

Analysis of clinical significance and prospective molecular mechanism of main elements of the JAK/STAT pathway in hepatocellular carcinoma

XIANGKUN WANG¹, XIWEN LIAO¹, TINGDONG YU¹, YIZHEN GONG², LINBO ZHANG³, JIANLU HUANG⁴, CHENGKUN YANG¹, CHUANGYE HAN¹, LONG YU^{1,5}, GUANGZHI ZHU¹, WEI QIN¹, ZHENGQIAN LIU¹, XIN ZHOU¹, JUNQI LIU¹, QUANFA HAN¹ and TAO PENG¹

Departments of ¹Hepatobiliary Surgery, ²Colorectal and Anal Surgery, ³Health Management and Division of Physical Examination, The First Affiliated Hospital of Guangxi Medical University, Nanning, Guangxi Zhuang Autonomous Region 530021; ⁴Department of Hepatobiliary Surgery, Third Affiliated Hospital of Guangxi Medical University, Nanning, Guangxi Zhuang Autonomous Region 530031; ⁵Department of Hepatobiliary and Pancreatic Surgery, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan 450000, P.R. China

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Abstract. Hepatocellular carcinoma (HCC) is one the most common malignancies and has poor prognosis in patients. The aim of the present study is to explore the clinical significance of the main genes involved in the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway in HCC. GSE14520, a training cohort containing 212 hepatitis B virus-infected HCC patients from the Gene Expression Omnibus database, and data from The Cancer Genome Atlas as a validation cohort containing 370 HCC patients, were used to analyze the diagnostic and prognostic significance for HCC. Joint-effect analyses were performed to determine diagnostic and prognostic significance. Nomograms and risk score models were constructed to predict HCC prognosis using the two cohorts. Additionally, molecular mechanism analysis was

performed for the two cohorts. Prognosis-associated genes in the two cohorts were further validated for differential expression using reverse transcription-quantitative polymerase chain reaction of 21 pairs of hepatitis B virus-infected HCC samples. JAK2, TYK2, STAT3, STAT4 and STAT5B had diagnostic significance in the two cohorts (all area under curves >0.5; $P \leq 0.05$). In addition, JAK2, STAT5A, STAT6 exhibited prognostic significance in both cohorts (all adjusted $P \leq 0.05$). Furthermore, joint-effect analysis had advantages over using one gene alone. Molecular mechanism analyses confirmed that STAT6 was enriched in pathways and terms associated with the cell cycle, cell division and lipid metabolism. Nomograms and risk score models had advantages for HCC prognosis prediction. When validated in 21 pairs of HCC and non-tumor tissue, STAT6 was differentially expressed, whereas JAK2 was not differentially expressed. In conclusion, JAK2, STAT5A and STAT6 may be potential prognostic biomarkers for HCC. JAK2, TYK2, STAT3, STAT4 and STAT5B may be potential diagnostic biomarkers for HCC. STAT6 has a role in HCC that may be mediated via effects on the cell cycle, cell division and lipid metabolism.

Correspondence to: Professor Tao Peng, Department of Hepatobiliary Surgery, The First Affiliated Hospital of Guangxi Medical University, Nanning, Guangxi Zhuang Autonomous Region 530021, P.R. China
E-mail: pengtaogmu@163.com

Abbreviations: HCC, hepatocellular carcinoma; JAK, Janus kinase; STAT, signal transducer and activator of transcription; HBV, hepatitis B virus; TCGA, The Cancer Genome Atlas; BCLC, Barcelona Clinic Liver Cancer; KEGG, Kyoto Encyclopedia of Genes and Genomes; GSEA, gene set enrichment analysis; BP, biological process; CC, cellular component; MF, molecular function; GO, gene ontology; OS, overall survival; MST, median survival time; CI, confidence interval; HR, hazard ratio; ROC, receiver operating characteristic; PPI, protein-protein interaction

Key words: hepatocellular carcinoma, genome-wide analysis, molecular mechanism, clinical significance, signalling pathway, JAK2, STAT6, Believe

Introduction

Liver cancer is considered the sixth most prevalent tumor type worldwide (1). Hepatocellular carcinoma (HCC) accounts for nearly all (70-85%) primary liver cancers (2). Many factors, including hepatitis B or C virus (HBV) infection, type 2 diabetes, alcohol intake and metabolic syndrome are risk factors for the development of HCC (3). Presently, therapeutic remedies for HCC treatment tend to rely on the Barcelona Clinic Liver Cancer (BCLC) staging system (4), and include surgical resection, locoregional and systemic approaches (2). Surgical approaches consist of liver resection and transplantation for early stage HCC and the application of these procedures is dependent on the cirrhosis, tumor extension and co-morbidities in patients (2). Locoregional

therapies used in intermediate-stage HCC consist of surgery, ablation and different types of transcatheter arterial chemo-embolization (5,6). Even with these treatments, patients still have an unsatisfactory prognosis, with a 5-year survival rate <10%, which makes liver cancer the second leading cause of cancer-related death worldwide (7). In addition, tumor recurrence was observed in ~70% of the cases within the first 5 years after tumor resection (5).

HCC is phenotypically and genetically heterogeneous (2). Other than the many important mutations in the telomerase promoter (8,9), tumor suppressor gene TP53 (10), Catenin β -1, AT-rich interaction domain 1 and axin 1, HCC pathogenesis is often characterized disruption in a series of signaling networks (2). The Janus kinase (JAK)-signal transducer and activator of transcription (STAT) signaling pathway is activated by over 40 cytokines and growth factors, and is associated with cell proliferation, differentiation and apoptosis (11,12). In the signaling pathway, cytokines induce phosphorylation of JAK1-3 and tyrosine kinase 2 (TYK2), followed by the activation of STAT1-6 (11,12). Furthermore, nuclear localization of STATs causes the activation of target genes among three families of inhibitory proteins: Protein inhibitors of activated STATs, the SH2-containing phosphatases and suppressors of cytokine signaling (13-15). JAK1 was also previously identified as a novel therapeutic target in patients with inflammatory HCC in the gp130 mutant population (16). Meanwhile, B7-H3 was reported to promote aggression and invasion of HCC by regulating epithelial-mesenchymal transition via JAK2/STAT3/Slug signaling (17). Calvisi *et al* (18) demonstrated that high expression of STAT3 was a marker of poor prognosis for HCC patients. Additionally, angiotensin II induces angiogenic factors production partly via an angiotensin II type 1/JAK2/STAT3/suppressors of cytokine signaling 3 (SOCS3) signaling pathway in MHCC97-H cells (18). He and Karin (19) suggested that STAT6 was involved in HCC and may be a poor predictor for HCC prognosis. Nuclear localization of STATs can lead to the activation of STAT target genes, such as the SOCS proteins (14). Inactivation of SOCS1 has been identified in many malignancies, including HCC (19).

At the same time, some members of JAK/STAT signaling pathway have been reported to be associated with HCC, while other factors are not. The current study investigated the clinical significance of mRNA expression of JAK/STAT signaling pathway for HCC diagnosis and prognosis.

Materials and methods

Ethical approval. This study was approved by the Ethical Review Committee of the First Affiliated Hospital of Guangxi Medical University [approval no. 2015 (KY-E-032)]. Informed consent was provided by all HCC patients.

HCC tissue collection and reverse transcription-quantitative PCR (RT-qPCR). A total of 21 HCC tissues and non-tumor tissues (>1 cm margin from tumor) of patients with HBV infection were collected between the December 2015 and July 2016 (The First Affiliated Hospital of Guangxi Medical University). Inclusion criteria were as follows: All the patients were determined to have HCC via pathological diagnosis.

Exclusion criteria: Patients of age <20 and >70 years. HCC and non-tumor tissues were cut into pieces and stored in RNastore Reagent (DP408; Tiangen Biotech Co., Ltd.) at 4°C overnight and transferred to -80°C for long-term storage. Total RNA was extracted from above tissues and reversed into cDNA (Takara Bio, Inc.). Primers for *GAPDH*, *JAK2*, *STAT5A* and *STAT6* were synthesized by Sangon Biotech Co., Ltd. with the following sequences (5'-3'): *GAPDH*, forward GTCAGCCGC ATCTTCTTT, reverse CGCCCAATACGACCAAT; *JAK2*, forward AGCCTATCGGCATGGAATATCT, reverse TAA CACTGCCATCCCAAGACA; *STAT5A*, forward GCAGAG TCCGTGACAGAGG, reverse CCACAGGTAGGGACAGAG TCT; *STAT6*, forward GTTCCGCCACTTGCCAATG, reverse TGGATCTCCCCTACTCGGTG. DEPC-treated water was purchased from Sangon Biotech Co., Ltd. FastStart Universal SYBR Green Master (ROX) was used for RT-qPCR (Roche Diagnostics). The conditions were as follows: 50°C for 2 min, 95°C for 10 min, 95°C for 15 sec, 60°C for 1 min (40 cycles), 95°C for 15 sec, 60°C for 1 min, 95°C for 30 sec, 60°C for 15 sec. The $2^{-\Delta\Delta C_q}$ method was used to calculate the relative expression of mRNAs (20).

Data source. Data from a total of 212 HCC patients with HBV infection were accessed from the GSE14520 dataset in the Gene Expression Omnibus database as a training cohort (platform GPL3921; ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE14520; accessed Sept 3rd, 2018) (22,22). Additionally, data from 370 HCC patients were accessed from the Cancer Genome Atlas (TCGA) database as a validation cohort (<https://cancergenome.nih.gov/>; accessed Sept 3rd, 2018).

Body map and protein expressions. In addition, expressions of genes in JAK/STAT pathway were collected from Gene Expression Profiling Interactive Analysis website (gepia.cancer-pku.cn/index.html; accessed Sept 8th, 2018), while the protein expressions were obtained from the Human Protein Atlas website (proteintlas.org/; accessed Sept 8th, 2018) (23).

Diagnosis, prognosis and joint-effect analysis. Diagnostic receiver operating characteristic (ROC) curves were constructed by use of mRNA expressions of tumor and non-tumor tissue samples (GraphPad version 7.0; GraphPad Software, Inc.). The mRNA expressions of tumor tissues were categorized into low and high expressions using the median expression level as the cut-off. Meanwhile, the overall survival was calculated using Kaplan-Meier and Cox proportional hazards regression model (SPSS version 16.0; SPSS, Inc.). Prognosis-related clinical factors, including tumor grade, tumor size, α -fetoprotein (AFP), cirrhosis, BCLC stage and radical resection, in two cohorts were adjusted for using the multivariate Cox proportional hazards regression model. Prognosis-related genes were combined for joint-effect analysis.

Gene set enrichment analysis (GSEA). Potential mechanisms, including biological processes and metabolic pathways of JAK/STAT pathway genes, were explored using GSEA (gsea2-2.2.4; software.broadinstitute.org/gsea/index.jsp). Datasets c2.cp.kegg.v6.1.symbols.gmt, c5.bp.b6.1.symbols.gmt, c5.cc.v6.1.symbols.gmt and c5.mf.v6.1.symbols.gmt

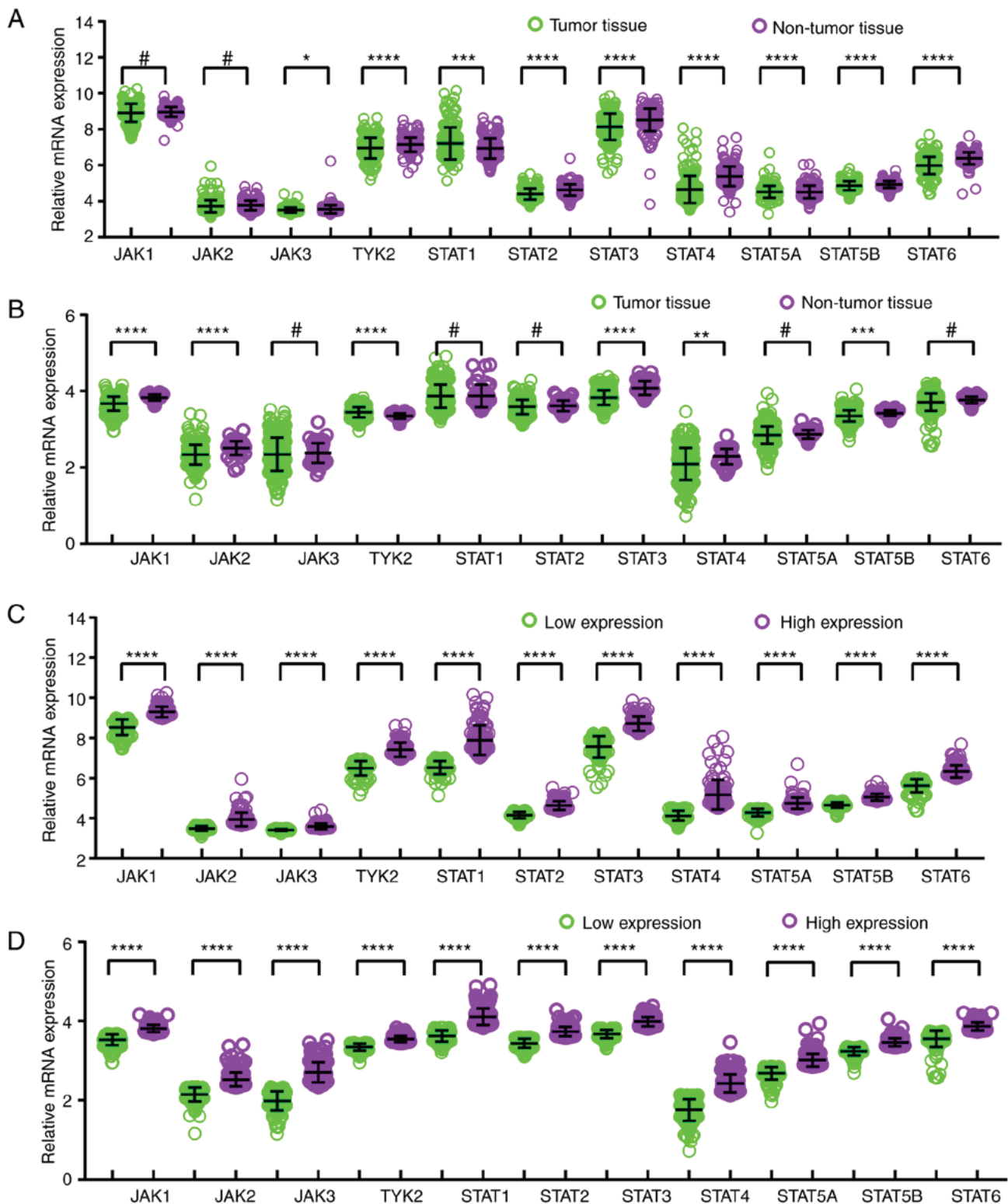


Figure 1. Relative mRNA expressions of key genes of JAK/STAT pathway. Relative mRNA expressions of key genes of JAK/STAT pathway in tumor and non-tumor tissue of the (A) GSE14520 cohort and (B) TCGA cohort. Analysis of low and high expressions of key genes of JAK/STAT pathway in the (C) GSE14520 cohort and (D) TCGA cohort. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$, # $P > 0.05$. TCGA, The Cancer Genome Atlas; JAK, Janus kinase; TYK2, tyrosine kinase 2; STAT, signal transducer and activator of transcription.

were then analyzed to obtain statistically significant Gene Ontology (GO) terms, including biological process (BP), cellular component (CC) and molecular function (MF), and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (24,25).

Risk score model and nomogram construction. For further examination of prognosis-related genes for HCC survival, risk score models were constructed for prognosis prediction. In addition, risk scores, patient survival status, expression heatmap and prognostic ROC curves were constructed in the

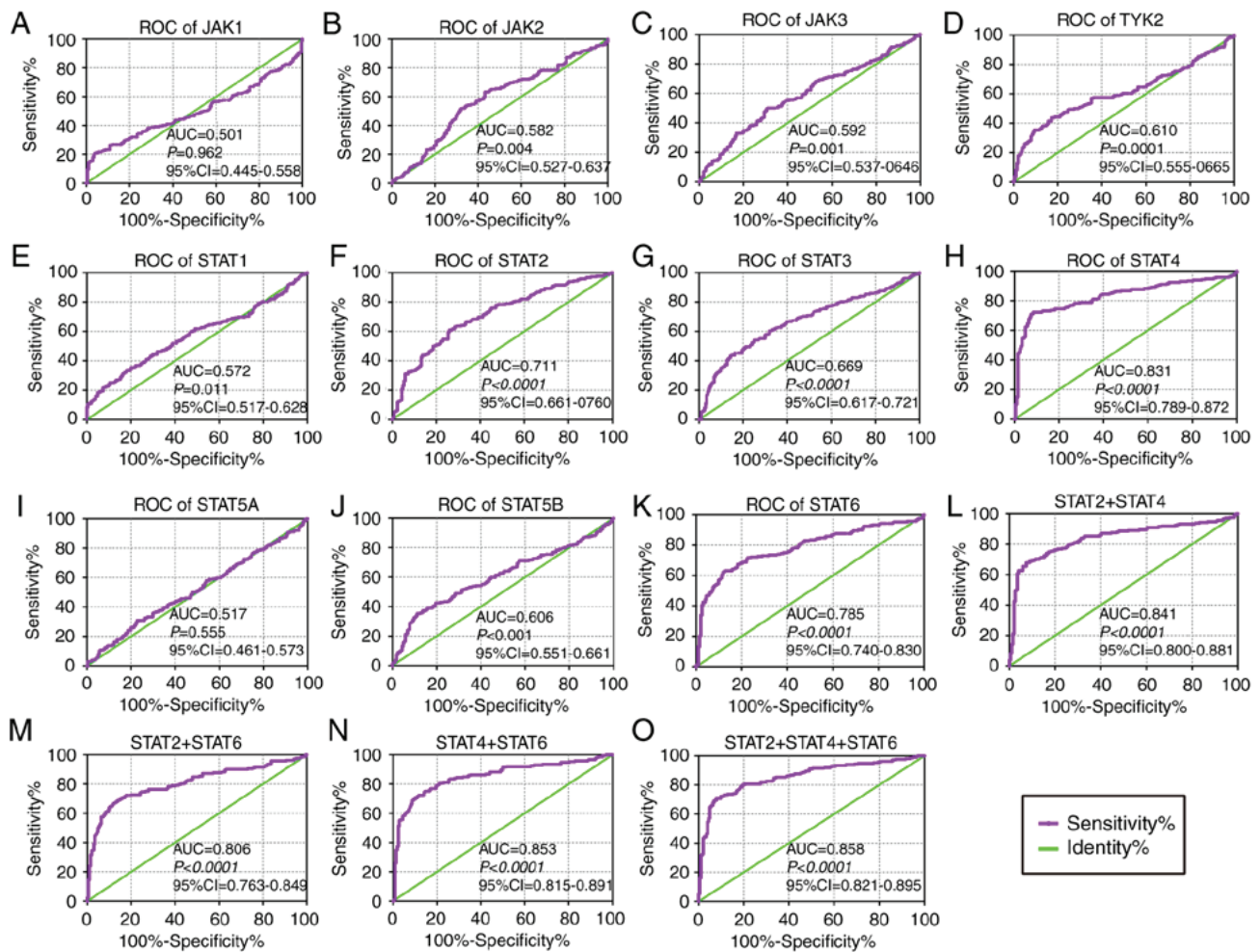


Figure 2. Diagnostic ROC curves of key genes of JAK/STAT pathway using the GSE14520 dataset. ROC curves of JAK/STAT pathway members: (A) JAK1, (B) JAK2, (C) JAK3, (D) TYK2, (E) STAT1, (F) STAT2, (G) STAT3, (H) STAT4, (I) STAT5A, (J) STAT5B and (K) STAT6. ROC curves of combinations: (L) STAT2 + 4, (M) STAT2 + 6, (N) STAT4 + 6 and (O) STAT2 + 4 + 6, respectively. ROC, receiver operating characteristic; AUC, area under the curve; CI, confidence interval; JAK, Janus kinase; TYK2, tyrosine kinase 2; STAT, signal transducer and activator of transcription.

model. The formula of the model is as follows: Risk score = expression of gene₁ × β_1 of gene₁ + expression of gene₂ × β_2 of gene₂ + ... + expression of gene_n × β_n of gene_n (26-28).

Contribution coefficients (β) originated from the multivariate Cox proportional hazards regression model. Nomograms were generated using clinical factors and genes for predicting patient survival probability at 1, 3 and 5 years.

Interaction analysis. Pearson correlation matrixes among JAK/STAT pathway genes were constructed using R version 3.5.0 (r-project.org/). An interactive gene-gene co-expression network was then constructed using the geneMANIA Cytoscape plugin (version 3.5.1) and the GO enrichment analysis was visualized using the BiNGO plugin of Cytoscape software version 3.6.0 (29-31). A protein-protein interaction (PPI) network was constructed using STRING (<https://string-db.org/cgi/input.pl>; accessed Sept 20th, 2018) (32).

Statistical analysis. Kaplan-Meier and box plots were produced using GraphPad software version 7.0 (GraphPad Software, Inc.). Survival analysis was performed by using SPSS software version 16.0 (SPSS, Inc.). The median survival time and Log-rank P-values were calculated using the Kaplan-Meier

method; 95% confidence interval (CI) and hazard ratio (HR) were calculated by univariate and multivariate Cox proportional hazards regression model. $P \leq 0.05$ was considered to indicate a statistically significant difference.

Results

Demographic characteristics of GSE14520 and TCGA cohorts.

In this study, a total of 212 HBV-related HCC patients were enrolled in training cohort, whereas 370 HCC patients were enrolled in the validation cohort. Tumor size, AFP, cirrhosis status and BCLC stage were associated with overall survival (OS; all $P \leq 0.05$; Table SI) in the GSE14520 cohort. Additionally, HBV infection, tumor stage and radical resection were associated with overall survival (all $P \leq 0.05$; Table SII) in the TCGA cohort.

mRNA, protein and BodyMap expression analysis. In comparison between tumor and normal tissues, JAK3, TYK2, STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6 genes exhibited differentially expressed levels in the GSE14520 cohort (all $P \leq 0.05$; Fig. 1A), and JAK1, JAK2, TYK2, STAT3, STAT4 and STAT5B demonstrated differentially expressed levels in the TCGA cohort (all $P \leq 0.05$; Fig. 1B). Meanwhile, in the

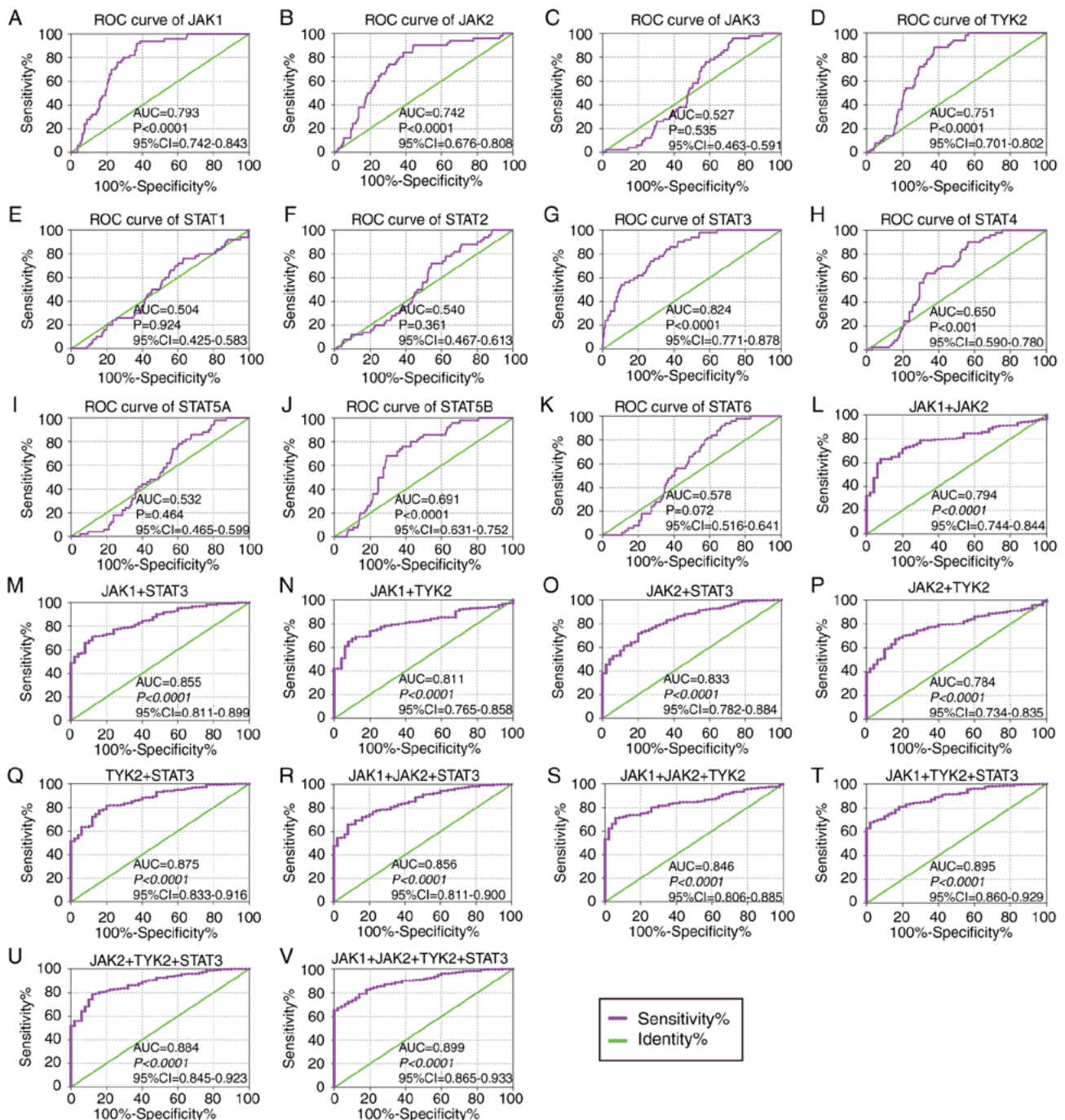


Figure 3. Diagnostic ROC curves of key genes of JAK/STAT pathway using data from The Cancer Genome Atlas. ROC curves of JAK/STAT pathway members: (A) JAK1, (B) JAK2, (C) JAK3, (D) TYK2, (E) STAT1, (F) STAT2, (G) STAT3, (H) STAT4, (I) STAT5A, (J) STAT5B and (K) STAT6. ROC curves of combination of (L) JAK1 + 2, (M) JAK1 + STAT3, (N) JAK1 + TYK2, (O) JAK2 + STAT3, (P) JAK2 + TYK2, (Q) STAT3 + TYK2, (R) JAK1 + 2 + STAT3, (S) JAK1 + 2 + TYK2, (T) JAK1 + STAT3 + TYK2, (U) JAK2 + STAT3 + TYK2 and (V) JAK1 + 2 + STAT3 + TYK2. ROC, receiver operating characteristic; AUC, area under the curve; CI, confidence interval; JAK, Janus kinase; TYK2, tyrosine kinase 2; STAT, signal transducer and activator of transcription.

comparison between low and high expression groups, all the genes showed differently expressed levels in both GSE14520 and TCGA cohorts (all $P \leq 0.05$; Fig. 1C and D). BodyMap expression of JAK/STAT pathway genes in tumor and normal tissues is illustrated in Fig. S1. Protein expression data from the Human Protein Atlas confirmed that STAT3 is the highest expressed of the JAK/STAT pathway proteins in HCC (Fig. S2).

Diagnosis and prognosis analysis. In the diagnostic analysis of the GSE14520 cohort (Fig. 2), JAK2, JAK3, TYK2, STAT1,

STAT2, STAT3, STAT4, STAT5B and STAT6 genes all indicated diagnostic significance for HCC [Fig. 2B-H, J-K; $P \leq 0.05$; area under curve (AUC) > 0.5]. Others did not show any diagnostic significance (Fig. 2A and I). Of them, STAT2, STAT4 and STAT6 genes showed higher diagnostic ability (AUC > 0.7). Their combination also illustrated a higher diagnosis ability compared with using just one gene (Fig. 2L-O). In the diagnostic analysis of TCGA cohort (Fig. 3), JAK1, JAK2, TYK2, STAT3, STAT4 and STAT5B indicated diagnostic significance for HCC (Fig. 3A, B, D, G, H and J;

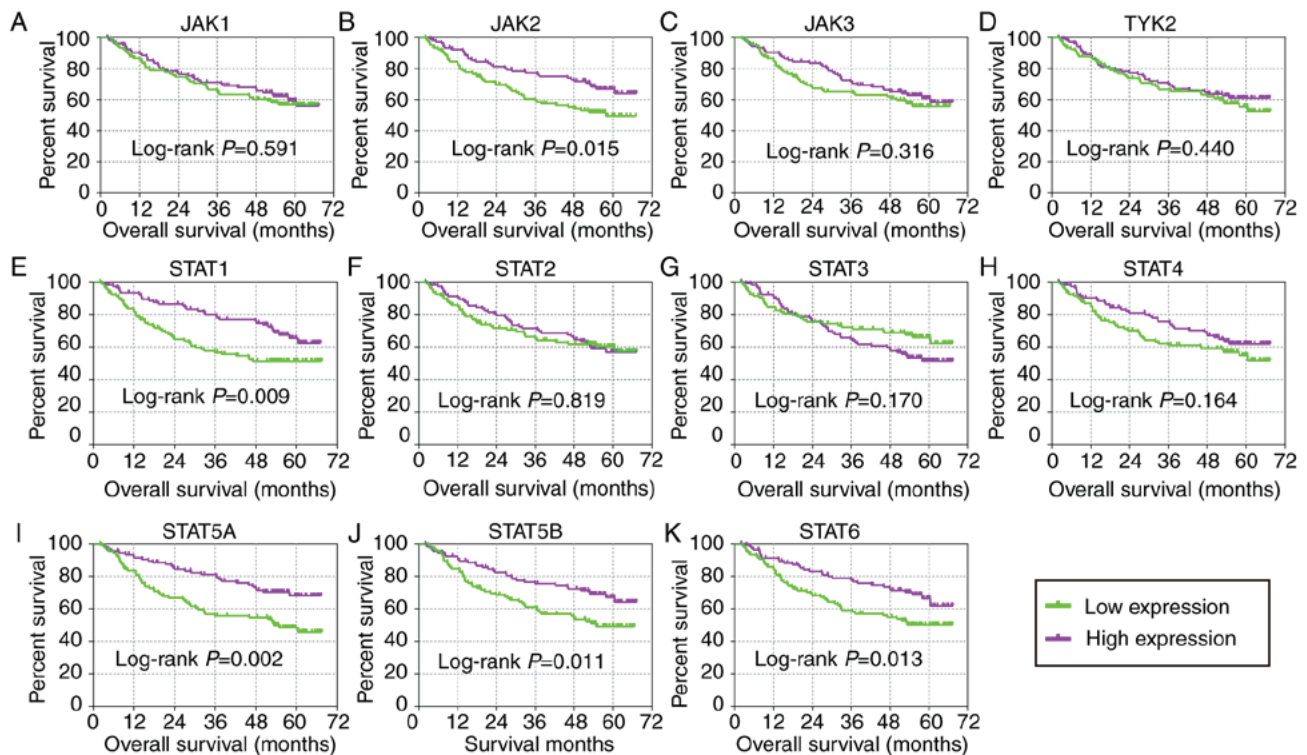


Figure 4. Kaplan-Meier plots of key genes of JAK/STAT pathway in the GSE14520 cohort. (A) JAK1, (B) JAK2, (C) JAK3, (D) TYK2, (E) STAT1, (F) STAT2, (G) STAT3, (H) STAT4, (I) STAT5A, (J) STAT5B and (K) STAT6. JAK, Janus kinase; TYK2, tyrosine kinase 2; STAT, signal transducer and activator of transcription.

$P \leq 0.05$; AUC > 0.5). Others did not show any diagnostic significance (Fig. 3C, E, F, I and K). Of them all, JAK1, JAK2, TYK2 and STAT3 displayed higher diagnosis ability (AUC > 0.7). A combination of all genes demonstrated higher diagnosis abilities as compared with just one gene (Fig. 3L-V). In the prognosis analysis of the GSE14520 cohort (Fig. 4), univariate analysis indicated that JAK2, STAT1, STAT5A, STAT5B and STAT6 were associated with OS (all adjusted $P \leq 0.05$; Table I; Fig. 4B, E and I-K). Others did not show any prognostic significance (Fig. 4A, C, D and F-H). Meanwhile, JAK2, STAT1, STAT3, STAT5A and STAT6 expressions were associated with OS after adjustment for tumor size, cirrhosis, AFP and BCLC stage (all adjusted $P \leq 0.05$; Table I). In the prognosis analysis of TCGA cohort, univariate analysis showed that STAT4, STAT5A, STAT5B and STAT6 were all associated with OS (all adjusted $P \leq 0.05$; Table II; Fig. 5H-K). Others did not show any prognostic significance (Fig. 5A-G). Meanwhile, JAK1, JAK2, STAT5A, STAT5B and STAT6 expressions were associated with OS after adjustment for radical resection, tumor stage and HBV infection (all adjusted $P \leq 0.05$; Table II).

Joint-effect analysis. JAK2, STAT5A and STAT6 demonstrated prognostic significance in both GSE14520 and TCGA cohorts (Tables I and II) and were additionally used in for joint-effect analysis. In the multivariate analysis, prognostic significance was observed among all the groups in the GSE14520 and TCGA cohorts (all adjusted $P \leq 0.05$; Tables III and IV). Groups 1, I, A, i, ■, ★, ● and ▲, with most of the poor prognosis indicators, indicated the worst prognosis. However, groups 3, III, C, iiiii, ■■■, ★★, ●● and ▲▲▲▲ showed the best prognosis (Fig. 6).

These results are in line with the fact that combination analysis has advantage over any gene alone.

GSEA results. STAT6 was further explored in analysis of GO terms and KEGG pathways (Figs. 7 and 8). Enriched STAT6 GO terms in both the GSE14520 and TCGA cohorts were enriched 'cell cycle checkpoint', 'cell cycle G1 S phase transition', 'regulation of cell division', 'meiotic cell cycle' and 'cell division' (Figs. 7A-L and 8A-L). Enriched KEGG pathways of STAT6 using the GSE14520 dataset included 'cell cycle', 'nucleotide excision repair', 'DNA replication' and 'homologous recombination', among others (Fig. 7M-P). Meanwhile, enriched KEGG pathways of STAT6 from The Cancer Genome Atlas data included 'drug metabolism cytochrome P450', 'PPAR signaling pathway', 'fatty acid metabolism' and 'metabolism of xenobiotics by cytochrome P450' (Fig. 8M-P).

Construction of nomogram and risk score model. Clinical factors, JAK2, STAT5A and STAT6 were analyzed using nomogram for the GSE14520 (Fig. 3A) and TCGA (Fig. 3B) cohorts. High expressions often cause low points in the two cohorts. The same points indicated highest probability of survival at 1 year and the lowest probability of survival at 5 years. Survival probability at 3 years was in the middle.

In addition, risk score models were constructed for prognosis prediction as presented in Figs. 9A and 10A. Meanwhile, coefficient β in the model was obtained from Cox proportional hazards regression model (Table V). The risk score model included risk score ranking, living status, gene expression heat maps and survival plot. The prognostic ROC curves and survival plots indicated that risk score models have values

Table I. Prognostic analysis of genes in JAK/STAT signaling pathway for overall survival in GSE14520 dataset.

Gene	No. of patients (n=212)	Overall survival					
		No. of events	MST (month)	HR (95% CI)	Crude P-value	HR (95%CI)	Adjusted P-value ^a
JAK1							
Low expression	106	43	NA	Ref.		Ref.	
High expression	106	39	NA	0.888 (0.576-1.370)	0.591	0.913 (0.588-1.419)	0.687
JAK2							
Low expression	106	49	57.9	Ref.		Ref.	
High expression	106	33	NA	0.578 (0.372-0.900)	0.015 ^b	0.585 (0.370-0.925)	0.022 ^b
JAK3							
Low expression	106	43	NA	Ref.		Ref.	
High expression	106	39	NA	0.801 (0.519-1.236)	0.316	1.019 (0.651-1.595)	0.934
TYK2							
Low expression	106	44	NA	Ref.		Ref.	
High expression	106	38	NA	0.843 (0.546-1.301)	0.440	0.831 (0.535-1.290)	0.409
STAT1							
Low expression	106	49	NA	Ref.		Ref.	
High expression	106	33	NA	0.555 (0.357-0.863)	0.009 ^b	0.617 (0.394-0.966)	0.035 ^b
STAT2							
Low expression	106	40	NA	Ref.		Ref.	
High expression	106	42	NA	0.951 (0.616-1.467)	0.819	1.184 (0.749-1.873)	0.469
STAT3							
Low expression	106	34	NA	Ref.		Ref.	
High expression	106	48	NA	1.360 (0.877-2.111)	0.170	1.650 (1.065-2.576)	0.028 ^b
STAT4							
Low expression	106	46	NA	Ref.		Ref.	
High expression	106	36	NA	0.734 (0.474-1.135)	0.164	0.683 (0.440-1.060)	0.089
STAT5A							
Low expression	106	52	53.3	Ref.		Ref.	
High expression	106	30	NA	0.487 (0.311-0.764)	0.002 ^b	0.498 (0.313-0.791)	0.003 ^b
STAT5B							
Low expression	106	49	54.8	Ref.		Ref.	
High expression	106	33	NA	0.563 (0.361-0.876)	0.011 ^b	0.696 (0.441-1.098)	0.119
STAT6							
Low expression	106	50	NA	Ref.		Ref.	
High expression	106	32	NA	0.569 (0.365-0.887)	0.013 ^b	0.529 (0.337-0.831)	0.006 ^b

^aP-values were adjusted for tumor size, cirrhosis, α -fetoprotein and Barcelona Clinic Liver Cancer stage using multivariate Cox proportional hazards regression model; ^bsignificant P-values and 95% CI. JAK, Janus kinase; STAT, signal transducer and activator of transcription; NA, not available; MST, median survival time; HR, hazard ratio; 95% CI, 95% confidence interval; Ref., reference; TYK2, tyrosine kinase 2.

for prognosis in the GSE14520 (Fig. 9B and C) and TCGA (Fig. 10B and C) cohorts.

Co-expression interactions, PPI network and enrichment analysis. Pearson correlation matrices of JAK/STAT pathway genes were constructed to show the correlations among them (Fig. 11A and B). Additionally, co-expression interaction and pathways along with PPI networks demonstrated complex interactions at the gene and protein levels (Fig. 11C and D).

Enrichment analysis identified BP, CC and MF GO terms associated with JAK/STAT pathway (Fig. S4). A schematic of the JAK/STAT pathway is presented in Fig. S5. In particular, the mechanisms of these genes involved in JAK/STAT signaling pathway were consistent with the GSEA results produced in the present study.

Validation of expressions of JAK2, STAT5A and STAT6 using RT-qPCR. A total of 21 pairs of HBV-infected HCC

Table II. Prognostic analysis of genes in JAK/STAT signaling pathway for overall survival in The Cancer Genome Atlas database.

Gene	Patients (n=370)	No. of event	MST (days)	Overall survival			
				HR (95% CI)	Crude P-value	HR (95% CI)	Adjusted P-value ^a
JAK1							
Low expression	185	70	1,685	Ref.		Ref.	
High expression	185	60	2,116	0.790 (0.557-1.119)	0.184	0.542 (0.364-0.807)	0.003 ^b
JAK2							
Low expression	185	69	1,622	Ref.		Ref.	
High expression	185	61	2,116	0.781 (0.552-1.105)	0.163	0.574 (0.388-0.848)	0.005 ^b
JAK3							
Low expression	185	69	1,622	Ref.		Ref.	
High expression	185	61	1,694	0.859 (0.609-1.213)	0.389	0.816 (0.551-1.208)	0.310
TYK2							
Low expression	185	69	1,490	Ref.		Ref.	
High expression	185	61	2,456	0.824 (0.583-1.165)	0.274	0.799 (0.541-1.180)	0.260
STAT1							
Low expression	185	69	1,694	Ref.		Ref.	
High expression	185	61	1,685	0.874 (0.618-1.237)	0.448	0.909 (0.609-1.358)	0.641
STAT2							
Low expression	185	70	1,423	Ref.		Ref.	
High expression	185	60	2,486	0.744 (0.526-1.053)	0.095	0.715 (0.481-1.063)	0.098
STAT3							
Low expression	185	68	1,622	Ref.		Ref.	
High expression	185	62	2,116	0.851 (0.602-1.203)	0.360	0.727 (0.495-1.069)	0.105
STAT4							
Low expression	185	73	1,423	Ref.		Ref.	
High expression	185	57	2,486	0.693 (0.490-0.981)	0.038 ^b	0.730 (0.487-1.095)	0.128
STAT5A							
Low expression	185	72	1,147	Ref.		Ref.	
High expression	185	58	2,456	0.626 (0.442-0.888)	0.009 ^b	0.648 (0.435-0.967)	0.033 ^b
STAT5B							
Low expression	185	78	1,271	Ref.		Ref.	
High expression	185	52	2,456	0.594 (0.418-0.845)	0.004 ^b	0.604 (0.408-0.895)	0.012 ^b
STAT6							
Low expression	185	75	1,372	Ref.		Ref.	
High expression	185	55	2,456	0.594 (0.418-0.843)	0.004 ^b	0.531 (0.357-0.790)	0.002 ^b

^aP-values were adjusted for radical resection, tumor stage and hepatitis B infection; ^bsignificant P-values and 95% CI. JAK, Janus kinase; STAT, signal transducer and activator of transcription; NA, not available; MST, median survival time; HR, hazard ratio; 95% CI, 95% confidence interval; Ref., reference; TYK2, tyrosine kinase 2.

tissue used to validate the mRNA expression levels of JAK2, STAT5A and STAT6 in tumor and non-tumor tissues. Due to the low sample size available for validation, it is clear that not all results were consistent with the data from the GSE14520 HBV infected-HCC cohort (Fig. S6). STAT6 was differentially expressed between tumor and non-tumor tissues (P=0.013), whereas JAK2 was not (P=0.245), which is in line with the GSE14520 cohort. For *STAT5A*, the results were not in accordance with the GSE14520 cohort, as it was not differentially

expressed between tumor and non-tumor tissues in the 21 tissue pairs (P=0.08); however, this is in line with the result of the TCGA cohort.

Discussion

In the present study, the diagnostic and prognostic clinical significance of the JAK/STAT signaling pathway in HCC was investigated using GSE14520, HBV-infected cohort and

Table III. Joint-effect analysis of prognosis-related genes for overall survival in the GSE14520 cohort.

Group	Expression			Overall survival			
	JAK2	STAT5A	STAT6	Events/total	MST (months)	Adjusted HR (95% CI)	Adjusted P-value ^a
1	Low	Low		34/61	32.6	Ref.	0.002 ^b
2	Low	High		33/90	NA	0.572 (0.340-0.961)	0.035 ^b
	High	Low					
3	High	High		15/61	NA	0.339 (0.181-0.638)	0.001 ^b
I	Low		Low	31/56	32.6	Ref.	<0.001 ^b
II	Low		High	37/100	NA	0.443 (0.269-0.728)	0.001 ^b
	High		Low				
III	High		High	14/56	NA	0.326 (0.171-0.619)	0.001 ^b
A		Low	Low	33/61	33.0	Ref.	0.001 ^b
B		Low	High	36/90	NA	0.569 (0.350-0.927)	0.023 ^b
		High	Low				
C		High	High	13/61	NA	0.287 (0.149-0.544)	<0.001 ^b
i	Low	Low	Low	21/34	28.2	Ref.	<0.0001 ^b
ii	Low	Low	High	35/76	NA	0.485 (0.277-0.851)	0.012 ^b
	Low	High	Low				
	High	Low	Low				
iii	Low	High	High	18/64	NA	0.263 (0.135-0.513)	<0.0001 ^b
	High	Low	High				
	High	High	Low				
iiii	High	High	High	8/38	NA	0.208 (0.090-0.478)	<0.001 ^b

^aP-values were adjusted for tumor size, cirrhosis, α -fetoprotein and Barcelona Clinic Liver Cancer stage; ^bsignificant P-values and 95% CI. JAK2, Janus kinase 2; STAT, signal transducer and activator of transcription; NA, not available; MST, median survival time; HR, hazard ratio; 95% CI, 95% confidence interval; Ref., reference.

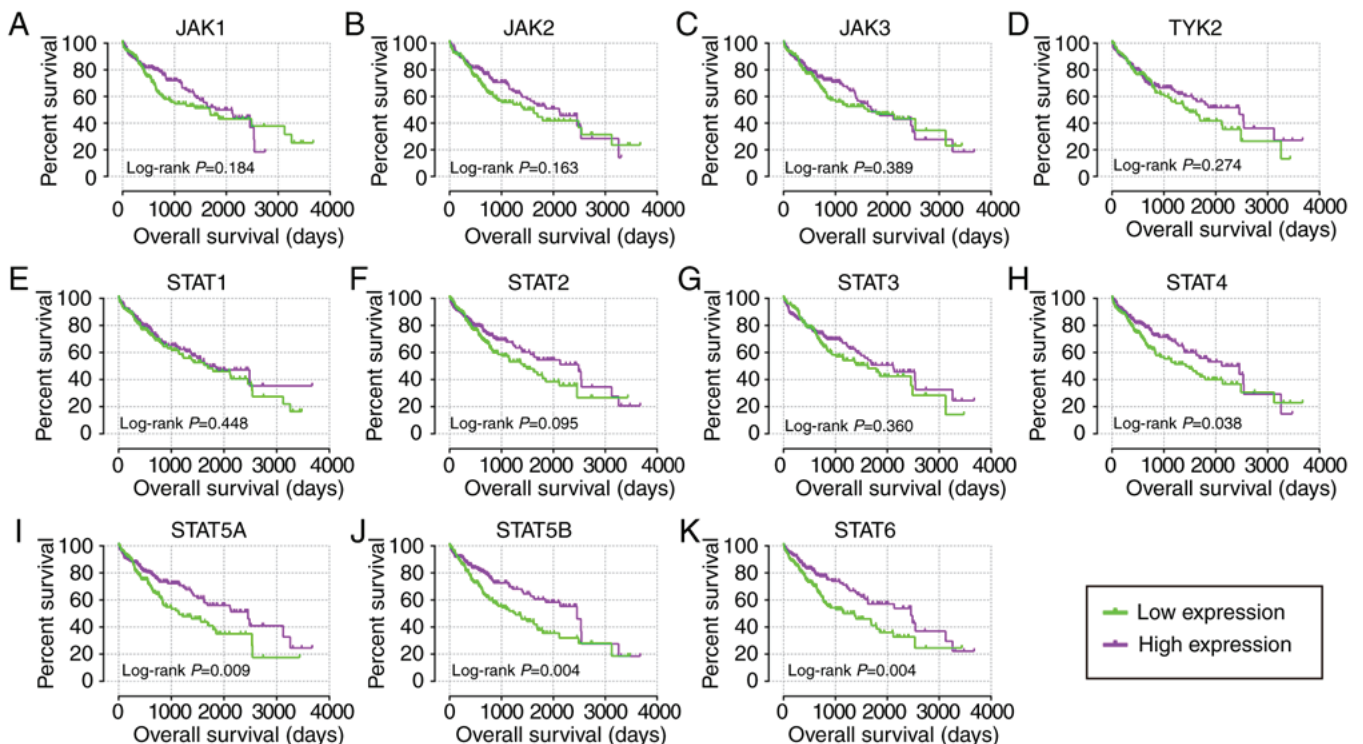


Figure 5. Kaplan-Meier plots of key genes of JAK/STAT pathway in The Cancer Genome Atlas cohort. (A) JAK1, (B) JAK2, (C) JAK3, (D) TYK2, (E) STAT1, (F) STAT2, (G) STAT3, (H) STAT4, (I) STAT5A, (J) STAT5B and (K) STAT6. JAK, Janus kinase; TYK2, tyrosine kinase 2; STAT, signal transducer and activator of transcription.

Table IV. Joint-effect analysis of prognosis-related genes for overall survival in The Cancer Genome Atlas cohort.

Group	Expression			Overall survival			
	JAK2	STAT5A	STAT6	Events/total	MST (days)	Adjusted HR (95% CI)	Adjusted P-value ^a
■	Low	Low		39/100	1,005	Ref.	0.004 ^b
■ ■	Low	High		63/170	1,423	0.703 (0.453-1.090)	0.115
	High	Low					
■ ■ ■	High	High		28/100	2,456	0.390 (0.223-0.682)	0.001 ^b
★	Low		Low	38/91	1,005	Ref.	<0.001 ^b
★ ★	Low		High	68/188	1,423	0.536 (0.345-0.834)	0.006 ^b
	High		Low				
★ ★ ★	High		High	24/91	2,456	0.285 (0.158-0.515)	<0.0001 ^b
●		Low	Low	45/94	802	Ref.	0.001 ^b
● ●		Low	High	57/182	1,685	0.542 (0.348-0.843)	0.007 ^b
		High	Low				
● ● ●		High	High	28/94	2,486	0.402 (0.238-0.677)	0.001 ^b
▲	Low	Low	Low	24/46	768	Ref.	<0.001 ^b
▲ ▲	Low	Low	High	50/147	1,852	0.516 (0.308-0.863)	0.012 ^b
	Low	High	Low				
	High	Low	Low				
▲ ▲ ▲	Low	High	High	44/123	1,560	0.357 (0.204-0.625)	<0.001 ^b
	High	Low	High				
	High	High	Low				
▲ ▲ ▲ ▲	High	High	High	12/54	3,258	0.208 (0.099-0.438)	<0.0001 ^b

^aP-values were adjusted for radical resection, tumor stage and hepatitis B infection; ^bsignificant P-values and 95% CI. JAK2, Janus kinase 2; STAT, signal transducer and activator of transcription; MST, median survival time; HR, hazard ratio; 95% CI, 95% confidence interval.

TCGA cohorts. JAK2, JAK3, TYK2, STAT1, STAT2, STAT3, STAT4, STAT5B and STAT6 genes had diagnostic significance for HCC in the GSE14520 cohort. Meanwhile, JAK1, JAK2, TYK2, STAT3, STAT4 and STAT5B showed diagnostic significance in the TCGA cohort. This indicates that JAK2, TYK2, STAT3, STAT4 and STAT5B may be diagnostic biomarkers for HCC. JAK2, STAT1, STAT3, STAT5A and STAT6 in the GSE14520 cohort, and JAK1, JAK2, STAT5A, STAT5B and STAT6 in TCGA cohort, were correlated with HCC prognosis, indicating that JAK2, STAT5A and STAT6 may be potential prognostic biomarkers for HCC. The gene combination analyses for both diagnosis and prognosis have advantages over using any one gene alone. Additionally, molecular mechanism exploration using GSEA indicated that STAT6 was enriched in terms associated with the cell cycle and cell division, and the 'fatty acid metabolism', 'drug metabolism cytochrome P450' and 'PPAR signaling pathway' KEGG pathways. Additionally, the nomograms and risk score models were constructed and showed advantage for prediction of HCC prognosis. Furthermore, validation of expression of the prognosis-related genes from the two cohorts (*JAK2*, *STAT5A* and *STAT6*) using tumor and non-tumor tissues samples. *STAT6* was differentially expressed between tumor and non-tumor tissues, but *JAK2* was not.

The JAK/STAT signaling pathway is a principal signaling transduction pathway stimulated by many crucial cytokines involved in sepsis, including interferon- γ , and interleukin-4,

-6, -10 and -12 (33-38). By binding with the corresponding receptor, several cytokines activate JAK kinases, which can selectively phosphorylate STATs (33). Subsequently, the STAT proteins translocate to the nucleus and mediate the transcription of target genes (33). The JAK/STAT signaling pathway consists of the JAK and STAT gene families. The JAK gene family is composed of JAK1, JAK2, JAK3 and TYK2, while the STAT gene family is made up of STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6 (33). Three members of the JAK family, JAK1, JAK2 and JAK3, are expressed universally, whereas TYK2 was found to be mainly expressed in the hematopoietic cells (39,40). The Src-homology 2 (SH2) domain is an ~100-residue motif that binds to phosphotyrosine residues and provides a mechanism for reading the code (41). Additionally, it has a crucial role in the recruitment of the cytokine receptors and recognition of specific receptor phosphotyrosine motifs. The SH2 domain is involved in JAK activation and STAT dimerization (42,43).

Complete deficiency of STAT3, STAT5A and STAT5B is a lethal event in mice, which is also consistent with the broad and crucial roles of these proteins (44). Other members, STAT1, STAT2, STAT4, and STAT6, have more restricted functions. They tend to have important roles in host defense and immunoregulation (44). More specifically, STAT3 localizes to mitochondria where it promotes oxidative phosphorylation and membrane permeability (45). This effect is dependent on serine phosphorylation, not tyrosine phosphorylation. STATs

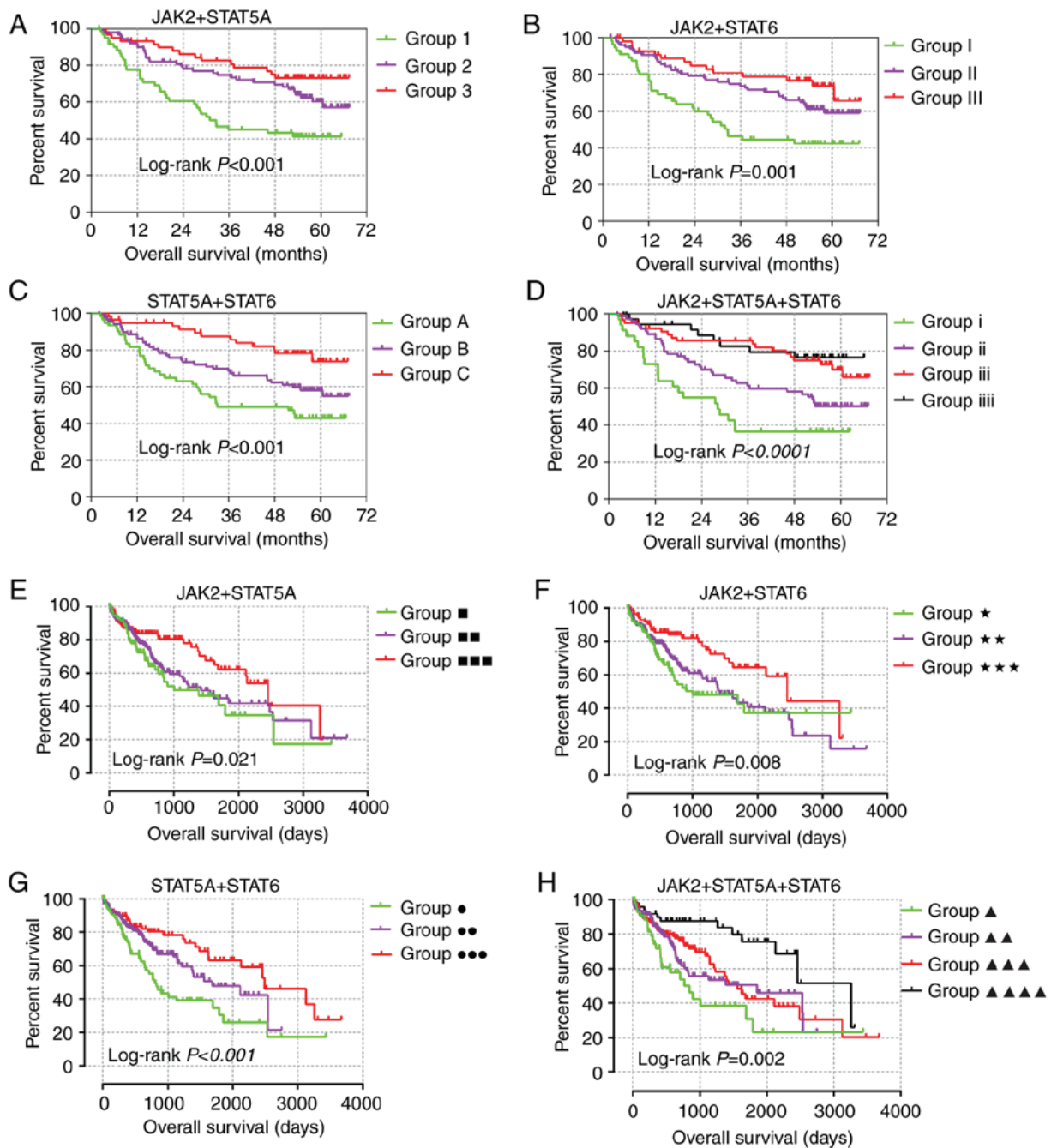


Figure 6. Joint-effect analysis of prognosis-related genes in two cohorts. Joint-effect analysis of (A) JAK2 + STAT5A, (B) JAK2 + STAT6, (C) STAT5A + 6, (D) JAK2 + STAT5A + 6 in the GSE14520 cohort; Joint-effect analysis of (E) JAK2 + STAT5A, (F) JAK2 + STAT6, (G) STAT5A + 6 and (H) JAK2 + STAT5A + 6 in The Cancer Genome Atlas cohort, respectively.

are considered to be associated with environments where cellular respiration is changed, including cellular stress and cancer (46).

Consecutive activation of JAKs and STATs was discovered and shown to be associated with malignancies in the 1990s (47). STAT hyperactivity, typically including STAT3 and STAT5, is considered to be able to induce cellular transformation downstream of classic oncogenic markers, such as BCR-ABL, Src and Ras. STAT hyperactivity is also recognized as a defining characteristic of most solid and blood malignancies (48). JAK1 mutation was revealed to be associated with the development of acute myeloid leukemia (49). Following knockdown of leukemia inhibitory factor receptor,

phosphorylation of JAK1 was increased and promoted HCC metastasis (50). Long non-coding RNA 00152 was reported to activate the JAK2-STAT3 signaling pathway to promote the development of HCC (51). JAK2/STAT3 signaling was reported to be activated by long non-coding RNA HOST2 and linked to epithelial-mesenchymal transition, proliferation, migration and invasion capacity of HCC cells (52). An increased protein level of JAK3 was observed when evaluating the anti-tumor immunostimulatory activity of polysaccharides from *Salvia chinensis* Benth in mice bearing HCC cells (53). TYK2/STAT3 signaling was previously reported to be associated with poor prognosis of HCC via high expression of claudin-17 (54). STAT1 was reported reduce

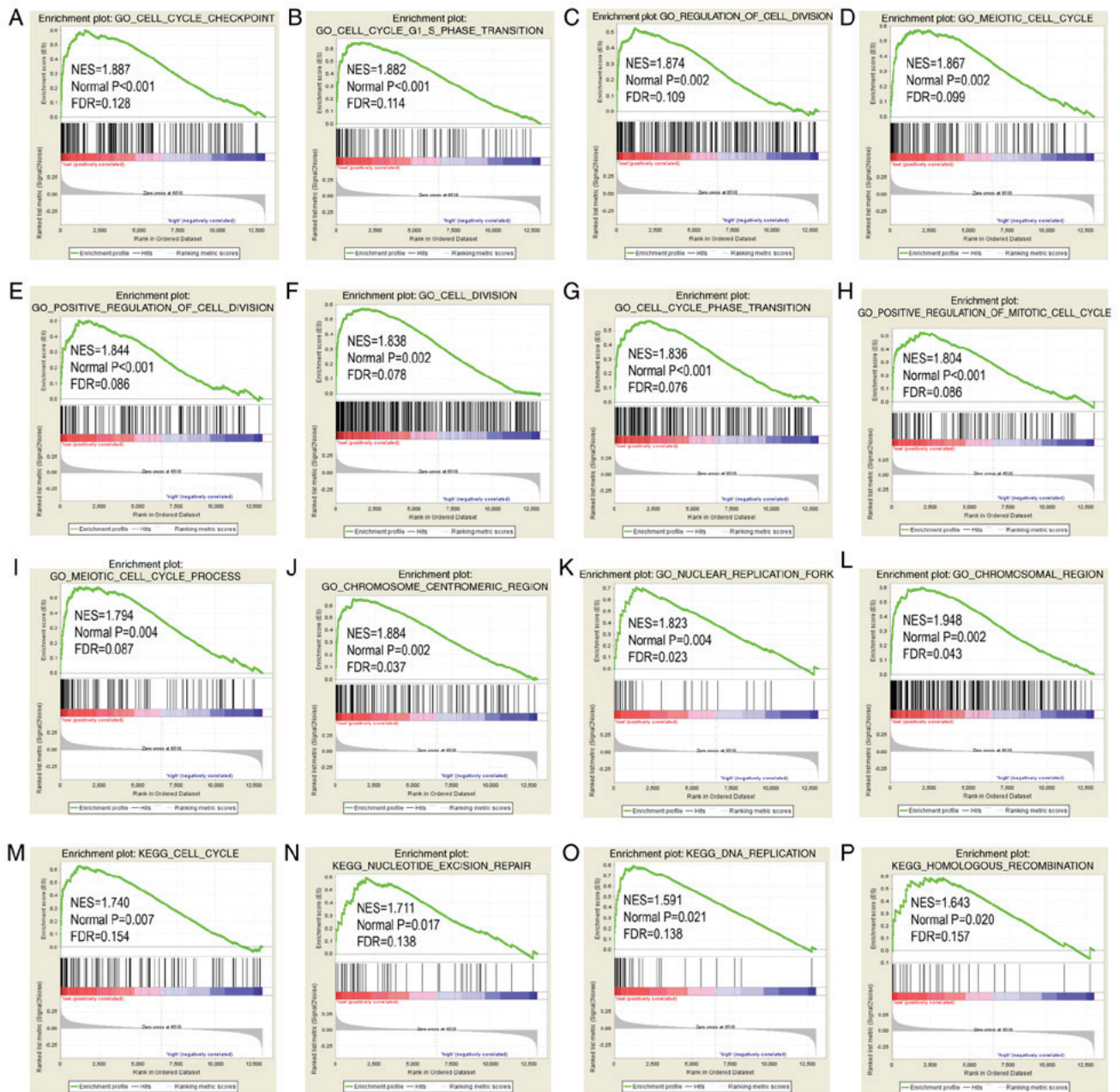


Figure 7. GO and KEGG pathway results of STAT6 in the GSE14520 cohort. (A-L) GO term and (M-P) KEGG pathways in gene set enrichment analysis. STAT6, signal transducer and activator of transcription 6; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

the growth ability of HepG2 cell by regulating p53-mediated cell cycling and apoptosis (55). STAT1 and STAT2 were demonstrated to decrease the growth capacity of HepG2 cells via increased expression stimulated by phosphatidylethanolamine (56). The rs7574865 polymorphism in STAT4 was found to be a risk factor for HCC in a meta-analysis (57). Additionally, somatic mutation of JAK2 was found to be associated with a number of hematologic tumors (58,59), while gain-of-function JAK2 mutations are involved in myeloproliferative malignancies (60). JAK3 mutations were found to be associated with leukemia and lymphoma (58), and secondary mutations in JAK3 were identified in juvenile myelomonocytic leukemia. Furthermore, JAK3 is considered to be associated with disease progression (61). Mutations in JAK3 and TYK2 have been reported to be associated

with primary immunodeficiency, such as severe combined immune deficiency (44). While patients with STAT1 mutations are susceptible to mycobacterial and virus infections, STAT2 mutations predispose patients to virus infection, and individuals with STAT3 mutations are susceptible to fungal infection (44). It is well established that STATs can directly bind to DNA and, thus, function as classical transcription factors (62). This is in line with the GSEA results of the present study that showed that STATs are involved in DNA replication and replication fork.

Next-generation sequencing has led to an explosion of genome-wide association studies (GWAS) in which disease occurrence has been associated with single nucleotide polymorphisms (SNPs) in affected populations (63). As such, GWAS technology has identified SNPs within STAT genes,



Figure 8. GO and KEGG pathway results of STAT6 in The Cancer Genome Atlas cohort. (A-L) GO term and (M-P) KEGG pathways in gene set enrichment analysis. STAT6, signal transducer and activator of transcription 6; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

with STAT3 SNPs associated with Crohn's disease, psoriasis and ankylosing spondylitis, STAT4 SNPs associated with rheumatoid arthritis, Crohn's disease, systemic lupus erythematosus and Sjogren's syndrome, and STAT6 SNPs associated with asthma (62). SNPs in STAT3 have been identified to be associated with Crohn's disease, Sjogren's syndrome and systemic lupus erythematosus, while SNPs in STAT6 have been associated with asthma (64,65). JAK2/STAT5A/B signaling tends to promote metastatic progression of prostate cancer by inducing epithelial-mesenchymal transition and stem cell properties (66). Additionally, interleukin 17 stimulates a REG3 β /JAK2/STAT3 inflammatory pathway seems to promote the transition from chronic pancreatitis to pancreatic cancer (67).

GSEA in the current study explored specific mechanism, demonstrating that STAT6 is involved in the cell cycle, DNA

replication and lipid metabolism, which is in agreement with results of KEGG pathway. High expression of STAT3 indicated a poor prognosis for HCC in multivariate analysis, which is in line with exiting studies (68-70). Existing evidence also indicates that STAT6 is involved in the development of HCC and could serve as a predictor of poor prognosis for patients with HCC (71). In the present study, high expression of STAT6 serves as a predictor of good prognosis. Unlike the results of Shi *et al* (72) in which JAK2 deficiency in mice had a protective role against HCC, the results of the current study indicated that high expression of JAK2 actually provides protection against HCC. Therefore, further studies are needful to further explore specific mechanisms of JAK2 in cancer. In addition, diagnostic ability analysis was performed to demonstrate that JAK2, TYK2, STAT3, STAT4 and STAT5B may serve as

Table V. Risk score model construction of genes in GSE14520 dataset and TCGA database.

A, GSE14520					
Variable	β	SE	Wald	HR (95% CI)	P-value
Tumor size	0.038	0.269	0.020	1.039 (0.613-1.761)	0.886
Cirrhosis	-1.072	0.736	2.122	0.342 (0.081-1.448)	0.145
BCLC stage					
0			28.571		<0.0001
A	1.440	0.726	3.938	4.223 (1.018-17.518)	0.047
B	1.828	0.789	5.362	6.219 (1.324-29.209)	0.021
C	2.976	0.780	14.551	19.612 (4.25-90.496)	0.0001
AFP	0.194	0.230	0.717	1.214 (0.774-1.904)	0.397
JAK2	-0.500	0.239	4.398	0.606 (0.380-0.968)	0.036
STAT5A	-0.592	0.239	6.127	0.553 (0.346-0.884)	0.013
STAT6	-0.616	0.231	7.101	0.540 (0.343-0.850)	0.008
B, TCGA					
Variable	β	SE	Wald	HR (95% CI)	P-value
Tumor stage					
I			8.832		0.012
II	0.249	0.265	0.881	1.283 (0.763-2.159)	0.348
III + IV	0.669	0.227	8.673	1.952 (1.251-3.045)	0.003
Radical resection	0.266	0.360	0.548	1.305 (0.645-2.642)	0.459
HBV infection	-0.854	0.266	10.305	0.426 (0.253-0.717)	0.001
JAK2	-0.567	0.200	8.045	0.567 (0.383-0.839)	0.005
STAT5A	-0.320	0.206	2.405	0.726 (0.485-1.088)	0.121
STAT6	-0.631	0.205	9.507	0.532 (0.356-0.795)	0.002

TCGA, The Cancer Genome Atlas; SE, standard error; HR, hazard ratio; 95% CI, 95% confidence interval; BCLC, Barcelona Clinic Liver Cancer; HBV, hepatitis B virus; JAK2, Janus kinase 2; STAT, signal transducer and activator of transcription.

potential diagnostic biomarkers, which has not been examined previously.

In the validation cohort analyzed via RT-qPCR, three genes, *JAK2*, *STAT5A* and *STAT6*, consistently showed elevated expression in tumor tissues compared with in non-tumor tissues. Nonetheless, these results were not consistent with previous results in the GSE14520 and TCGA cohorts. Racial difference, patient numbers and unknown differences in the clinicopathological characteristics may be the main factors causing this inconsistency.

The present study had some limitations that should be recognized. Firstly, there should be additional datasets used to validate these findings. Additionally, further investigation should use a multi-centre and multi-racial validation cohort to explore clinical significance. Finally, there is a need to designate for functional trials to investigate the mechanisms that *JAK2*, *STAT5A* and *STAT6* are involved in, including the proliferation, invasion and metastasis ability of HCC.

The present study revealed that genes in JAK/STAT signaling pathway have diagnostic and prognostic clinical significance for HCC. In particular, *JAK2*, *TYK2*, *STAT3*,

STAT4 and *STAT5B* may be potential diagnostic biomarkers for HCC. Meanwhile, *JAK2*, *STAT5A* and *STAT6* may be potential prognostic biomarkers for HCC. A combination analysis of multiple genes for diagnosis and prognosis has advantages over any one gene alone. Additionally, molecular mechanism exploration using GSEA indicated that *STAT6* was enriched in cell cycle, cell division, fatty acid metabolism, drug metabolism cytochrome P450 and in the PPAR signaling pathway. Nomograms and risk score models were generated for HCC prognosis prediction. *STAT6* was differentially expressed between tumor and non-tumor tissues in the validation cohort, which confirmed the result from the GSE14520 cohort. In future studies, well-designed functional trials are required to confirm the mechanisms by which these genes are involved in HCC development, particularly *STAT6*.

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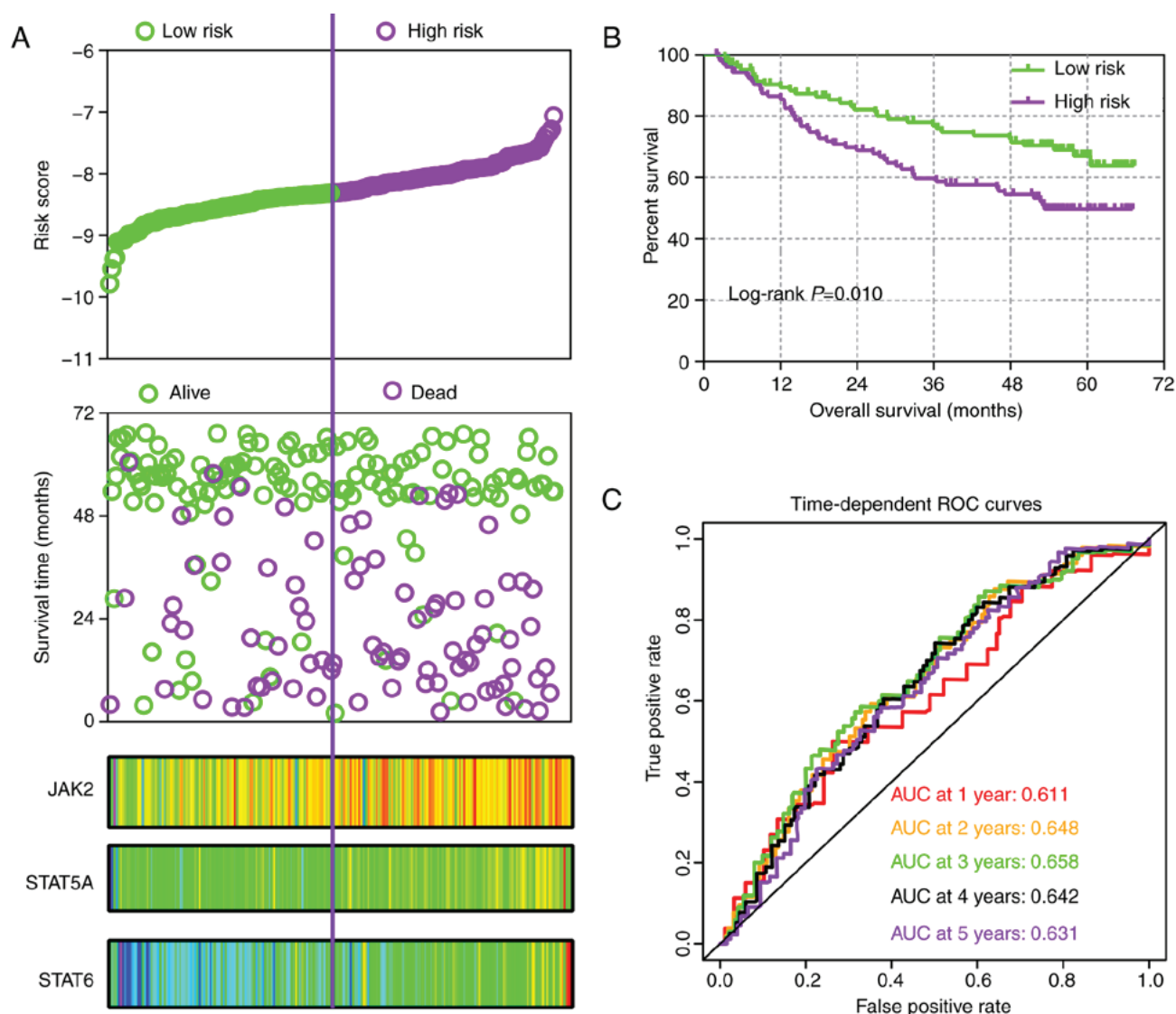


Figure 9. Risk score model, Kaplan-Meier plot, and survival ROC curves in GSE14520 cohort. (A) Risk score model including risk score ranking, survival status, and JAK2, STAT5A and STAT6 heat map. (B) Kaplan-Meier plot of low and high risk score groups. (C) Time-dependent ROC curves at 1, 2, 3, 4 and 5 years. JAK, Janus kinase; STAT, signal transducer and activator of transcription; ROC, receiver operating characteristic; AUC, area under the curve.

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Availability of data and materials

The datasets analyzed during the current study are available from the corresponding author on reasonable request.

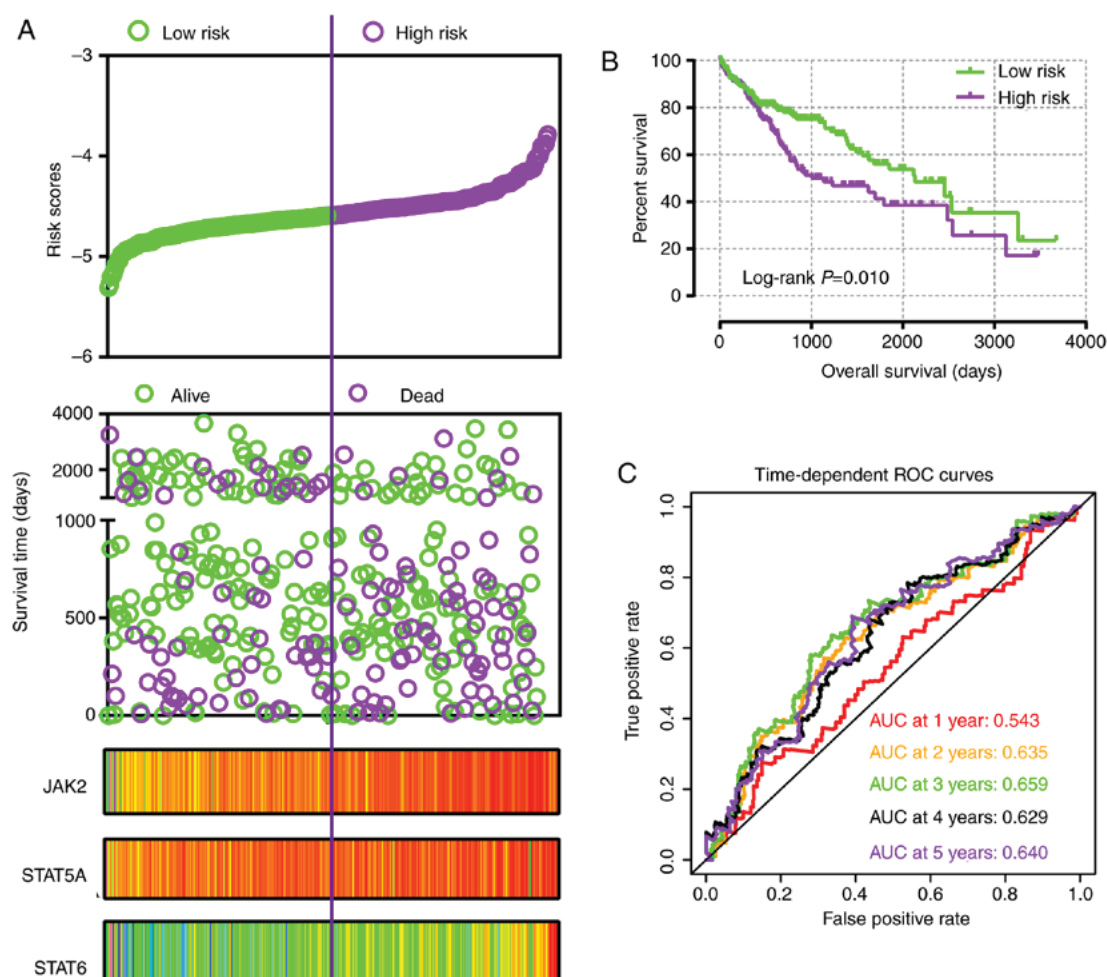


Figure 10. Risk score model, Kaplan-Meier plot, and survival ROC curves in The Cancer Genome Atlas cohort. (A) Risk score model including risk score ranking, survival status, and JAK2, STAT5A and STAT6 heat map. (B) Kaplan-Meier plot of low and high risk score groups. (C) Time-dependent ROC curves at 1, 2, 3, 4 and 5 years. JAK, Janus kinase; STAT, signal transducer and activator of transcription; ROC, receiver operating characteristic; AUC, area under the curve.

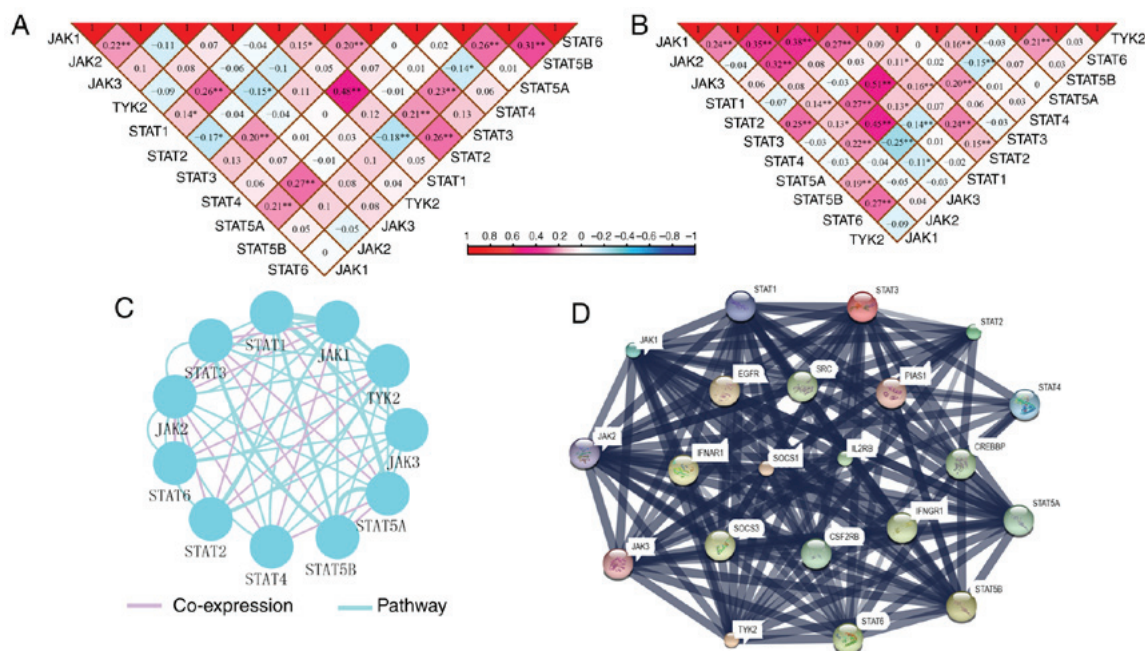


Figure 11. Correlation matrix and interactive networks among JAK/STAT pathway members. Correlation matrix of key genes of JAK/STAT pathway in the (A) GSE14520 and (B) The Cancer Genome Atlas cohorts. (C) Co-expression and pathway interactions of key genes of JAK/STAT pathway. (D) Protein-protein interaction network. * $P \leq 0.05$, ** $P \leq 0.01$. JAK, Janus kinase; TYK2, tyrosine kinase 2; STAT, signal transducer and activator of transcription.

Author contributions

XW and TP designed this manuscript; XL, TY, YG, LZ, CH, JH, LY, CY, GZ, WQ, ZL, XZ, JL, QH and TP conducted the study and analyzed the data. XZ wrote the manuscript and TP guided the writing.

Ethics approval and patient consent to participate

This study was approved by the Ethical Review Committee of the First Affiliated Hospital of Guangxi Medical University [approval no. 2015 (KY-E-032)]. Informed consent was signed by all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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