and FASTKD5 were linked to dismal prognosis in

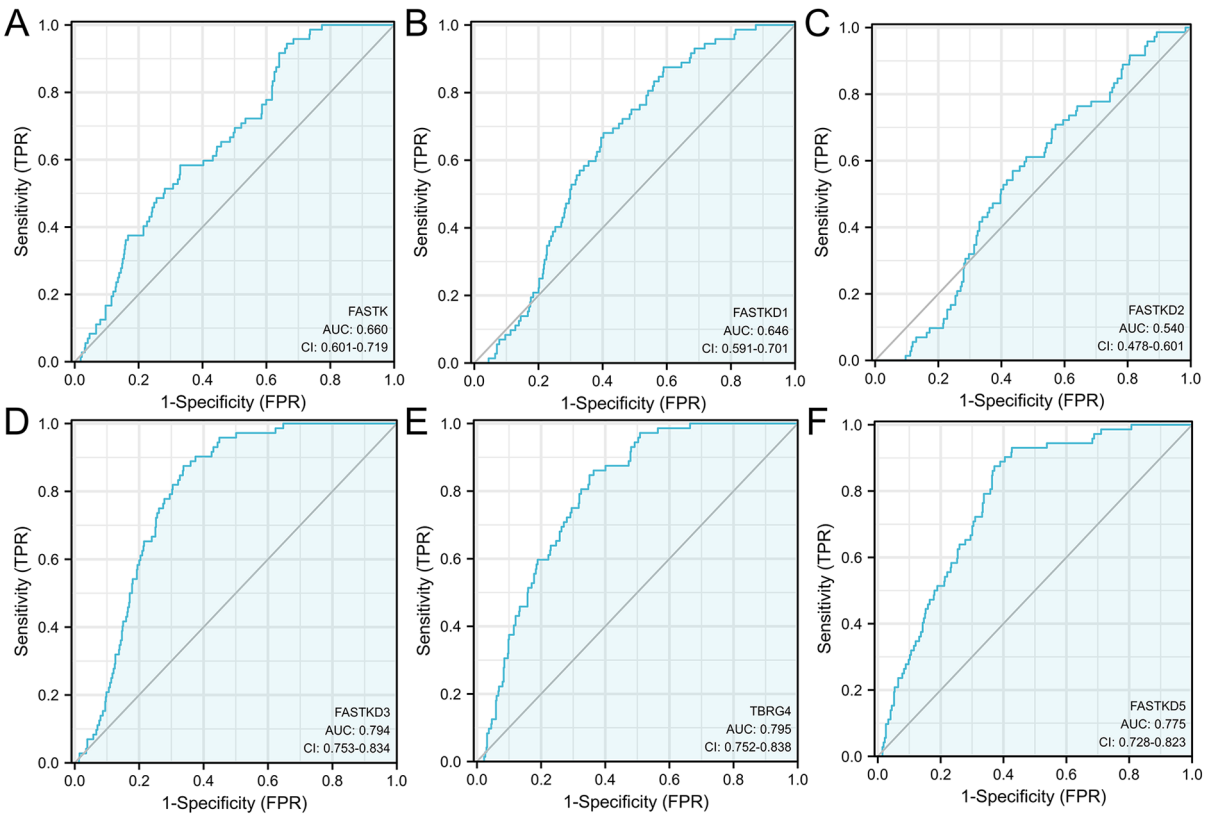

Figure 10. ROC analysis of FASTK family members in KIRC. (A-F) FASTKD3, TBRG4, FASTKD5 shows promising discrimination power between normal and ccRCC tissues (AUC > 0.7).

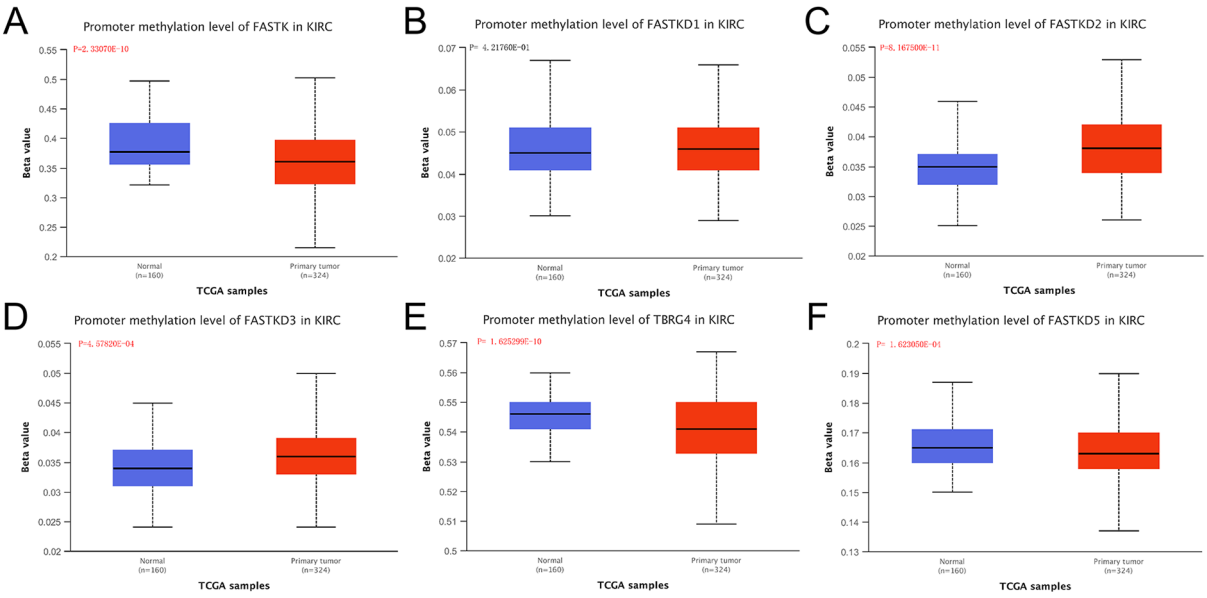

Figure 11. The DNA methylation analysis of FASTK family members in KIRC. The promoter methylation level of (A) FASTK in KIRC tissues; (B) FASTKD1 in KIRC tissues; (C) FASTKD2 in KIRC tissues; (D) FASTKD3 in KIRC tissues; (E) TBRG4 in KIRC tissues; (F) FASTKD5 in KIRC tissues.

KIRC. However, in other cancers such as pancreatic cancer, FASTKD2 expression was upregulated and was associated with an unfavorable prognosis.[16] Nonetheless, additional research is required to confirm this finding.

FASTK family with its potential oncogenic role in KIRC encouraged us to further probe into the correlation of the expression of FASTK family genes with clinical parameters in KIRC. Here, KIRC patients accompanied with distant

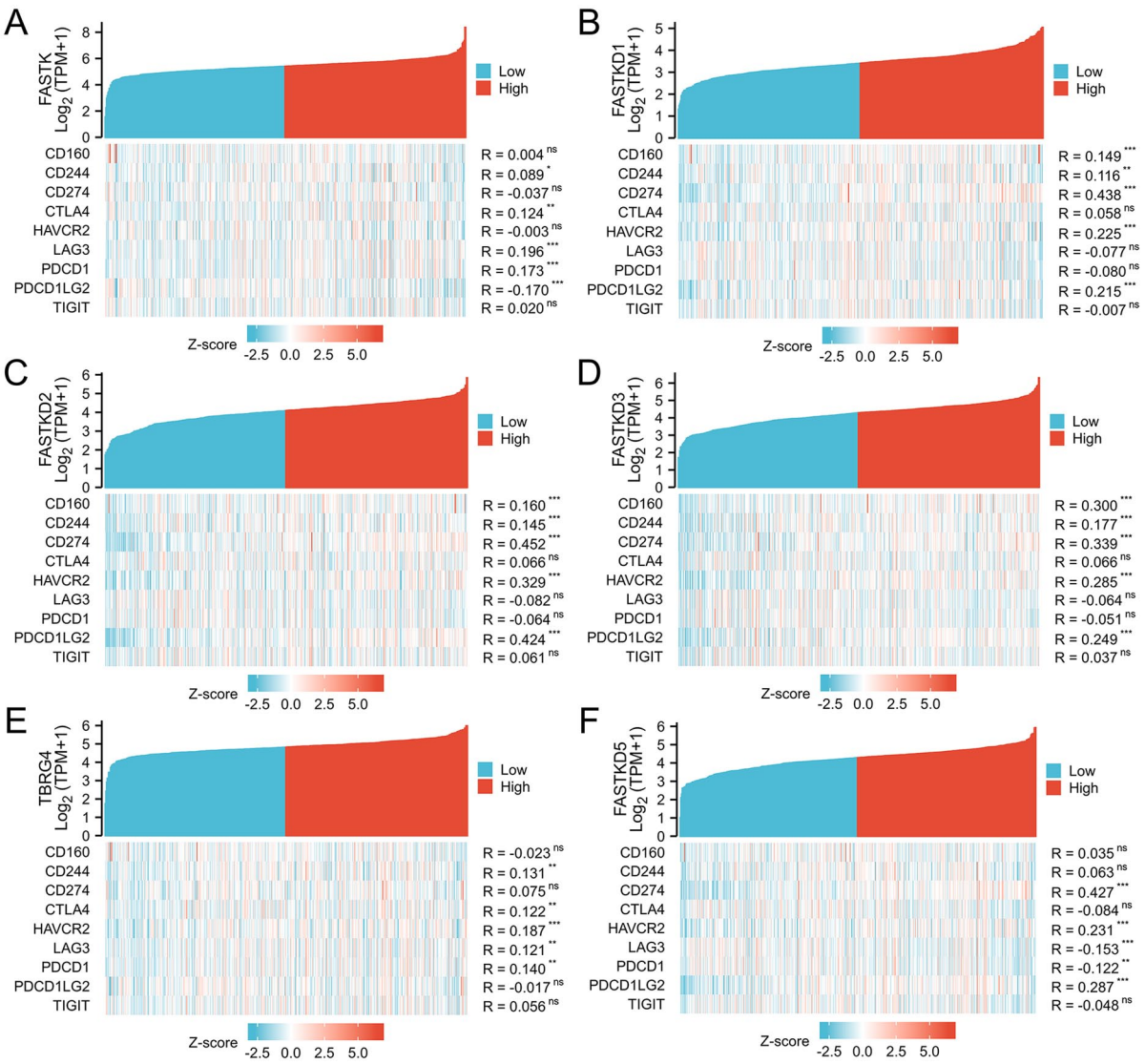

Figure 12. Immune checkpoints and the expression of members of the FASTK family in KIRC. (A) FASTK expression was shown to be favorably linked to CD244, CTLA4, and LAG3, PDCD1 and inversely linked to PDCD1LG2. (B) Expression of FASTKD1 was positively linked to CD160, CD244, CD274, HAVCR2, and PDCD1LG2. (C) HAVCR2, CD274, CD160, CD244, and PDCD1LG2 were favorably linked to FASTKD2 expression. (D) The expression of FASTKD3 was associated favorably with CD160, CD244, CD274, PDCD1LG2 and HAVCR2. (E) The expression levels of TBRG4 were favorably linked to CD244, CTLA4, LAG3, HAVCR2, and PDCD1. (F) FASTKD5 expression was positively linked to CD274, PDCD1LG2, and HAVCR2, and inversely linked to PDCD1 and LAG3.

metastasis or lymph node metastasis often showed a higher level of FASTK. FASTKD1 level was observed to be lower in those with high-grade, late-pathological stage, late-T stage. Similarly, the high-grade, late-T stage, and late-pathological stage and KIRC patients were normally accompanied with a lower FASTKD5 level. The TBRG4 level was higher in the high-grade and late-pathological stage KIRC patients with distant metastasis. The development process of tumorigenesis involves the important epigenetic alteration of DNA methylation. Hypomethylation often takes place on the promoter of proto-oncogenes to activate and induce cell carcinogenesis. This study observed that compared with normal tissues, FASTK, TBRG4 and FASTKD5 showed a significantly lower level of the promoter methylation in KIRC tissues.

This study used GeneMANIA, STRING, and CancerSEA to analyze the data and draw inferences on the functions of FASTK family. The FASTK family was shown to regulate DNA damage, cell differentiation, proliferation, invasion, and EMT in a CancerSEA study. This suggested that the FASTK family could affect the onset and progression of KIRC. Relevant research evidence has demonstrated alterations in FASTKD2 expression in human pancreatic ductal adenocarcinoma (PDAC), and that upregulation of FASTKD2 has a tumorigenic influence on PDAC in vitro.[16] To determine whether FASTK genes have the potential to serve as treatment targets, it will be promising to investigate the effect of knocking down FASTK genes on the proliferation and migration of the cell lines originating from various kinds of malignancies.

Additionally, the immune infiltration in KIRC was shown to be closely linked to the FASTK family in this study. Immune cell infiltration into the tumor microenvironment (TME) is crucial in tumor onset, progression, and metastasis.[30,31] We found that FASTK expression exhibited a close negative relationship with Tgd cells ($r=-.312$, $P<.001$), Mast cells ($r=-.292$, $P<.001$), Tcm cells ($r=-.275$, $P<.001$) and Macrophages ($r=-.242$, $P<.001$). This research also validated the link between FASTK expression and tumor-infiltrating lymphocyte (TIL)-related marker genes in KIRC. Furthermore, correlation analysis revealed that multiple FASTK members, including FASTKD1, FASTKD2, FASTKD3, and FASTKD5, were adversely correlated with the accumulation of these tumor-infiltrating immune cells (TIICs), in particular with Treg cells, which are T cell subsets with negative regulatory functions and are essential for preserving immunological homeostasis.[32] The activation and function of effector T cells is suppressed and immune escape is promoted by enriched and infiltrated Treg cells surrounding the tumor cells in the microenvironment of KIRC.[33] Poor survival outcomes in KIRC are usually related to high levels of tumor-infiltrating Treg cells.[34] Taken together, the immunosuppression from Treg cells could be suppressed and the efficacy of immunotherapy could be increased by targeting FASTKD1, FASTKD2, FASTKD3, and FASTKD5.

In addition, markers of T-cell exhaustion (LAG3, PD-1, CTLA4) were substantially linked to FASTK upregulation. To a large extent, immunotherapy relies on immune checkpoint blockade. It is known that PD1/PD-L1, a crucial component of the immune checkpoint, modulates the activity of TILs. Patients suffering from multiple cancers, including mRCC, are routinely administered with PD1/PD-L1 checkpoint blockade medication.[35] However, numerous studies have shown that PD-1 has a crucial function in the tolerance of tumor antigens, as a result, some patients receiving PD1 treatment have less therapeutic benefits.[36,37] Hence, enhancing the tumor cell responsiveness to ICIs and cytokines should be regarded as a top priority. Based on these findings, targeting FASTK could be an option for developing more efficient immunotherapies. Although FASTK is involved in the recruitment and modulation of TILs in KIRC, additional research is therefore encouraged to study both the molecular mechanism underlying its modulation of the TME and the function of such a modulation.

Our investigation had some limitations. All the data evaluated in this study were derived from electronic databases. Therefore, our findings need to be validated by additional research such as cell experiments and clinical studies to fully investigate the underlying mechanism, molecule interplay, and clinical significance of various FASTKs in KIRC.

Conclusion

In summary, we conducted an in-depth exploration on the differentially expressed FASTK family members in KIRC and their potential prognostic significance. This study found that compared with normal tissues, KIRC tissues showed upregulated FASTK and TBRG4 and downregulated FASTKD1, FASTKD2, and FASTKD5. Moreover, the upregulation of FASTK and TBRG4 mRNA and the downregulation of FASTKD1, FASTKD2, FASTKD3, and FASTKD5 mRNA were substantially related to an unfavorable OS and DSS of KIRC patients. In addition, the roles of the FASTKs with differential expression were mostly linked to DNA damage, cell differentiation, cell proliferation, invasion, and EMT. It was shown that the expression of FASTKs in KIRC was substantially associated with the infiltration levels of various immunocytes, including TReg, Cytotoxic cells, NK CD56dim cells. Our research offers innovative insights and conclusions that can be used to select predictive biological markers from the members in the FASTK family in KIRC.

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Author Contributions

YF L and GH Z wrote the main manuscript and DL W prepared figures 1–4, HP C prepared figures 5–8, LF Y prepared figures 9–12, and Q X prepared supplementary figures 1–5 and tables 1–3,s1. T W supervised this work. All the authors have read and approved the manuscript.

Ethics Approval and Consent to Participate

All procedures performed in this study involving human genes were in accordance with the Declaration of Helsinki (as revised in 2013).

Consent for Publication

Not applicable.

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Availability of Data and Materials

The entire RNA-seq profile data and the clinical data of KIRC patients in this study come from The Cancer Genome Atlas (TCGA, https://cancergenome.nih.gov/) database, and Gene expression omnibus (GEO, GSE66271, https://www.ncbi.nlm.nih.gov/gds/) database. The datasets supporting the conclusions of this article are included within the article.

Supplemental Material

Supplemental material for this article is available online.

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