Original Article

Prognostic value of JAK3 promoter methylation and mRNA expression in clear cell renal cell carcinoma

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HIGHLIGHTS

- JAK3 promoter methylation was correlated with JAK3 expression in ccRCC.
- JAK3 promoter methylation was associated with clinicopathological characteristics in ccRCC.
- JAK3 promoter methylation was associated with overall survival in ccRCC.
- JAK3 promoter methylation was correlated with immune cell infiltration in ccRCC.
- JAK3 promoter methylation was correlated with the expression of immune checkpoint molecules in ccRCC.
- JAK3 promoter methylation was a potential biomarker for predicting responses to immune checkpoint inhibitors in ccRCC.

GRAPHICAL ABSTRACT
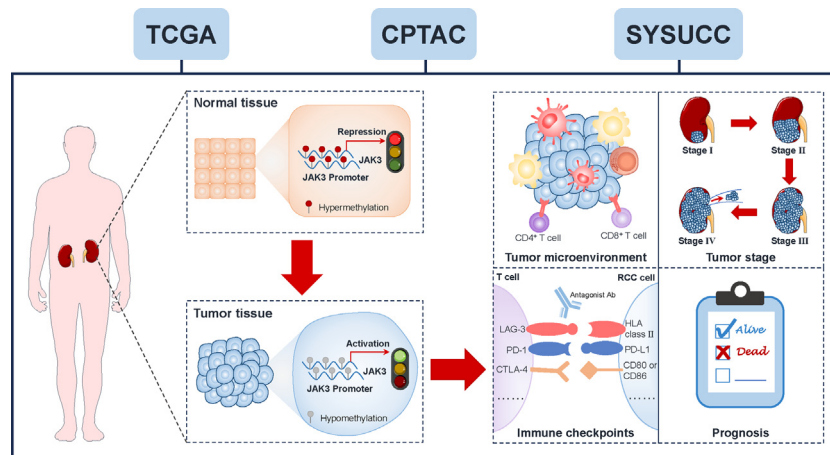
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ABSTRACT

Introduction: Janus kinase 3 (JAK3) is a well-established oncogene in clear cell renal cell carcinoma (ccRCC). The methylation status of oncogene promoters has emerged as biomarkers for cancer diagnosis and prognosis.
Objective: This study aims to investigate the biological and clinical significance of JAK3 promoter methylation in ccRCC.

Methods: We analyzed the relationship of JAK3 promoter methylation with its mRNA expression, overall survival, and immune cell infiltration in a cohort obtained from The Cancer Genome Atlas (TCGA), which was further validated by another independent cohort. We further validated correlations of JAK3 promoter methylation with JAK3 expression, overall survival, and immune cell infiltration in an independent ccRCC cohort (Sun Yat-sen University Cancer Center (SYSUCC) cohort) by methods of immunohistochemistry (IHC) and pyrosequencing.

Results: We found JAK3 promoter was significantly hypomethylated in tumor tissues compared to normal adjacent tissues in ccRCC, and JAK3 promoter hypomethylation was strongly correlated with high JAK3 mRNA expression in all three ccRCC cohorts we examined. JAK3 promoter hypomethylation predicted advanced clinicopathological characteristics and shorter overall survival (TCGA cohort and SYSUCC cohort). Furthermore, we found that JAK3 promoter methylation was significantly associated with immune cell infiltration and expression of immune checkpoint molecules (TCGA cohort and CPTAC cohort). Finally, our SYSUCC cohort validated that JAK3 promoter methylation was correlated with CD4$^+$ and CD8$^+$ T cell infiltration in ccRCC tumor tissues.

Conclusion: Our data demonstrated that the crucial role of JAK3 promoter methylation in its expression regulation and tumor microenvironment. JAK3 promoter methylation and expression are associated with clinicopathological characteristics, overall survival, and immune cell infiltration in ccRCC. We propose a rationale for further validation of JAK3 promoter methylation as a molecular biomarker for predicting responses to immune checkpoint inhibitors in ccRCC.

Introduction

Kidney and renal pelvis cancer is estimated to have 48,780 or 27,300 new cases in males and females, which ranks among the top 10 of all cancers, according to the cancer statistics of 2021 [1]. Renal cell carcinoma (RCC) is the most common type of kidney and renal pelvis cancer, and mainly consists of clear-cell RCC, papillary RCC, and chromophobe RCC, of which clear-cell RCC accounts for ∼70% of cases [2,3]. RCC is considered to be an immunotherapy responsive tumor characterized by high infiltration of immune cells [4]. However, infiltration of immune cells is always accompanied by an elevation of immune suppressive factors such as immune checkpoint molecules, thus leading to the inactivation of anti-tumor immune responses. Based on these findings, immune checkpoint inhibition therapies, which target the immune suppressive microenvironment have revolutionized the treatment of RCC [2]. Unfortunately, only a limited portion of patients developed durable benefits to immune checkpoint inhibitors, which limited the application of these promising strategies in clinical practice [5]. Hence, it is extremely necessary to identify reliable molecular biomarkers for predicting responses to checkpoint blockade to improve the clinical efficacy of these therapies.

JAK3 is a member of the Janus kinase (JAK) family of tyrosine kinases, which consist of JAK1/2/3 and TYK2 [6]. Janus kinase-signal transducer and activator of transcription (JAK-STAT) signaling is one of the most crucial pathways in immune regulation, which mediates almost all immune regulatory processes [7]. Despite its well-known oncogenic role in hematologic malignancies, JAK3 expression was also reported to be significantly elevated and associated with poor prognosis in various solid tumors, including stomach adenocarcinoma [8], breast cancer [9], colon cancer [10,11], and renal cancer [12,13]. In view of the crucial role of JAKs in the regulation of immune responses, many drugs targeting JAKs have been developed, which are mainly used in immune-related disorders [7]. Recently, a lot of studies have demonstrated the indispensable role of the JAK-STAT pathway in modulating tumor immune microenvironment [6]. Based on these findings, JAK inhibitors are now being tested in cancers in combination with immune checkpoint inhibitors [7], which shows a promising future.

Epigenetic mechanisms including DNA methylation play a crucial role in gene expression regulation not only in tumor cells but also in various immune cells [14]. A series of studies have indicated that DNA methylation status at specific promoters may alter the cell phenotype and reshape the tumor microenvironment, leading to cell survival and immune evasion [15]. Generally, aberrant DNA methylation is considered as an epigenetic hallmark of cancer that silences the expression of tumor suppressor genes [16]. In addition, accumulating evidence has demonstrated that specific DNA CpG sites located in the genes of some immune checkpoint molecules, including LAG3, PD-L2, and CTLA4, are reliable biomarkers for predicting responses to immune checkpoint inhibitors [17].

Although JAK3 has been reported to serve as an oncogene in RCC, comprehensive investigation of JAK3 promoter methylation in its expression regulation and tumor immune microenvironment in RCC remains to be explored. In the present study, we aimed to explore the association of JAK3 promoter methylation with gene expression, clinicopathological characteristics, immune correlation, and survival outcome in ccRCC.

Material and methods

Data and resources

TCGA cohort

We downloaded gene expression data, methylation data, and associated clinical data for ccRCC from the Cancer Genome Atlas data portal (TCGA, http://cancergenome.nih.gov/). R package 'ChAMP' was used to process the methylation data of Infinium HumanMethylation450 BeadChip beads. For differential methylation site analysis, data of 323 tumor tissues and 160 adjacent normal tissues from Infinium HumanMethylation450 BeadChip beads were included. For the combined methylation and transcriptome analysis, 317 tumor tissues with both methylation and expression data were selected. Clinical pathological information of TCGA patients was supplemented in Table S1.

CPTAC cohort and GEO datasets

Gene expression data and methylation data for validation, which included 100 ccRCC samples, were downloaded from The National Cancer Institute's Clinical Proteomic Tumor Analysis Consortium (CPTAC). The expression and methylation data were processed in the same way as the data from the TCGA cohort. Raw data of CPTAC patients were supplemented in Table S2. Methylation data of GSE61441 and GSE105260 were downloaded from Gene Expression Omnibus (GEO) database for validation of differential JAK3 promoter methylation. Raw data of GSE61441 and

GSE105260 patients were supplemented in Table S3 and Table S4, respectively.

SYSUCC cohort

For validation of cg04557677 methylation in ccRCC, a total of 98 samples including 14 adjacent normal tissues and 84 tumor tissues were collected from Sun Yat-sen University Cancer Center (SYSUCC). The patients underwent surgery from 2009 to 2015 in SYSUCC, and clinical follow-up information was collected for analysis. Raw data of SYSUCC patients were supplemented in Table S5. Basic information of all datasets included in our study was supplemented in Table S6.

Antibodies, reagent, and immunohistochemistry (IHC)

Anti-JAK3 antibody was purchased from Abcam (ab45141), anti-CD4 antibody from Servicebio (GB11064), anti-CD8 antibody from Servicebio (GB13429), 5-Azacytidine from Shelleck (S1782). SYSUCC samples were prepared in a tissue microarray. Briefly, a hollow needle was used to acquire tissue cores as small as 0.6 mm in diameter from paraffin-embedded surgical specimens of renal cancer patients. These tissue cores are then inserted in a recipient paraffin block in a precisely spaced, array pattern. Sections from this block are cut using a microtome, mounted on a microscope slide, and then analyzed by any method of immunohistochemistry (IHC). Immunohistochemistry (IHC) was performed on paraffin tissue sections. Briefly, we deparaffinized and rehydrated the paraffin section followed by antigen retrieval procedures. Next, we incubated sections with primary antibodies against JAK3 (dilution: 1:100), CD4 (dilution: 1:100), or CD8 (1:300) at 4 °C overnight in a humidified container (We chose the dilution ratio of different primary antibodies according to their recommended dilution ratio suggested in manufacturer instructions. Also, we conducted preliminary test for each primary antibody to determine the optimal dilution ratio.). Subsequently, the tissues were incubated at room temperature for 50 min. The IHC slides were scanned and digitalized using 3DHISTECH (Hungary). The IHC data of JAK3, CD4, and CD8 were expressed as a density score. Briefly, five random areas of each IHC picture (1 mm^2 each) were selected for each marker to count the number of positive cells per high power field (20X magnification), and the average of the five fields was taken, and then the data were expressed as a density score (total number of positive cells per 1 mm^2 area).

Cell lines and cell culture

The human RCC cell lines 786-O and ACHN were obtained from American Type Culture Collection (ATCC, Manassas, VA) and cultured in RPMI-1640 (Invitrogen, Carlsbad, CA). All cell lines were maintained in an incubator with a humidified atmosphere of 95% air and 5% CO2 at 37 °C.

RNA extraction and RT-qPCR

Total RNA was extracted using RaPure Total RNA Micro Kit from Magen (R4012-03) following the instructions. cDNA was synthesized using HiScript II One-Step RT-PCR Kit from Vazyme (R312-01) following the instructions. qPCR was performed using ChamQ SYBR qPCR Master Mix from Vazyme (Q311-02) following instructions. β-actin was used as internal control. The primers were displayed as follows. JAK3: forward primer: 5′-TTCGGGCTACGCAAGGATTTG-3′, reverse primer: 5′-AGGCTGAGACACTCACCCT-3′; β-actin: forward primer: 5′- CGTCTTCCCCTCCATCGT-3′, reverse primer: 5′- GAAGGTGTGGTGCCAGATTT-3′.

Methylation analysis

Methylation data of the TCGA ccRCC cohort and CPTAC ccRCC cohort were obtained from TCGA and CPTAC database, respectively. Beta value was approximately considered percent methyla-

tion. For differential analysis of methylation sites, R package 'limma' was used by the standard of adjusted *P* value < 0.05 and log (fold change) > 0.05.

For validation of cg04557677 methylation in the SYSUCC cohort, we used pyrosequencing for examining the percentage of its methylation. Briefly, DNA was extracted using TIANamp Genomic DNA Kit (DP304) and then followed by bisulfite conversion according to the instructions. Purified bisulfite conversed DNA was amplified by PCR using specific primers covering the cg04557677 CpG sites. The PCR products were turned into pyrosequencing using the PyroMark Q96 pyrosequencing and quantification platform following the manufacturer's instructions (Shanghai Biotechnology Corporation). The primers for amplification of target region are forward: 5′- AGATGGGTAAATTGAGGTAATAAGG −3′; reverse: 5′- CTCAACCCACTCTAAACCCACTTATTAAAT −3′.

Gene set variation analysis (GSVA) and estimation of tumor microenvironment immune cell infiltration

We performed GSVA enrichment analysis for immune cell infiltration and biological pathways using R packages 'GSVA'. The GSVA method is both non-parametric and unsupervised, and bypasses the conventional approach of explicitly modeling phenotypes within the enrichment scoring algorithm [18]. The gene sets of other biological processes were obtained from the study of Powles [19].

ssGSEA (single-sample gene-set enrichment analysis) algorithm was used to quantify immune cells infiltration in the tumor microenvironment. The gene sets of immune cell types were obtained from the study of Zhou [20] (Table S7). The enrichment scores calculated by ssGSEA analysis were considered as the relative abundance of each tumor microenvironment infiltrating immune cell in each sample. Relative abundance of tumor microenvironment cells in each sample of TCGA and CPTAC cohort was supplemented in Table S8 and S9, respectively.

Correlation between JAK3 expression/promoter methylation and immune-related pathways

Previous researchers constructed a panel of gene sets that stored genes associated with some biological processes closely related to the tumor immune microenvironment [19,20,21,22] (Table S10). Relative pathway enrichment scores of each sample of TCGA and CPTAC cohort were supplemented in Table S11 and S12, respectively. We then investigated the enrichment scores of each biological process in the high and low groups of JAK3 expression and promoter methylation, respectively.

Statistics

Statistical analyses were conducted using SPSS, version 23.0, GraphPad Prism (version 8), and R version 4.0.3 software. The correlation coefficients in our study were calculated using Spearman's rank correlation. *T*-test or Mann Whitney test was used to conduct difference comparisons between two groups. For survival analysis, the optimal cut-off values were calculated by the R package 'Survminer'. Kaplan-Meier survival analysis was used to analyze the association of JAK3 expression and promoter methylation with overall survival in ccRCC. All statistical *P* values were from a two-tailed *t*-test, with *P* < 0.05 as statistically significant. (*P* < 0.05, **P* < 0.01, ***P* < 0.001, ****P* < 0.0001).

Ethics statement

The use of the public data was approved by TCGA and CPTAC. The clinical specimens were obtained from the Tumor Bio-bank of Sun Yat-sen University Cancer Center. The study protocol for the SYSUCC cohort was approved by the Institutional Research Ethics Committee of Sun Yat-sen University Cancer Center (B2020-310-01).

Results

JAK3 promoter is hypomethylated in tumor versus normal adjacent tissues in ccRCC

To investigate the methylation of JAK3 promoter in ccRCC, we downloaded the methylation data (Infinium HumanMethylation450 Bead-Chip) of TCGA ccRCC cohort from the UCSC Xena browser (http:// xena.ucsc.edu, ccRCC n = 323, NAT n = 160). According to the annotation of Infinium HumanMethylation450 Bead-Chip, there are 33 probes located in JAK3 gene and five (cg03039294, cg11624708, cg18145683, cg21718276, cg24896366) with missing values. Among all valid probes, 15 probes are located in TSS1500, TSS200, or 5′UTR of JAK3. First, we conducted a differential analysis of all CpG sites located in JAK3 between tumor and normal adjacent tissues in ccRCC by the standard of adjusted P value < 0.05 and log (fold change) > 0.05. 19 CpG sites were significantly differentially methylated, and eight of them were located in JAK3 TSS1500, TSS200, or 5′UTR (Fig. 1A, 1B). To validate JAK3 promoter hypomethylation in tumor tissues, we analyzed eight sites in JAK3 promoter in two independent GEO datasets. Seven sites were significantly hypomethylated in tumor versus normal tissues in both datasets, and only cg11249283 was not significantly hypomethylated (Fig. 1C, 1D). These results demonstrated that JAK3 promoter was abnormally hypomethylated in ccRCC.

JAK3 promoter methylation correlates with JAK3 expression in ccRCC

JAK3 was a well-established oncogene in various cancers. To investigate the role of JAK3 in ccRCC, we analyzed JAK3 expression in tumor tissues and normal adjacent tissues. We found that JAK3 expression was significantly elevated in tumor tissues (Fig. 2A). Gene promoter methylation plays an important role in gene transcription regulation. To elucidate the role of JAK3 promoter methylation in regulating JAK3 expression, we analyzed the correlation between the eight differentially methylated sites and JAK3 expression. Except for the site cg11249283, other sites were all significantly negatively correlated with JAK3 expression (Fig. 2B-2I). To further validate our conclusion, we analyzed the correlation between these sites and JAK3 expression using another independent cohort from the CPTAC database. The results were exactly consistent with our conclusion from TCGA data (Fig. S1A-1H). To further explore the role of JAK3 promoter methylation in its expression, we conducted vitro 5-Azacytidine experiments in ACHN and 786-O cells. The result showed that JAK3 expression was significantly elevated after treatment with 5-Azacytidine for 48 h (Fig. S2). These results indicated that JAK3 promoter hypomethylation was probably the leading cause of abnormal high expression of JAK3 in ccRCC.

JAK3 promoter methylation and expression predict overall survival in ccRCC

To explore the prognostic value of JAK3 expression and promoter methylation in ccRCC, we first conducted a Kaplan-Meier survival analysis of JAK3 expression in the TCGA ccRCC cohort. We found that high JAK3 expression was associated with poor overall survival (Fig. 3A), which was consistent with the conclusion of previous studies by other teams [12,13]. Subsequently, we analyzed the association of eight differentially methylated CpG sites with overall survival in ccRCC. The results showed that hypomethylation of seven out of eight sites was associated with poor overall survival (Fig. 3B-3H), only hypomethylation of cg11249283 predicted a favorable overall survival (Fig. 3I), but

there was no correlation between cg11249283 methylation and JAK3 expression (Fig. 2E). These results showed that JAK3 expression and promoter methylation were potential biomarkers in predicting prognosis in ccRCC.

Promoter hypomethylation and high expression of JAK3 were associated with aggressive clinical phenotypes in ccRCC

To further explore the clinical significance of JAK3 expression and promoter methylation in ccRCC, we analyzed the association between JAK3 expression and promoter methylation with clinical-pathological characteristics of ccRCC patients. We found that JAK3 expression was significantly elevated in patients with advanced T, M, AJCC stage, and pathological grade (Fig. 4A). There was no significant association between N stage and JAK3 expression, and this was probably due to the small number of patients with lymph node metastasis (only eight in the cohort). Next, we analyzed the association of JAK3 promoter methylation with clinical-pathological characteristics of ccRCC patients. Our results showed that except cg11249283, the patients with advanced AJCC and T stage tended to have hypomethylated JAK3 promoter (Fig. 4B, 4D). Regarding pathological grade, cg03491584 and cg11249283 had no association with pathological grade, while hypomethylation of other JAK3 promoter CpG sites was correlated with advanced pathological grade in ccRCC (Fig. 4C). Further analysis of JAK3 promoter methylation with N and M stage of ccRCC patients revealed that only cg04557677 hypomethylation was associated with advanced N and M stage (Fig. 4E, 4F). These results demonstrated the clinical significance of JAK3 promoter hypomethylation in predicting TNM stage and pathological grade.

JAK3 expression and promoter methylation were correlated with immune cell infiltration in ccRCC

The tumor microenvironment is not only infiltrated by tumor cells but also various stromal and immune cells, which play important roles in tumor progression. Considering the critical roles of JAK3 in immune cell functions and immune regulation, we assumed that JAK3 expression was correlated with tumor microenvironment immune cell infiltration. Based on our assumption, we correlated RNA-seq signatures of 23 immune cells, including CD4 and CD8 T cells, with JAK3 expression and promoter methylation. We found that high JAK3 expression and promoter hypomethylation (excluding cg11249283) were associated with higher infiltration of immune cells. Among the sites examined, cg04557677, cg0327225, and cg01089639 hypomethylation were most significantly correlated with infiltration of immune cells (Fig. 5A). To further validate our result, we analyzed the correlation of JAK3 expression and promoter methylation with infiltration of immune cells using another independent cohort from the CPTAC database, and the result was in exact accordance with our analysis of the TCGA cohort (Fig. S3). To further investigate the role of JAK3 expression and promoter methylation in key immune-related pathways, we analyzed the relationship between them and 17 biological pathways, respectively. We found that high JAK3 expression was correlated with a lower angiogenesis score, indicating that tissues/patients with high JAK3 expression were probably insensitive to anti-angiogenesis therapy. Also, high JAK3 expression was associated with higher scores of CD8 T effector, antigen processing machinery, cytolytic activity, and MHC HLA signature, which indicate abundant infiltration of immune cells in the tumor microenvironment. In addition, higher JAK3 expression was significantly associated with higher immune checkpoint activity, co-inhibition APC and co-inhibition T cell signature (Fig. 5B). These results showed that although tumors with high JAK3 expression were abundant in the infiltration of immune cells, they also bore stron-
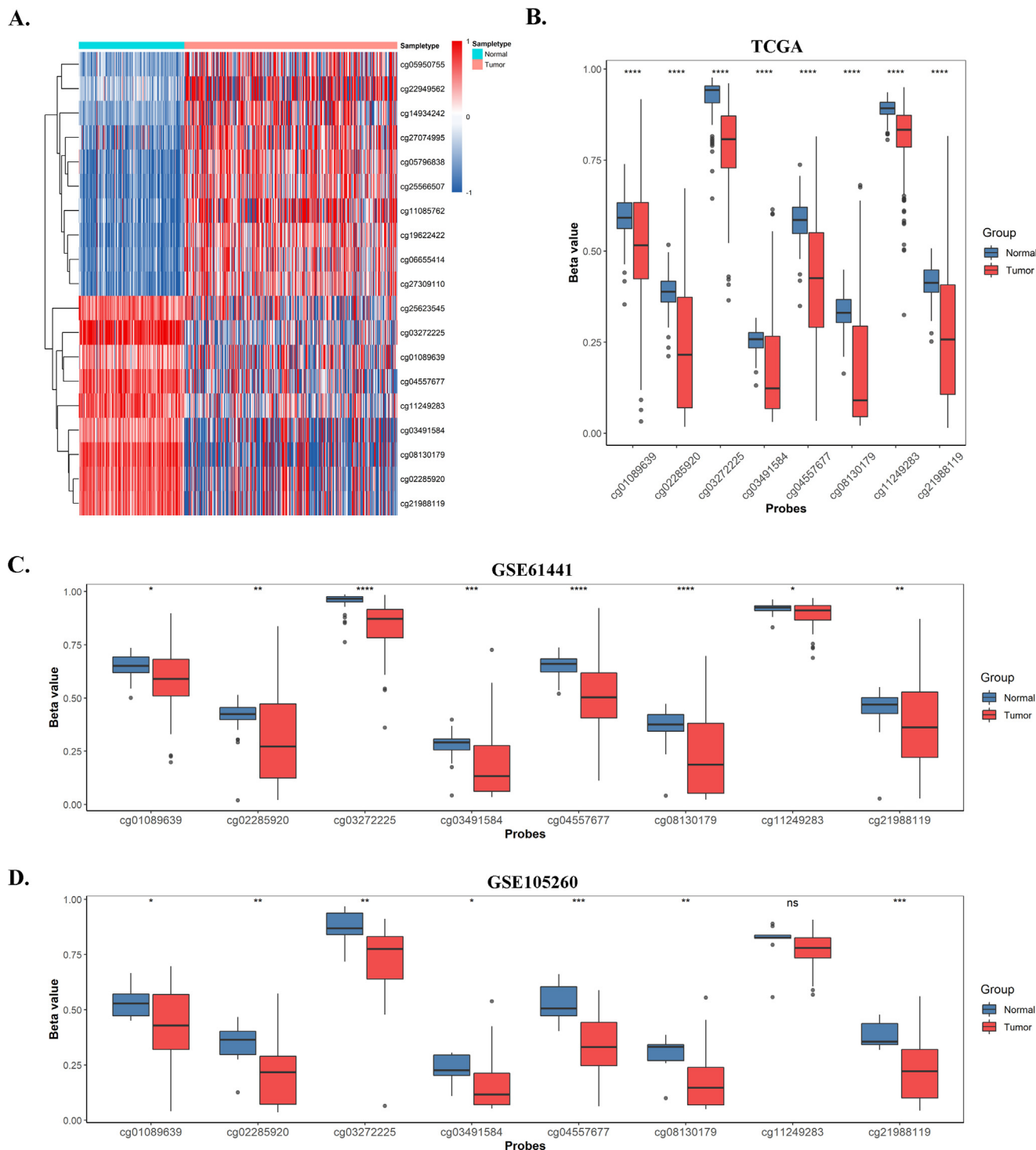
A.

B.

C.

D.

Fig. 1. JAK3 promoter is hypomethylated in tumor versus normal adjacent tissues in ccRCC. A. The heatmap of differentially methylated CpG sites located in JAK3 between tumor and adjacent normal tissues using data from TCGA cohort; B. Relative methylation of eight differentially methylated CpG sites (cg01089639, cg02285920, cg03272225, cg03491584, cg04557677, cg08130179, cg11249283, and cg21988119) located in JAK3 promoter between tumor and adjacent normal tissues of TCGA cohort; C. Relative methylation of eight differentially methylated CpG sites between tumor and adjacent normal tissues of GSE61441; D. Relative methylation of eight differentially methylated CpG sites between tumor and adjacent normal tissues of GSE105260.

ger immune checkpoint signatures, thus leading to an immune suppressive microenvironment. Next, we analyzed the correlation of JAK3 promoter methylation with 17 immune-related pathways. cg04557677, cg0327225, and cg01089639 hypomethylation were associated with lower angiogenesis score and higher scores of CD8 T effector, antigen processing machinery, cytolytic activity,

and MHC HLA signature, higher immune checkpoint activity, co-inhibition APC, and co-inhibition T cell signature (Fig. 5C, 5D, and Fig. S4A), which was consistent with our analysis of JAK3 expression. Other JAK3 promoter CpG sites had no significant association with these pathways (Fig. S4B-4F). Our analysis of JAK3 expression and promoter methylation with immune-related pathways using
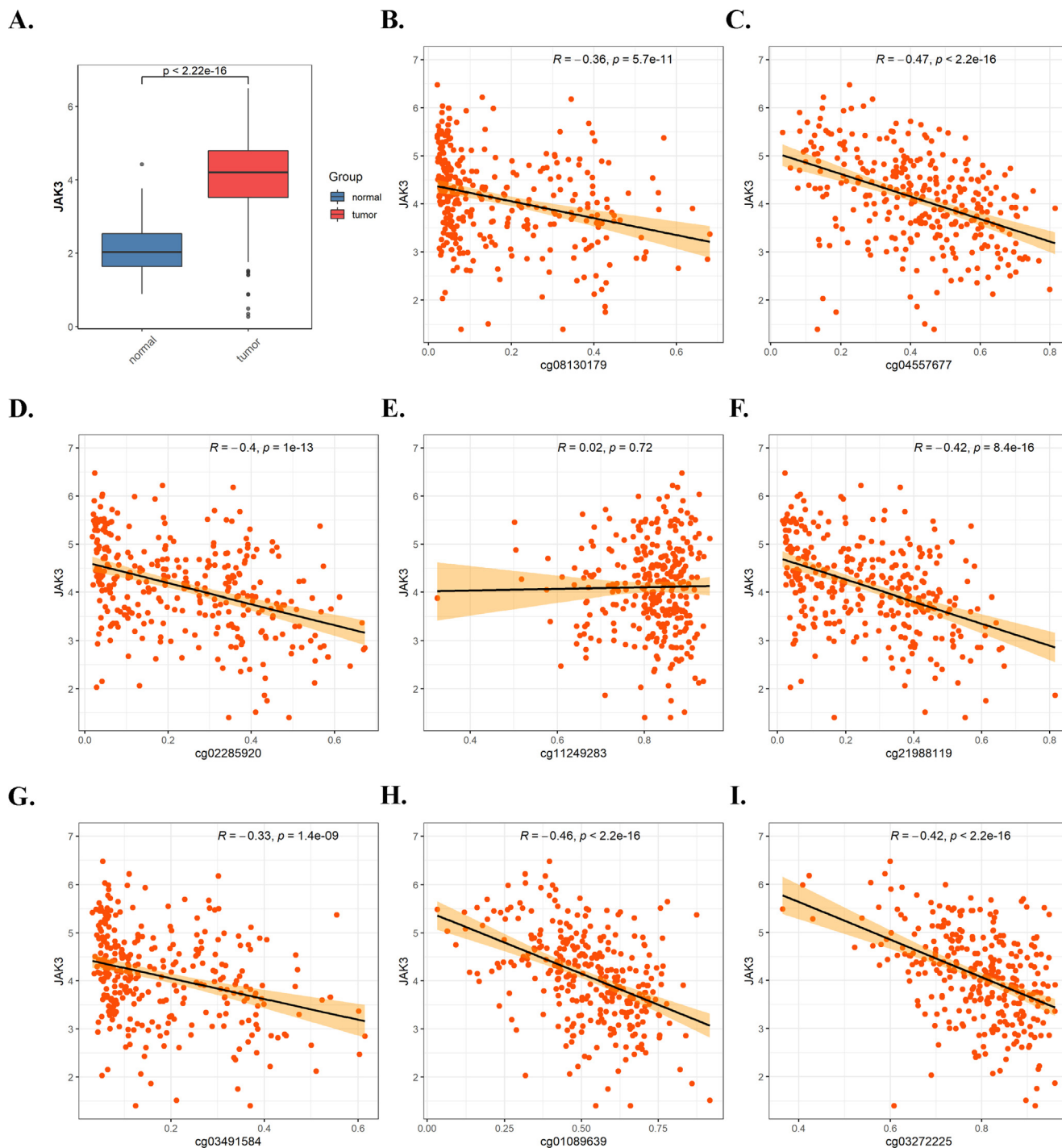
Fig. 2. JAK3 promoter methylation correlates with JAK3 expression in ccRCC. A. JAK3 expression between tumor and normal adjacent tissues of TCGA cohort; B-I. The correlation of cg08130179 cg04557677, cg02285920, cg11249283, cg21988119, cg03491584, cg01089639 and cg03272225 methylation with JAK3 expression in TCGA tumor tissues.

the CPTAC cohort was exactly consistent with our above results (Fig. S5A-5I). These results demonstrated that high JAK3 expression and its promoter hypomethylation were positively correlated with immune cell infiltration, indicating immunoreactive "hot tumors". At the same time, these "hot tumors" were characterized by high enrichment of immune checkpoint molecules, which will probably respond to immune checkpoint inhibitors. Therefore, JAK3 expression and promoter methylation were potential molecular biomarkers for predicting responses to immune checkpoint inhibitors therapy.

JAK3 expression and promoter methylation were associated with the expression of key immunomodulators in ccRCC

The tumor immune microenvironment is precisely regulated by various membrane proteins and cytokines. To elucidate the relationship of JAK3 expression and promoter methylation with key molecules participating in anti-tumor immune responses, we analyzed the correlation of JAK3 expression and promoter methylation with 74 key immunomodulators using the TCGA cohort. We found that high JAK3 expression and hypomethylation of cg04557677
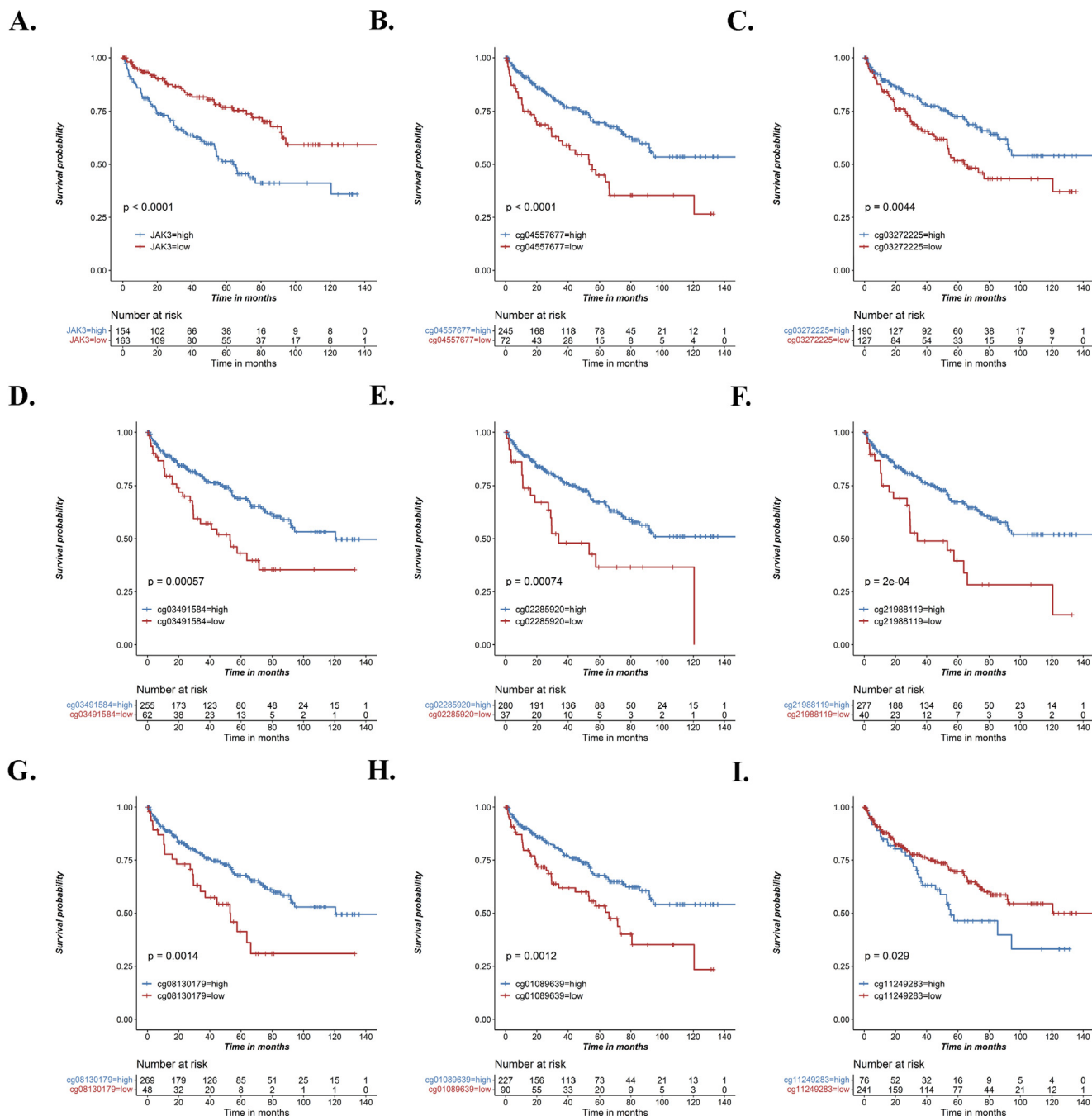
Fig. 3. JAK3 promoter methylation and expression predict overall survival in ccRCC. A-I. Kaplan-Meier survival analysis of JAK3 expression, cg04557677, cg03272225, cg03491584, cg02285920, cg21988119, cg08130179, cg01089639, and cg11249283 for overall survival of patients from TCGA ccRCC cohort, respectively.

and cg0327225 were correlated with the expression of a majority of key immunomodulators (Fig. 6A). Considering the promising therapeutic effect of immune checkpoint inhibitors in various tumors, we analyzed the association of JAK3 expression and cg04557677/cg0327225 methylation with immune checkpoint molecules (LAG3, PD1, PDL1, CTLA4, TIGIT, and PDL2), using expression data from the TCGA cohort. A significant positive correlation was found between JAK3 expression and expression of LAG3, PD1, CTLA4, TIGIT, and PDL2 but not PDL1 (Fig. 6B). On the contrary, cg04557677/cg0327225 methylation was significantly negatively associated with expression of LAG3, PD1, CTLA4, TIGIT, and PDL2 but not PDL1 (Fig. 6C, 6D). Our analysis of data from the CPTAC cohort also confirmed our results from the TCGA cohort

(Fig. S6A-6D). These results further proved that JAK3 expression and promoter methylation were associated with the immune suppressive microenvironment in ccRCC.

JAK3 promoter methylation is associated with overall survival and tumor infiltration of CD4/CD8 T cells in the SYSUCC validation cohort

Our previous results from TCGA and CPTAC cohorts proved that JAK3 promoter was hypomethylated in tumor tissues, and promoter hypomethylation was associated with poor survival and advanced TNM stage and pathological grade. Furthermore, JAK3 promoter hypomethylation was associated with infiltration of immune cells and the expression of immune checkpoint molecules.
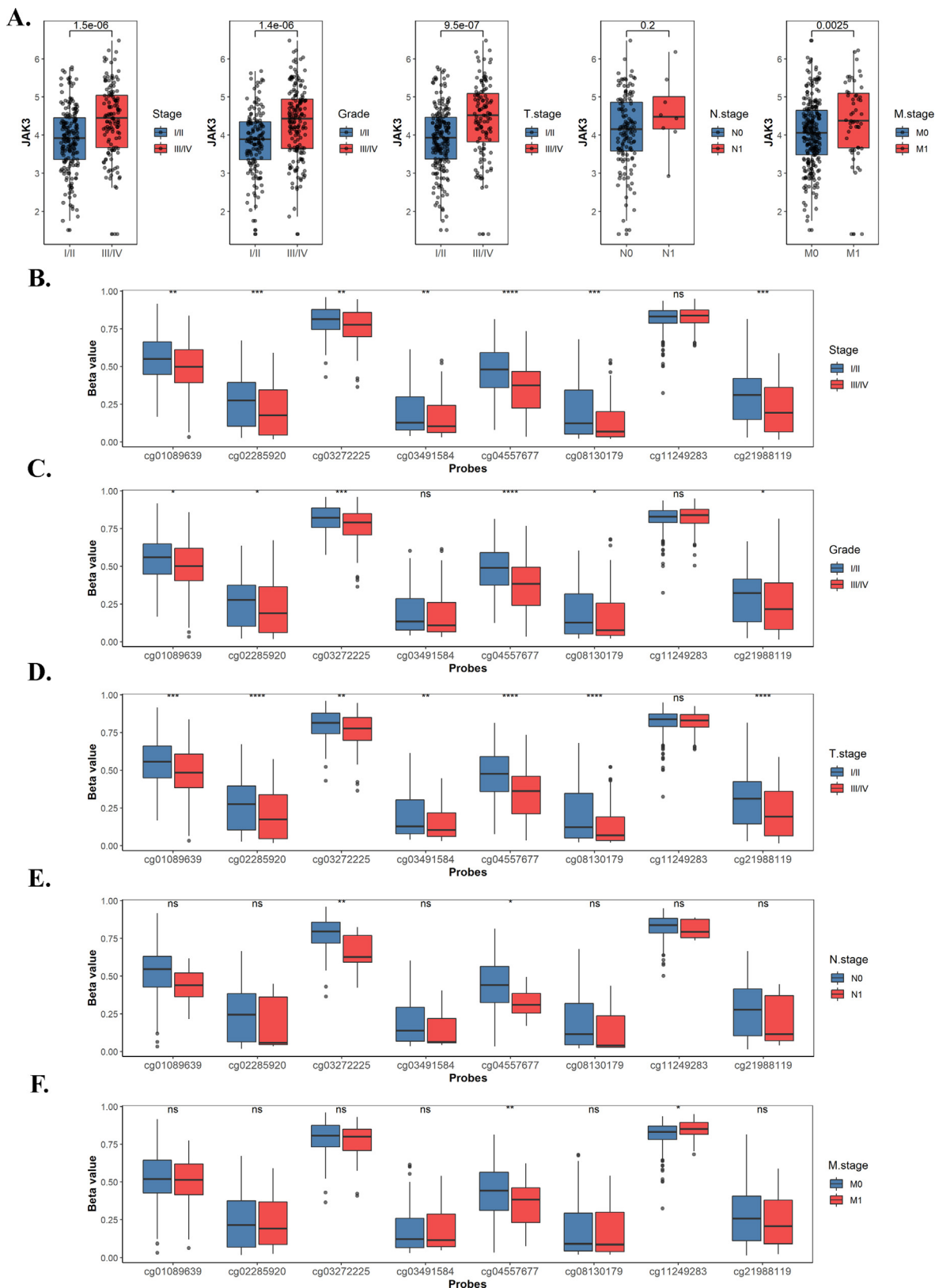
Fig. 4. Promoter hypomethylation and high expression of JAK3 were associated with aggressive clinical phenotypes in ccRCC. A. JAK3 expression in different TNM stages and pathological grades of patients from TCGA cohort; B-F. Relative methylation of cg01089639, cg02285920, cg03272225, cg03491584, cg04557677, cg08130179, cg11249283, and cg21988119 in different TNM stages and pathological grade of patients from TCGA cohort.
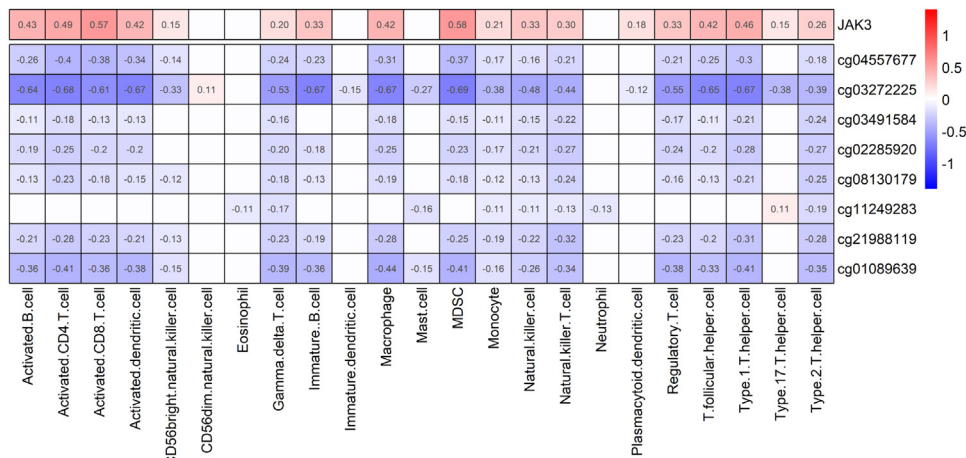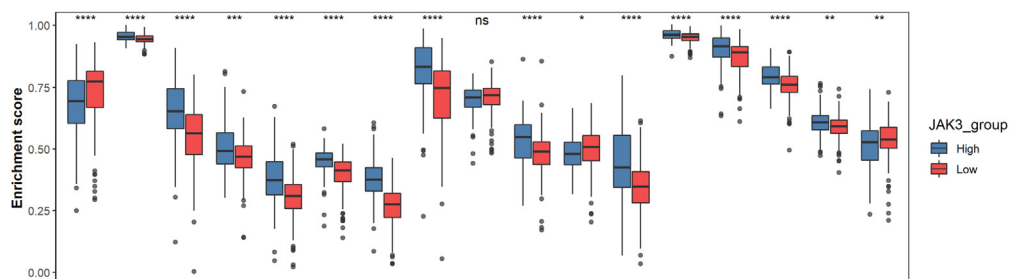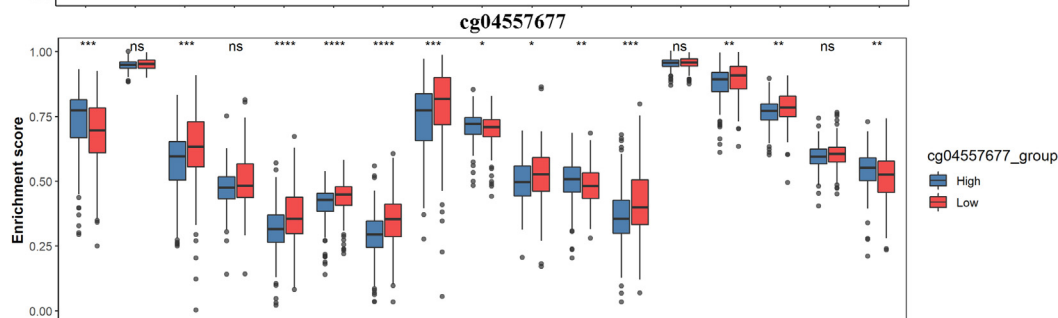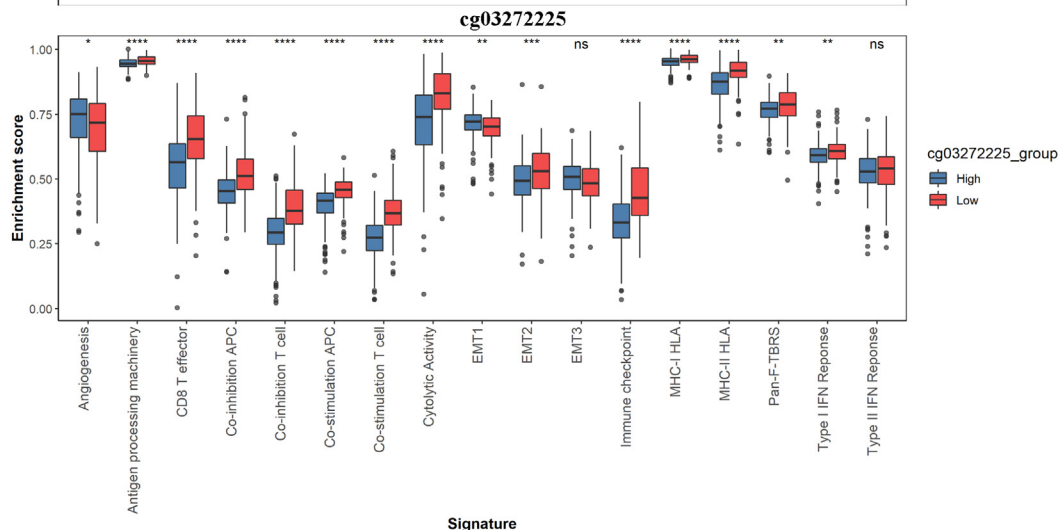
Fig. 5. JAK3 expression and promoter methylation were correlated with immune cell infiltration in ccRCC. A. The correlation heatmap of JAK3 expression and eight differentially methylated CpG sites with 23 types of immune cells in the TCGA ccRCC cohort, only statistically significant (*P* < 0.05) are shown in color and correlation coefficients. B-D. Relative enrichment scores of 17 immune-related pathways in the high and low groups according to the median of JAK3 expression and methylation of cg04557677 or cg03272225, respectively.
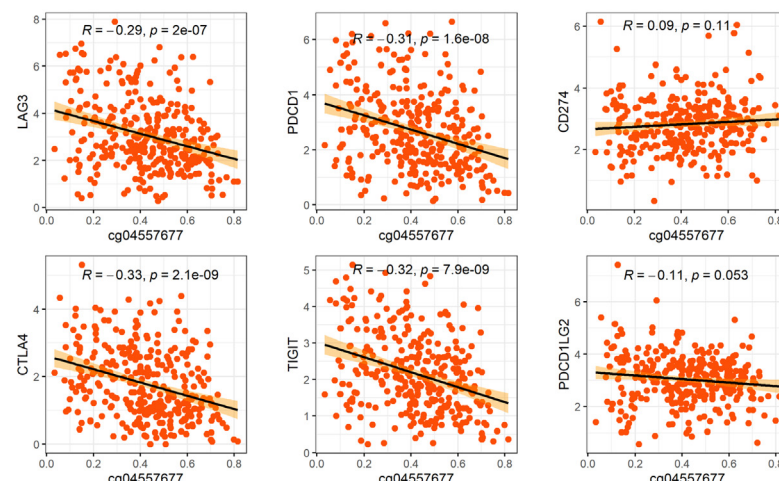
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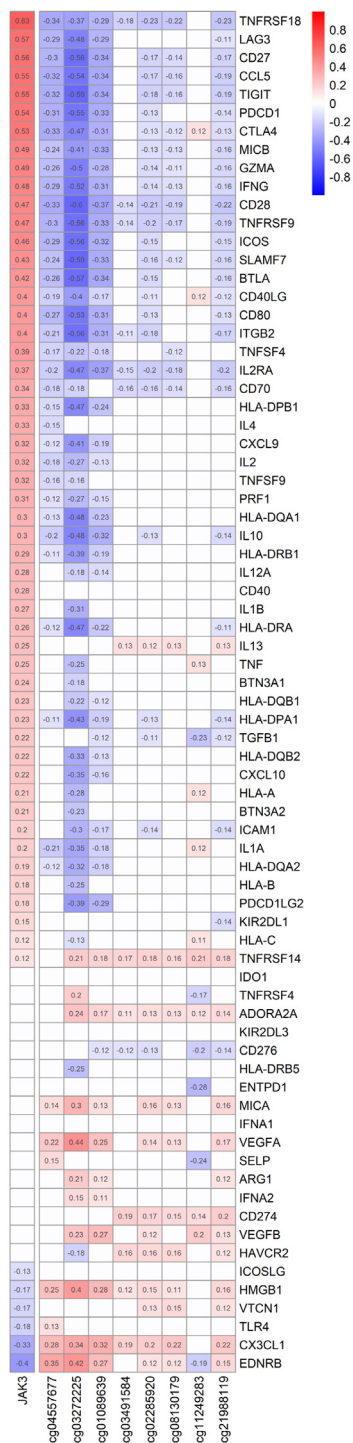
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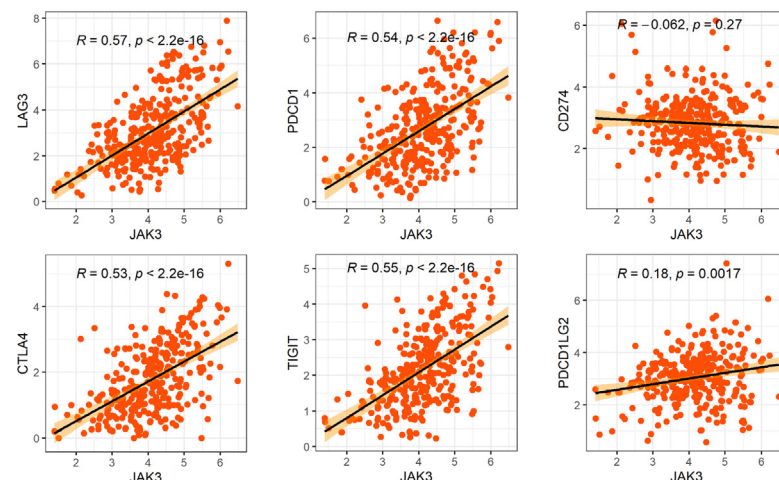
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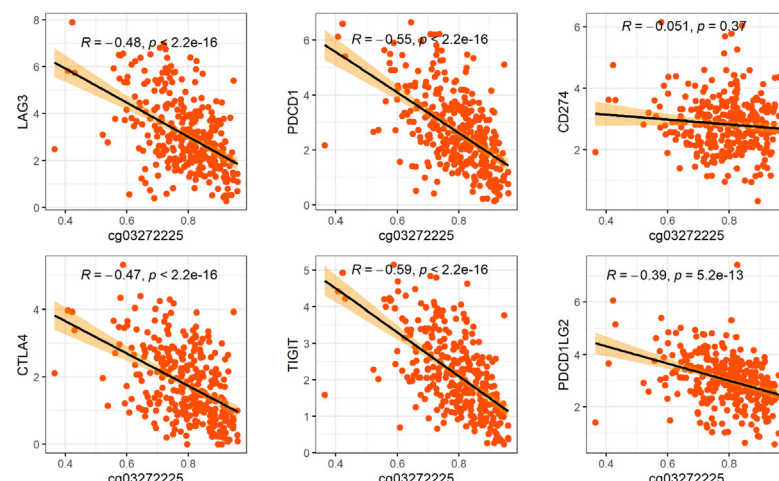
Fig. 6. JAK3 expression and promoter methylation were associated with the expression of key immunomodulators in ccRCC. A. The correlation heatmap of JAK3 expression and eight differentially methylated CpG sites with 74 key immunomodulators in the TCGA ccRCC cohort, only statistically significant (P < 0.05) are shown in color and correlation coefficients. B-D. The correlation of JAK3 expression and cg04557677 or cg03272225 methylation with immune checkpoint molecules LAG3, PD1, PDL1, CTLA4, TIGIT, and PDL2, respectively.

Considering that cg04557677 was among the most significant prognostic factors and its significant association with TNM stage, pathological grade, and infiltration of immune cell, we selected

CpG site cg04557677 for our verification in the SYSUCC validation cohort. We also found a significant positive correlation between cg04557677 and two promising CpG sites (cg03272225 and
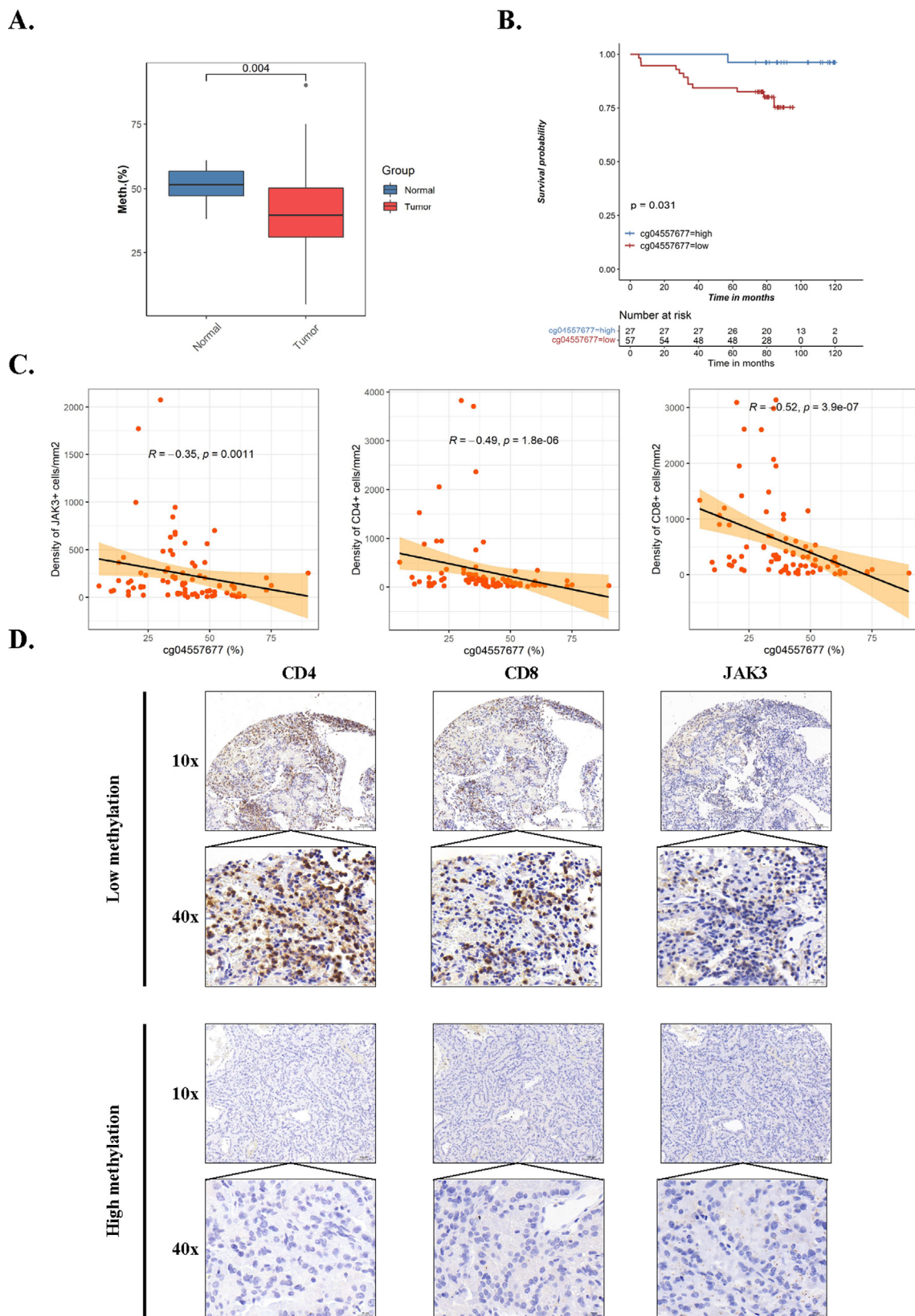
Fig. 7. JAK3 promoter methylation is associated with overall survival and tumor infiltration of CD4/CD8 T cells in the SYSUCC validation cohort. A. Relative cg04557677 methylation between tumor and normal adjacent tissues of SYSYCC cohort; B. Kaplan-Meier survival analysis of cg04557677 methylation in SYSUCC cohort; C. The correlation of cg04557677 methylation with CD4, CD8, and JAK3 expression in SYSUCC cohort, respectively. D. The representative IHC images of CD4, CD8, and JAK3 in high and low cg04557677 methylation group of SYSUCC cohort.

cg01089639) (Fig. S7A, 7B), indicating that cg04557677 was representative. First, we examined the methylation of cg04557677 by pyrosequencing. The results showed that methylation of cg04557677 was significantly decreased in tumors compared to normal adjacent tissues (Fig. 7A), and cg04557677 hypomethylation was associated with poor overall survival (Fig. 7B). CD4 and CD8 T cells are the final executors of anti-tumor immune responses, which are the most important immune cells in anti-tumor immunity. To verify the association of JAK3 promoter methylation with infiltration of immune cells, we examined the expression of CD4, CD8, and JAK3 in tumor tissues. We found that cg04557677 methylation was negatively correlated with CD4, CD8, and JAK3 expression (Fig. 7C, 7D), which was consistent with our previous results from TCGA and CPTAC cohorts. These results further validated that JAK3 promoter methylation was associated with overall survival and infiltration of immune cells in ccRCC.

Discussion

Aberrant DNA methylation is one of the hallmarks of many cancers, which usually occurs early during carcinogenesis [23]. DNA methylation profiling is becoming an increasingly important tool for cancer diagnosis, prognosis prediction, and therapeutic effect monitoring because it's highly stable and robust [24]. In the present study, we found that JAK3 promoter was generally hypomethylated in tumor tissues compared to normal tissues in ccRCC. Subsequently, we identified eight significantly differentially methylated CpG sites (cg03272225, cg08130179, cg04557677, cg02285920, cg11249283, cg21988119, cg03491584, and cg01089639), which were significantly hypomethylated in tumor tissues (Fig. 1A-1D). In general, hypermethylation of specific gene promoter often leads to gene silencing. On the contrary, gene promoter hypomethylation often goes along with gene activation due to more open chromatin structure [25]. The eight differentially methylated sites are all located in JAK3 promoter; hence it is reasonable that most of them are negatively correlated with JAK3 expression. Indeed, significant correlations were found by further analysis of JAK3 promoter (except cg11249283) with its expression (Fig. 2B-2I). Regarding cg11249283 not negatively correlated with JAK3 expression, we proposed that CpG sites located in different regions of JAK3 promoter might have different roles in regulating its expression. 5-Azacytidine experiments in renal cancer cells also showed that JAK3 expression was significantly elevated after treatment with 5-Azacytidine. These results demonstrated that aberrant JAK3 promoter hypomethylation was probably the leading genomic impetus of elevated expression of JAK3 in ccRCC. To further explore the clinical significance of JAK3 promoter methylation, we conducted a Kaplan-Meier survival analysis of JAK3 expression and promoter methylation using the optimal cut-off value. High expression of JAK3 was associated with poor survival for patients (Fig. 3A), which was consistent with previous studies by other teams [12,13]. Given that JAK3 promoter methylation was negatively correlated with JAK3 expression, it was logical that JAK3 promoter hypomethylation should be associated with worse overall survival. Our result supported our above speculation. Indeed, hypomethylation of most CpG sites we analyzed was associated with worse overall survival (except cg11249283) (Fig. 3B-3I). Among them, cg04557677 was the most statistically significant ($P < 0.0001$). However, cg11249283 hypomethylation was abnormally correlated with better over survival for ccRCC patients ($P = 0.029$). Based on our above results, we thought cg04557677 was the most prominent prognostic predictor for ccRCC patients. However, the paradoxical prognostic role of cg11249283 for ccRCC patients remained to be further validated by other independent cohorts in the future study. However, when we conducted Kaplan-Meier survival analysis of JAK3 promoter methylation

according to the median of each CpG site respectively, only methylation of cg04557677 and cg03272225 was significantly associated with overall survival for ccRCC patients (The data didn't show). Additionally, the JAK3 promoter methylation between high and low TNM stage or pathological grade showed that only cg04557677 was significantly hypomethylated in all advanced TNM stage and pathological grade (Fig. 4B-4F). The result indicated that cg04557677 methylation was the most prominent prognostic predictor for ccRCC patients.

Despite the currently non-negligible role of DNA methylation in cancer diagnosis and prognosis prediction, DNA methylation markers also appear to be accurate and promising predictors of patient outcomes with different kinds of therapies, including immunotherapy [26]. Dietrich's group has identified a series of DNA methylation biomarkers located in the promoter of immune-related genes (LAG3, CTLA4, PDL2, PD1, TNFRSF9, etc.), which predict immune cell infiltration and responses to immune checkpoint inhibitors in different types of cancers [27,28,29,30,31,32,33]. Considering the crucial role of JAK3 in immune regulation, we wondered whether JAK3 expression and promoter methylation could also predict immune cell infiltration in ccRCC. Therefore, we analyzed the association of JAK3 expression and promoter methylation with 23 immune cells in the tumor microenvironment, which were quantified by the ssGSEA algorithm with transcriptome data. We found that JAK3 expression and promoter hypomethylation were significantly positively correlated with all analyzed immune cells, especially CD4^{+} and CD8^{+} T cells, in both TCGA and CPTAC ccRCC cohorts (Fig. 5A and Fig. S2). Paradoxically, unlike other immunotherapy responsive solid tumors, high levels of tumor CD8^{+} T cell infiltration are associated with a worse prognosis in ccRCC patients [34], and this is consistent with our aforesaid results that high JAK3 expression and promoter hypomethylation were positively correlated with immune cell infiltration but predicted worse survival. An explanation for this paradox is that high infiltration of immune cells is always accompanied by an elevation of inhibitory immune checkpoint molecules, which leads to inactivation of anti-tumor immune responses of immune effector cells like CD4^{+} and CD8^{+} T cells [35]. Hence, we correlated JAK3 expression and promoter methylation with the key 74 immunomodulators using data from TCGA and CPTAC cohorts. The results showed that JAK3 expression and promoter hypomethylation (especially cg04557677 and cg03272225) were significantly correlated with the expression of immune checkpoint molecules CTLA4, LAG3, PD-1, TIGIT, and PDL2 (Fig. 6A-6D and Fig. S5A-5D). Finally, we selected cg04557677, the most prominent predictor of prognosis and immune cell infiltration, to further validate our above-mentioned results in the SYSUCC cohort. As expected, cg04557677 was hypomethylated in tumor tissues, which was associated with poor survival (Fig. 7A, 7B). Also, cg04557677 methylation was negatively correlated with CD4, CD8, and JAK3 expression according to our IHC data (Fig. 7C, 7D). These results further confirmed the association of JAK3 promoter methylation with JAK3 expression, overall survival, clinicopathological characteristics, and immune cell infiltration in ccRCC.

Noteworthily, our data from TCGA were derived from Infinium HumanMethylation450 BeadChip beads, which didn't cover all CpG sites of human genomic DNA. Therefore, apart from cg04557677, there are probably other CpG sites to be identified, which may better predict survival and immune cell infiltration in ccRCC. With the rapid development of technology in DNA methylation detection, Infinium HumanMethylation850 BeadChip beads, which are capable of covering almost all CpG sites in human genomic DNA, have been widely used in methylation analysis. In the near future, we may identify new CpG sites using the data from Infinium HumanMethylation850 BeadChip beads. Another issue that should

raise our attention was that our study lacks validation with a cohort that received immune checkpoint inhibitors therapy, though we have proved that JAK3 methylation was associated with immune cell infiltration ccRCC. This shall be further validated by retrospective and prospective studies in our future projects.

JAK/STAT3 pathway is aberrantly hyperactivated in many types of cancer, which is generally associated with a poor clinical prognosis [6]. JAKs have become important therapeutic targets and currently, several JAK inhibitors have been approved by the FDA for the treatment of both autoimmune diseases and hematological malignancies [36]. Based on our observation of the significantly positive correlation of JAK3 with multiple immune checkpoint molecules (LAG3, TIGIT, CTLA4, PD1, and PDL2), it is intriguing to investigate whether JAK3 is capable of regulating the expression of such immune checkpoint molecules. If so, the combination of JAK3 inhibitors and immune checkpoint inhibitors in solid tumors is a promising strategery. Taken together, our study demonstrated that JAK3 promoter methylation was not only associated with JAK3 expression, clinicopathological characteristics, and overall survival in ccRCC but also correlated with immune cell infiltration and expression of immune checkpoint molecules. Our study provided strong evidence for the application of JAK3 promoter methylation as molecular biomarkers for predicting prognosis and responses to immune checkpoint inhibitors, which will personalize cancer immunotherapy in ccRCC.

Data Sharing and Data Accessibility

All processed data generated or analyzed in this study are included in the additional files.

Compliance with Ethics Requirement

The use of the public data was approved by TCGA and CPTAC. The clinical specimens were obtained from the Tumor Bio-bank of Sun Yat-sen University Cancer Center. The study protocol for the SYSUCC cohort was approved by the Institutional Research Ethics Committee of Sun Yat-sen University Cancer Center (B2020-310-01).

CRediT authorship contribution statement

Qian Long: Conceptualization, Methodology, Software, Investigation, Writing – original draft, Visualization, Investigation. **Chunyu Huang:** Conceptualization, Methodology, Software, Investigation, Writing – original draft, Visualization, Investigation. **Jinsheng Huang:** Conceptualization, Methodology, Software, Investigation, Visualization, Investigation. **Qi Meng:** Conceptualization, Methodology, Software, Investigation, Writing – original draft, Visualization, Investigation. **Yanjun Cheng:** Formal analysis, Data curation. **Yilin Li:** Formal analysis, Data curation. **Liru He:** Formal analysis, Data curation, Resources, Software, Validation. **Miao Chen:** Formal analysis, Data curation, Software, Validation. **Changlin Zhang:** Formal analysis, Data curation, Software, Validation. **Xiaonan Wang:** Formal analysis, Data curation. **Wancui Zhu:** Resources. **Jin Peng:** Resources. **Dingbo Shi:** Resources. **Fufu Zheng:** Supervision, Writing – review & editing. **Pei Dong:** Supervision, Writing – review & editing. **Wuguo Deng:** Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jare.2021.11.016.

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