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Expressional and prognostic value of cytokine receptor-like factor 3 in liver hepatocellular carcinoma patients via integrated bioinformatics analyses and experiments

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Abstract

Background: Liver hepatocellular carcinoma is a highly prevalent and lethal malignancy. The orphan cytokine receptor-like factor 3, although evolutionarily conserved and implicated in hematopoiesis and neuroprotection, remains poorly characterized in liver hepatocellular carcinoma.

Objective: To investigate the expression pattern of cytokine receptor-like factor 3 in liver hepatocellular carcinoma and its association with clinicopathological characteristics, prognosis, and potential biological functions through bioinformatics analysis.

Methods: Cytokine receptor-like factor 3 mRNA and protein expression in liver hepatocellular carcinoma were evaluated using the cancer genome atlas, human protein atlas, immunohistochemistry, and qPCR. The association of cytokine receptor-like factor 3 expression with prognosis and clinicopathological features was assessed using Kaplan–Meier survival analysis, Cox regression, logistic regression, and receiver operating characteristic curves. Potential functional pathways associated with cytokine receptor-like factor 3 were explored using gene ontology, Kyoto encyclopedia of genes and genomes, gene set enrichment analysis, and ssGSEA.

Results: Cytokine receptor-like factor 3 expression was significantly elevated in liver hepatocellular carcinoma tissues and correlated with poorer overall survival, disease-specific survival, and progression-free interval. High cytokine receptor-like factor 3 expression was associated with advanced T stage, pathologic stage, histologic grade, and elevated alpha-fetoprotein levels, and emerged as an independent prognostic factor. Receiver operating characteristic curve analysis suggested a potential diagnostic value for cytokine receptor-like factor 3 in distinguishing liver hepatocellular carcinoma tissues from normal tissues. Enrichment analysis indicated that genes correlated with cytokine receptor-like factor 3 are enriched in pathways such as PI3K/Akt, Wnt, and JAK/STAT, as well as immune-related processes. Notably, cytokine receptor-like factor 3 expression showed a strong positive correlation with Th2 cell infiltration.

Conclusions: This study reveals that cytokine receptor-like factor 3 is overexpressed in liver hepatocellular carcinoma and is associated with poor patient prognosis and immune infiltration. These findings suggest that cytokine receptor-like factor 3

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may serve as a promising candidate prognostic biomarker and warrants further investigation as a potential immunotherapeutic target in liver hepatocellular carcinoma. The mechanistic hypotheses generated here provide a foundation for subsequent experimental validation.

Keywords
cytokine receptor-like factor 3 (*CRLF3*), liver hepatocellular carcinoma, overall survival, prognosis, diagnosis

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Introduction

With more than 800,000 fatalities each year, liver hepatocellular carcinoma (LIHC) is the fourth most common cause of cancer-related mortality worldwide.[1,2] It has been shown that developing countries have a higher prevalence of liver illnesses.[3] LIHC primarily arises in the context of chronic liver conditions, including hepatitis B and C virus infections, metabolic-associated fatty liver disease (formerly known as nonalcoholic fatty liver disease), and cirrhosis.[4] Currently, treatment outcomes for LIHC patients remain suboptimal, largely due to the high heterogeneity of the disease. This heterogeneity develops through tumor-initiating stem cells that undergo genetic and epigenetic changes, interact with alterations in the tumor microenvironment, and differentiate into diverse drug-resistant tumor cell populations, thereby affecting therapeutic efficacy.[5] Tumor heterogeneity refers to qualitative differences between tumors of the same type across different patients, as well as variations within a single tumor, including differences in genotype, phenotype, and function among regions and cells within the same patient.[5–7] Although significant progress has been made in the genomics and molecular classification of LIHC over the past decade, its heterogeneity has not been fully resolved due to the complexity of its regulatory mechanisms.[8–12] Currently, the clinical management of LIHC includes multiple therapeutic strategies, such as chemotherapy, immunotherapy, natural product-based treatments, and nanotechnology-based approaches. Despite these options, a definitive cure for LIHC remains elusive.[13] The integration of diverse tumor biomarkers, tailored to specific clinical contexts, is critical for accurate diagnosis, monitoring of treatment response, and prognostic assessment of primary liver cancer.[14] Therefore, the identification and validation of novel biomarkers continue to be essential for advancing these objectives.[15]

Currently used biomarkers for LIHC include alpha-fetoprotein (AFP), its isoform lectin-reactive AFP (AFP-L3), and des-gamma-carboxy prothrombin (DCP), also known as vitamin K-dependent protein induced by absence or antagonist-II.[16–18] However, the complex pathogenesis and heterogeneity of LIHC present significant challenges for its early detection. Consequently, only 20%–30% of LIHC patients are eligible for curative treatments, primarily due to the lack of reliable early-detection methods. This underscores the critical need for more dependable and precise biomarkers for LIHC.[19]

The cytokine receptor-like factor (*CRLF*) family consists of three members: *CRLF1*, *CRLF2*, and *CRLF3*. Evidence suggests that CRLF proteins are involved in the pathogenesis of various neoplastic conditions.[20–23] *CRLF3*, in particular, has been linked to multiple human diseases and is known to play roles in vertebrate hematopoiesis and neuroprotection in insects.[24] T studies have shown that *CRLF3* is essential for embryonic hematopoiesis during the early stages of zebrafish development.[25] In teleost fish, *CRLF3* promotes the degradation of TBK1, thereby negatively regulating antiviral immunity.[26] However, the precise molecular mechanisms by which *CRLF3* contributes to LIHC remain unclear.

In this study, we analyzed the transcriptional and protein expression patterns of *CRLF3* using the cancer genome atlas (TCGA) and human protein atlas (HPA) databases, and validated its expression in LIHC and adjacent normal tissues through immunohistochemistry (IHC) and qPCR. To further investigate the potential biological roles of *CRLF3*, we performed functional enrichment analyses using gene ontology (GO), Kyoto encyclopedia of genes and genomes (KEGG), and gene set enrichment analysis (GSEA). This study aims to investigate the potential mechanisms by which *CRLF3* may be involved in LIHC, with particular emphasis on its association with immune infiltration. Additionally, bioinformatics analyses were conducted to assess the diagnostic and prognostic significance of *CRLF3* in LIHC patients.

Materials and methods

Downloading and processing data from public databases

The dataset used in this study consisted of RNA-seq gene expression data from TCGA, including 374 LIHC tissues and 50 normal liver tissues. *CRLF3* protein expression levels in LIHC and normal liver tissues were analyzed using the HPA database (https://www.proteinatlas.org). Additionally, *CRLF3* expression in LIHC was evaluated using the HCCDB database (http://lifeome.net/database/hccdb). The prognostic value of *CRLF3* in LIHC was assessed in terms of overall survival (OS) using the Kaplan–Meier plotter (kmplot.com/analysis).

Functional enrichment analysis

The screened genes were subjected to functional enrichment analysis using the GO and KEGG databases.

GSEA evaluates the distribution patterns of genes within a predefined gene set across a ranked list of genes sorted by their association with a particular phenotype, in order to determine their contribution to that phenotype.[27] After converting the input data to standardized gene IDs, GSEA was performed using the clusterProfiler R package, with significance thresholds set at p adjust <0.05 and q-value <0.25.

Protein–protein interaction (PPI) network analysis

PPI network analysis was performed using the STRING database, with a minimum required interaction score set at 0.400.

Screening of differentially expressed genes (DEGs)

DEGs were identified using the DESeq2 R package, with significance thresholds set at adjusted p-value <0.05 and $|\log2(\text{fold change})| > 1$.[28] The raw counts matrix was first analyzed for variance using DESeq2 according to the standard protocol and subsequently normalized using the variance stabilizing transformation method provided by the DESeq2 package.

Immune cell infiltration of single-sample GSEA (ssGSEA)

The ssGSEA method was employed to quantify the infiltration levels of 24 immune cell types in LIHC tumor samples. Spearman's correlation test was used to assess the relationship between *CRLF3* expression and immune cell abundance.[29] Additionally, the Wilcoxon rank-sum test was applied to compare immune cell infiltration between high and low *CRLF3* expression groups.

Patients and samples

This study is an exploratory investigation that integrates systematic bioinformatics analyses with preliminary validation using clinical samples. All human tissue samples were obtained from the Department of Pathology at Hangzhou Xixi Hospital, comprising 25 tumor samples and 25 adjacent normal tissue samples. The study started in 2024 and ran for a duration of 10 months. Inclusion Criteria: Confirmed Diagnosis: Patients with LIHC confirmed by postoperative pathological examination. Surgical Type: Patients who underwent radical hepatectomy without prior anticancer treatment, including chemotherapy, radiotherapy, targeted therapy, or immunotherapy. Clinical Records: Availability of detailed clinicopathological information, including at minimum age, sex, tumor size, and differentiation grade. Exclusion Criteria: Pathology Type Mismatch: Exclusion of patients with mixed LIHC, intrahepatic cholangiocarcinoma, metastatic liver cancer, or other non-LIHC pathological types. Incomplete Clinical Information: Patients lacking essential clinicopathological data or follow-up information. Preoperative Treatment: Patients who received any antitumor therapy prior to surgery that could affect tumor antigen expression. Sample Quality Issues: Tissue sections unsuitable for accurate immunohistochemical analysis due to improper fixation, severe fading, damage, or insufficient tumor cell content.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from LIHC and adjacent normal tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Complementary DNA was synthesized using the PrimeScript RT reagent kit (Thermo Fisher Scientific, Waltham, MA, USA). qRT-PCR was performed using SYBR Green assays (Vazyme, Nanjing, China) on a StepOnePlus real-time PCR system (Life Technologies, Carlsbad, CA, USA). The primer sequences for *CRLF3* were as follows: forward 5′–GAAAGTGCATCACAGACAAGGG–3′ and reverse 5′–TCTGGCAGTCATCTAGTGGTTT–3′. GAPDH was used as the internal control. Relative *CRLF3* expression levels were calculated using the 2^−ΔΔCT method.

IHC and scoring analyses

IHC was performed to assess *CRLF3* protein expression and its prognostic significance. LIHC tissues and paired adjacent normal tissues were stained with anti-human *CRLF3* antibody (1:150; Rabbit; DF8931; Affinity, USA), followed by detection using a two-step universal kit (mouse/rabbit ultrasensitive polymer assay system; PV-800; ZSGB-BIO, Beijing, China). Stained samples were observed under a microscope and photographed for further analysis. Each LIHC sample was evaluated based on staining intensity and the percentage of positively stained cells. Staining intensity was scored as negative (0), mild (1), moderate (2), or strong (3). The proportion of positive cells was scored as $\leqslant$25% (1), $>$25% and $\leqslant$50% (2), $>$50% and $\leqslant$75% (3), or $>$75% (4). The sum of these two scores yielded the final IHC score for each sample. Immunohistochemical scoring was further validated using ImageJ software. A paired t-test was employed to compare *CRLF3* expression between LIHC tissues and matched nontumor tissues.

Single cell portal database analysis

The Single Cell Portal database (https://singlecell.broadinstitute.org/single_cell) is an online resource for the analysis and exploration of single-cell sequencing data.

Ethics approval and consent to participate

This study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of Hangzhou Xixi Hospital, Hangzhou Sixth People's Hospital, and Hangzhou Xixi Hospital affiliated with Zhejiang Chinese Medical University [Approval No. (2024)11]. Written informed consent was obtained from all participants.

Statistical analysis

All statistical analyses were performed using R software (version 4.2.1). The Wilcoxon rank-sum test and paired-samples *t*-test were applied to compare *CRLF3* expression between tumor and normal tissues. Univariate and multivariate analyses were conducted to evaluate the impact of clinical factors on OS. A p-value <0.05 was considered statistically significant.

Results

Protein and mRNA expression levels of CRLF3 in patients with LIHC

We initially assessed the expression levels and prognostic associations of *CRLF* family members in LIHC and corresponding adjacent tissues. *CRLF1, CRLF2*, and *CRLF3* were all significantly upregulated in LIHC tissues (Figure 1(a)). Notably, *CRLF3* showed both high expression in LIHC tissues and a significant correlation with poor OS (Figure 1(b)). Furthermore, *CRLF3* expression was elevated in LIHC tissues compared with paired adjacent or normal tissues (Figure 1(c) and (d)), leading us to select *CRLF3* as the primary focus of this study. CRLF3 protein expression was also higher in LIHC tissues than in normal tissues, as shown in the HPA database (Figure 1(e)), and these findings were further validated by immunohistochemical staining (Figure 1(f)). Receiver operating characteristic (ROC) curve analysis suggested a potential diagnostic value for *CRLF3*, yielding an area under the curve (AUC) of 0.823 (Figure 1(g)).

CRLF3 expression was also upregulated across multiple cancer types, suggesting a potential broad role in tumor biology (Figure 1(h)). Using the HCCDB database, we performed a comprehensive analysis of nine independent HCC cohorts. This analysis consistently showed a significant increase in *CRLF3* mRNA expression in HCC tissues compared with adjacent normal tissues (Figure 1(i)).

Association between CRLF3 expression and clinicopathologic characteristics in LIHC

The 374 LIHC samples were divided into high and low *CRLF3* mRNA expression groups. Analysis revealed significant associations between *CRLF3* expression and several clinicopathological parameters, including pathologic stage ($p=0.046$), age ($p=0.026$), histologic grade ($p<0.001$), and AFP levels ($p<0.001$). No significant associations were observed between *CRLF3* expression and TNM stage, gender, or BMI (Table 1).

Logistic regression analysis revealed significant associations between *CRLF3* expression and several clinical variables, including T stage (T2–T4 vs T1; OR = 1.701, $p=0.011$), pathologic stage (Stages II–IV vs Stage I; OR = 1.621, $p=0.025$), age (>60 vs ⩽60; OR = 0.629, $p=0.026$), and AFP levels (>400 vs ⩽400; OR = 3.562, $p<0.001$) (Table 2). Consistently, both Welch's *t*-test and Student's *t*-test analyses demonstrated significant correlations between *CRLF3* mRNA expression and clinical parameters such as AFP concentration, histologic grade, pathologic T stage, and overall pathologic stage (Figure 2(a)–(f)).

Prognostic value of CRLF3 expression in LIHC

We next evaluated the association between *CRLF3* mRNA expression and clinical outcomes in LIHC. High *CRLF3* expression was significantly associated with poorer (OS; [HR] = 1.51, $p=0.021$), disease-specific survival (DSS; HR = 1.62, $p=0.034$), and progression-free interval (HR = 1.39, $p=0.025$) (Figure 3(a)–(c)). Subgroup analysis based on clinicopathological characteristics revealed that *CRLF3* overexpression remained significantly associated with poorer OS in patients with advanced disease, including those at pathologic T3–T4 stages (HR = 1.75, $p=0.046$) and pathologic Stages III–IV (HR = 1.85, $p=0.037$) (Figure 3(d)–(k)). In contrast, no significant association was observed between *CRLF3* expression and AFP levels in LIHC patients (Figure 3(l)–(m)).

Univariate and multivariate Cox regression analyses demonstrated that high *CRLF3* expression was significantly associated with poorer (OS; HR = 1.507, $p=0.021$) and (DSS; HR = 1.622, $p=0.034$). Multivariate analysis further confirmed that elevated *CRLF3* expression serves as an independent prognostic factor in patients with LIHC (Tables 3 and 4). These findings suggest that *CRLF3* may serve as a potential prognostic biomarker for LIHC patients.

Predicted protein interaction network of CRLF3

To explore potential molecular associations of *CRLF3* in LIHC, we constructed a predicted PPI network using the STRING database (Figure 4(a)). Network analysis identified several potential interacting partners of *CRLF3* based on database predictions. The top-ranked interactors, based on composite confidence scores, included *UTP6* (score = 0.802), *TEFM* (score = 0.709), and *ATAD5* (score = 0.631). According to existing annotations, these proteins are involved in key cellular processes: *UTP6* in ribosome biogenesis, *TEFM* in mitochondrial transcription and energy metabolism, and *ATAD5* in DNA replication and genome stability. These predicted interactions suggest that *CRLF3* may be functionally

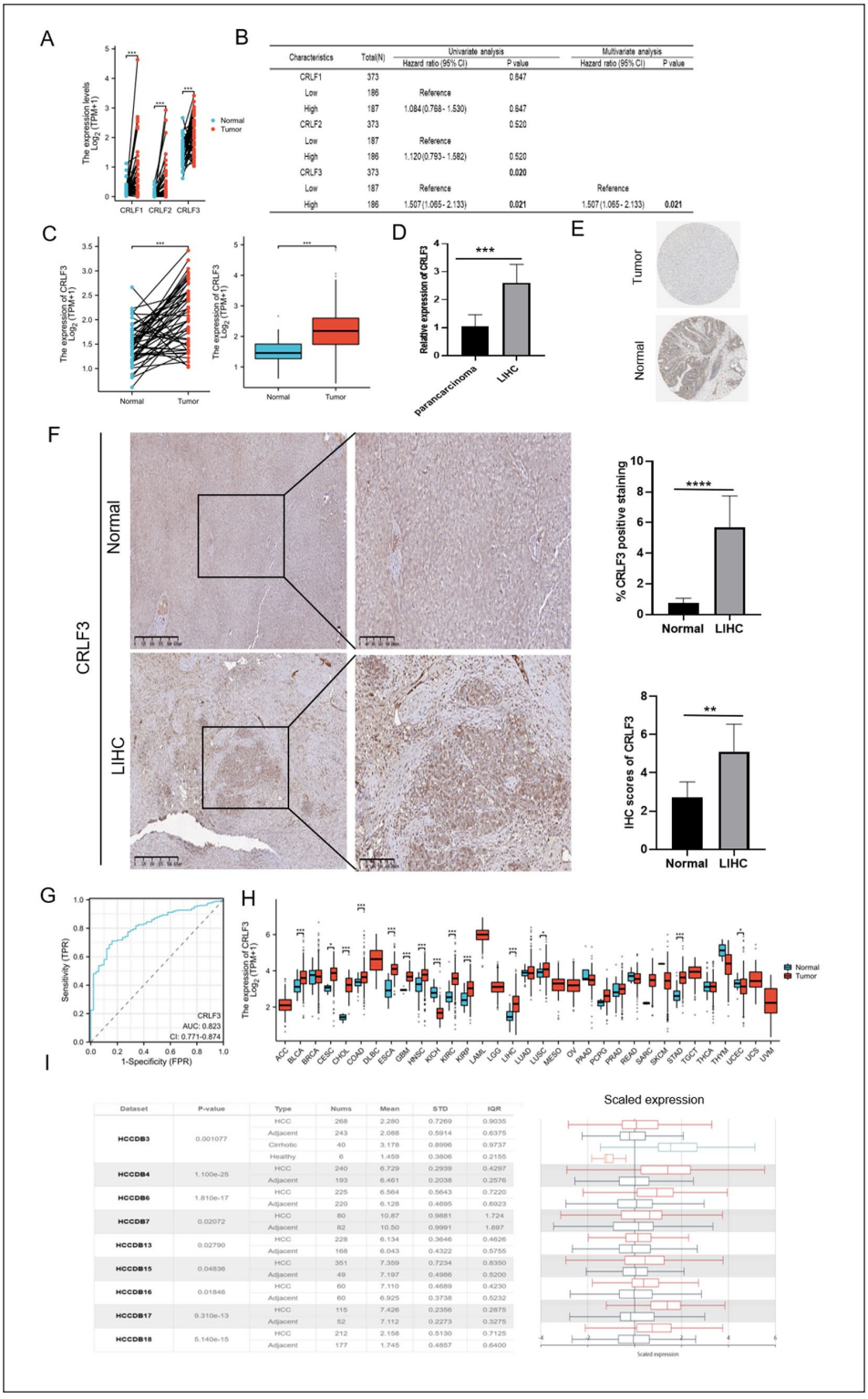

Figure 1. The expression of *CRLFs* in liver hepatocellular carcinoma tissues.

(a) The mRNA expression levels of each member of the CRLF family in liver hepatocellular carcinoma tissues. (b) Multivariate and Univariate Cox regression analysis of CRLF family members according to OS. (c) The mRNA expressions of *CRLF3* in LIHC samples and normal liver tissue. (d) Validation of *CRLF3* expression in cancer and paracancer tissues by q-PCR experiments. (e) The protein expression levels of *CRLF3* in LIHC tissues and normal tissues were analyzed using the HPA database. (f) Immunohistochemistry detecting the expression of *CRLF3* in LIHC and paired adjacent normal samples (Normal group: $n = 20$; Tumor group: $n = 20$). (g) The ROC curve for *CRLF3* in normal and LIHC samples. (h) Expression levels of *CRLF3* across several types of cancer. (i) In the Hepatromic cohort, the comparison was made between normal liver samples and LIHC samples. Data are mean ± SEM. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

CRLF3: cytokine receptor-like factor 3; LIHC: liver hepatocellular carcinoma; ROC: receiver operating characteristic.

Table 1. Relationship between the clinicopathologic features of LIHC and the expression of *CRLF3*.

Characteristics	Low expression of *CRLF3*	High expression of *CRLF3*	*p* Value
n	187	187	
Pathologic T stage, *n* (%)			0.089
T1	103 (27.8%)	80 (21.6%)	
T2	41 (11.1%)	54 (14.6%)	
T3	34 (9.2%)	46 (12.4%)	
T4	6 (1.6%)	7 (1.9%)	
Pathologic N stage, *n* (%)			0.625
N0	127 (49.2%)	127 (49.2%)	
N1	1 (0.4%)	3 (1.2%)	
Pathologic stage, *n* (%)			0.046
Stage I	98 (28%)	75 (21.4%)	
Stage II	40 (11.4%)	47 (13.4%)	
Stage III	35 (10%)	50 (14.3%)	
Stage IV	4 (1.1%)	1 (0.3%)	
Gender, *n* (%)			0.097
Male	134 (35.8%)	119 (31.8%)	
Female	53 (14.2%)	68 (18.2%)	
Age, *n* (%)			0.026
⩽60	78 (20.9%)	99 (26.5%)	
>60	109 (29.2%)	87 (23.3%)	
BMI, *n* (%)			0.851
⩽25	90 (26.7%)	87 (25.8%)	
>25	83 (24.6%)	77 (22.8%)	
Histologic grade, *n* (%)			<0.001
G1	36 (9.8%)	19 (5.1%)	
G2	100 (27.1%)	78 (21.1%)	
G3	45 (12.2%)	79 (21.4%)	
G4	4 (1.1%)	8 (2.2%)	
AFP (ng/ml), *n* (%)			<0.001
⩽400	128 (45.7%)	87 (31.1%)	
>400	19 (6.8%)	46 (16.4%)	

CRLF3: cytokine receptor-like factor 3; LIHC: liver hepatocellular carcinoma.

Table 2. The logistic regression analysis shows the relationship between clinicopathological characteristics and *CRLF3* expression.

Characteristics	Total (N)	OR (95% CI)	*p* value
Pathologic T stage (T2 and T3 and T4 vs T1)	371	1.701 (1.128–2.564)	0.011
Pathologic N stage (N1 vs N0)	258	3.000 (0.308–29.225)	0.344
Pathologic M stage (M1 vs M0)	272	0.349 (0.036–3.394)	0.364
Pathologic stages (Stage II and Stage III and Stage IV vs Stage I)	350	1.621 (1.063–2.472)	0.025
Histological type (Hepatocellular carcinoma and Hepatocholangiocarcinoma (mixed) vs Fibrolamellar carcinoma)	374	0.497 (0.045–5.532)	0.570
Histologic grades (G2 and G3 and G4 vs G1)	369	2.098 (1.154–3.817)	0.015
Tumor status (With tumor vs Tumor free)	355	1.465 (0.961–2.235)	0.076
Residual tumor (R1 and R2 vs R0)	345	1.681 (0.636–4.443)	0.295
Gender (Female vs Male)	374	1.445 (0.934–2.234)	0.098
Race (Black or African American and White vs Asian)	362	0.965 (0.637–1.462)	0.867
Age (>60 vs ⩽60)	373	0.629 (0.418–0.947)	0.026
AFP (ng/ml) (>400 vs ⩽400)	280	3.562 (1.955–6.490)	< 0.001
Vascular invasion (Yes vs No)	318	1.517 (0.953–2.413)	0.079
Adjacent hepatic tissue inflammation (mild and severe vs none)	237	1.250 (0.749–2.087)	0.393

AFP: alpha-fetoprotein; *CRLF3*: cytokine receptor-like factor 3.

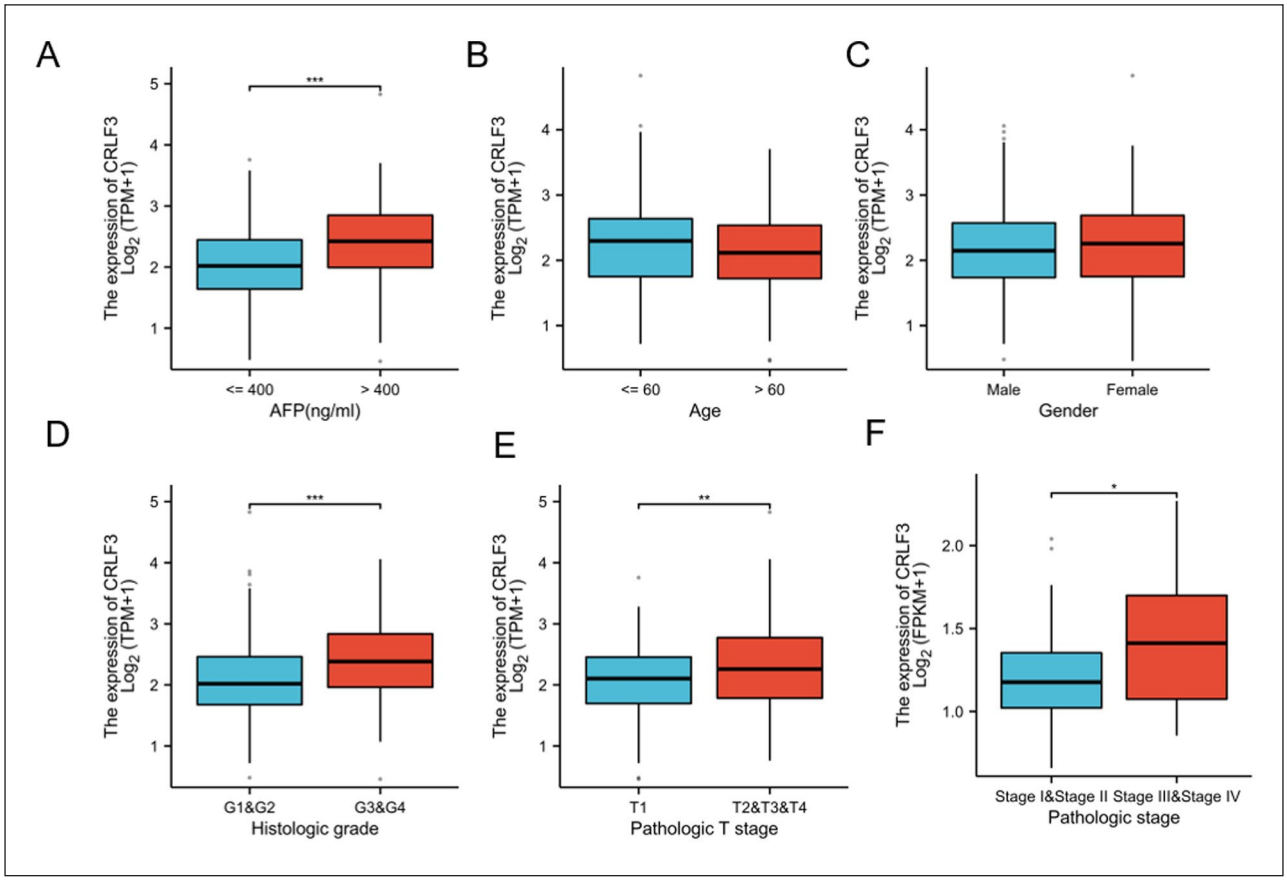

Figure 2. The box plots show different characteristics. According to the expression level of *CRLF3* mRNA. (a) AFP, (b) Age, (c) Gender, (d) histologic grade, (e) pathologic T stage, (f) Pathologic stage. Data are mean ± SEM. *$p < 0.05$; **$p < 0.01$;***$p < 0.001$.
AFP: alpha-fetoprotein; *CRLF3*: cytokine receptor-like factor 3.

linked to fundamental processes governing cell growth, proliferation, and survival, although this remains to be experimentally validated.

To assess the potential clinical relevance of these predicted CRLF3-interacting proteins, we analyzed their prognostic associations in LIHC using the Kaplan–Meier plotter database (Figure 4(b)–(d)). Survival analysis showed that high expression of most of these proteins, including *UTP6*, *TEFM*, and *ATAD5*, was significantly associated with poorer OS in LIHC patients. These findings suggest a potential coordinated prognostic role for this predicted network, but experimental studies are needed to confirm whether *CRLF3* functionally interacts with these proteins to influence LIHC progression.

Functional enrichment analysis

To further explore the potential biological processes and pathways associated with *CRLF3*, we performed GO, KEGG, and GSEA analyses using genes correlated with *CRLF3* expression. GO enrichment analysis showed that genes correlated with *CRLF3* were significantly enriched in immune-related processes (Figure 5(a)). KEGG pathway analysis indicated that these genes were predominantly enriched in four signaling pathways known to be closely linked to cancer progression (Figure 5(a)). GSEA further revealed significant enrichment of *CRLF3*-correlated genes in multiple pathways, including PI3K–Akt signaling, Wnt signaling, FcεRI-mediated NF-κB activation, activation of the intestinal immune network for IgA production, interactions between immune cells and microRNAs in the tumor microenvironment, and JAK/STAT signaling pathways (Figure 5(b)–(g)). These enrichment results suggest potential functional links between *CRLF3* and these pathways, providing hypotheses for future experimental investigation.

Correlation between CRLF3 *and immune cell infiltration*

The association between *CRLF3* expression and the infiltration levels of 24 immune cell types in LIHC was analyzed using the ssGSEA method (Figure 6(a)). *CRLF3* expression was significantly positively correlated with the infiltration levels of T helper cells ($R = 0.515$, $p < 0.001$) and Th2 cells ($R = 0.414$, $p < 0.001$) (Figure 6(b) and (c)).

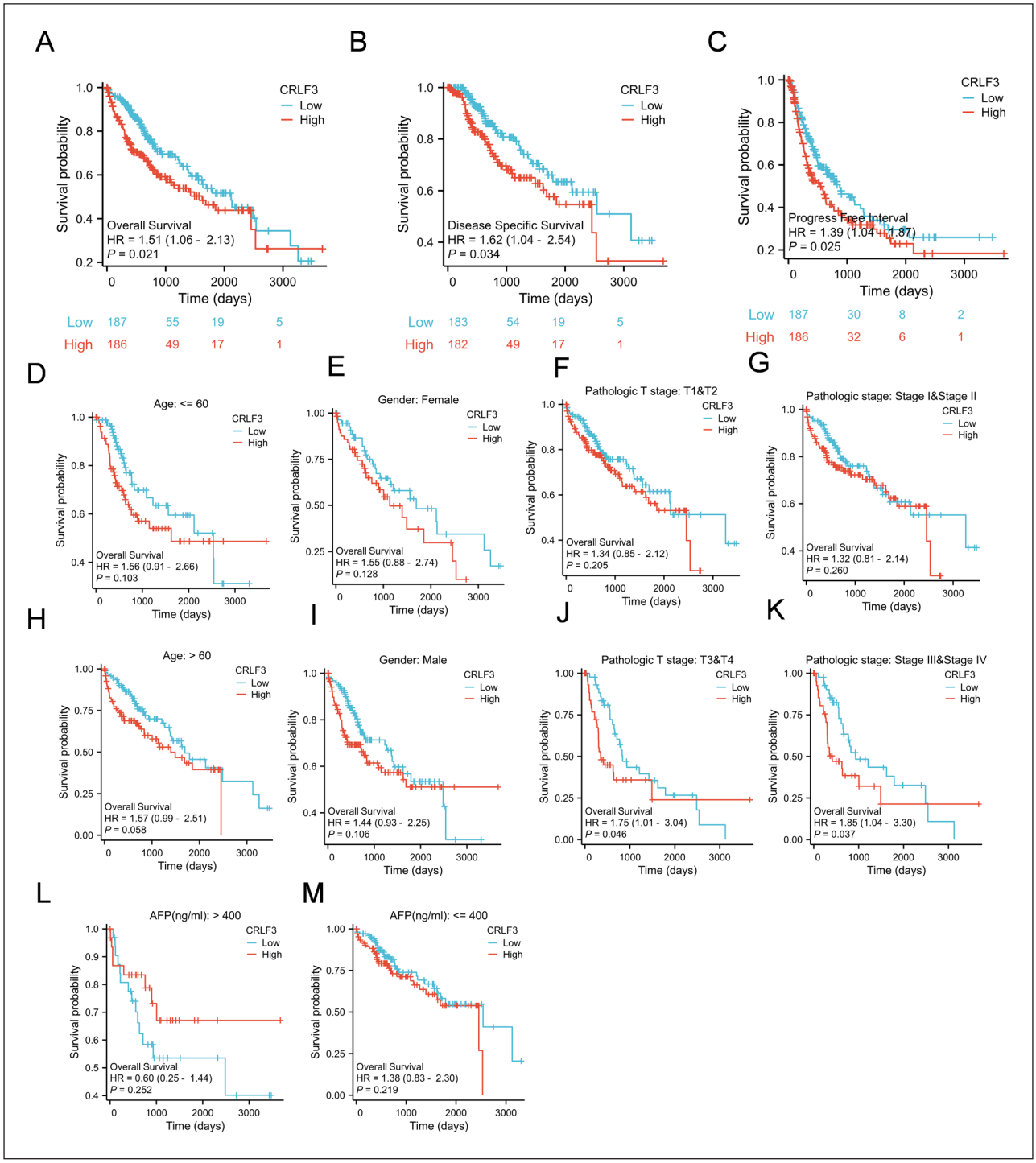

Figure 3. Correlations between *CRLF3* and prognosis in LIHC. (a) OS, (b) DSS,(c) PFI, (d) Age: ⩽60, (e) Gender: Female, (f) T stage: T1 andT2, (g) Pathologic stage: Stages I and II, (h) Age: >60, (i) Gender: Male, (j) T stage: T3 and T4, (k) Pathologic stage: Stages III–IV, (l) AFP (ng/ml): >400, (m) AFP (ng/ml): ⩽400.

AFP: alpha-fetoprotein; *CRLF3*: cytokine receptor-like factor 3; LIHC: liver hepatocellular carcinoma; OS: overall survival; PFI: progression-free interval.

The Wilcoxon rank-sum test was used to compare differences in immune cell infiltration between high and low *CRLF3* expression groups. The results confirmed that T helper cells and Th2 cells were significantly more abundant in the high *CRLF3* expression group (Figure 6(d) and (e)). Additionally, we examined *CRLF3* expression across immune cell subpopulations using single-cell datasets (Figure 6(f)).

Table 3. Cox proportional risk models with univariate and multivariate variables were used to analyze the factors influencing OS.

Characteristics	Total (N)	Univariate analysis		Multivariate analysis	
		Hazard ratio (95% CI)	*p* Value	Hazard ratio (95% CI)	*p* Value
Pathologic T stage	370				
T2 and T3 and T4	187	Reference		Reference	
T1	183	0.470 (0.328–0.675)	<0.001	0.726 (0.449–1.173)	0.191
Pathologic stage	349				
Stages III and IV	90	Reference		Reference	
Stages I and II	259	0.399 (0.275–0.579)	<0.001	0.489 (0.305–0.785)	0.003
Histologic grade	368				
G1 and G2	233	Reference			
G3 and G4	135	1.091 (0.761–1.564)	0.636		
Gender	373				
Male	252	Reference			
Female	121	1.261 (0.885–1.796)	0.200		
Age	373				
⩽60	177	Reference			
>60	196	1.205 (0.850–1.708)	0.295		
BMI	336				
⩽25	177	Reference			
>25	159	0.798 (0.550–1.158)	0.235		
AFP (ng/ml)	279				
⩽400	215	Reference			
>400	64	1.075 (0.658–1.759)	0.772		
Vascular invasion	317				
No	208	Reference			
Yes	109	1.344 (0.887–2.035)	0.163		
CRLF3	373				
Low	187	Reference		Reference	
High	186	1.507 (1.065–2.133)	0.021	1.466 (1.015–2.116)	0.041

AFP: alpha-fetoprotein; *CRLF3*: cytokine receptor-like factor 3; OS: overall survival.

Table 4. Cox proportional risk models with univariate and multivariate variables were used to analyze the factors influencing DSS.

Characteristics	Total (N)	Univariate analysis		Multivariate analysis	
		Hazard ratio (95% CI)	*p* Value	Hazard ratio (95% CI)	*p* Value
Pathologic T stage	362				
T2 and T3 and T4	182	Reference		Reference	
T1	180	0.353 (0.218–0.573)	<0.001	0.680 (0.344–1.343)	0.267
Pathologic stage	341				
Stages III and IV	87	Reference		Reference	
Stages I and II	254	0.263 (0.162–0.427)	<0.001	0.330 (0.175–0.622)	<0.001
Histologic grade	360				
G1 and G2	227	Reference			
G3 and G4	133	1.086 (0.683–1.728)	0.726		
Gender	365				
Male	247	Reference			
Female	118	1.230 (0.780–1.937)	0.373		
Age	365				
⩽60	174	Reference			
>60	191	0.846 (0.543–1.317)	0.458		

(continued)

Table 4. (continued)

Characteristics	Total (N)	Univariate analysis		Multivariate analysis	
		Hazard ratio (95% CI)	*p* Value	Hazard ratio (95% CI)	*p* Value
BMI	329				
⩽25	175	Reference			
>25	154	0.826 (0.512–1.330)	0.431		
AFP (ng/ml)	275				
⩽400	214	Reference			
>400	61	0.867 (0.450–1.668)	0.668		
Vascular invasion	309				
No	204	Reference			
Yes	105	1.277 (0.707–2.306)	0.418		
CRLF3	365				
Low	183	Reference		Reference	
High	182	1.622 (1.037–2.537)	0.034	1.671 (1.024–2.727)	0.040

AFP: alpha-fetoprotein; *CRLF3*: cytokine receptor-like factor 3; DSS: disease-specific survival.

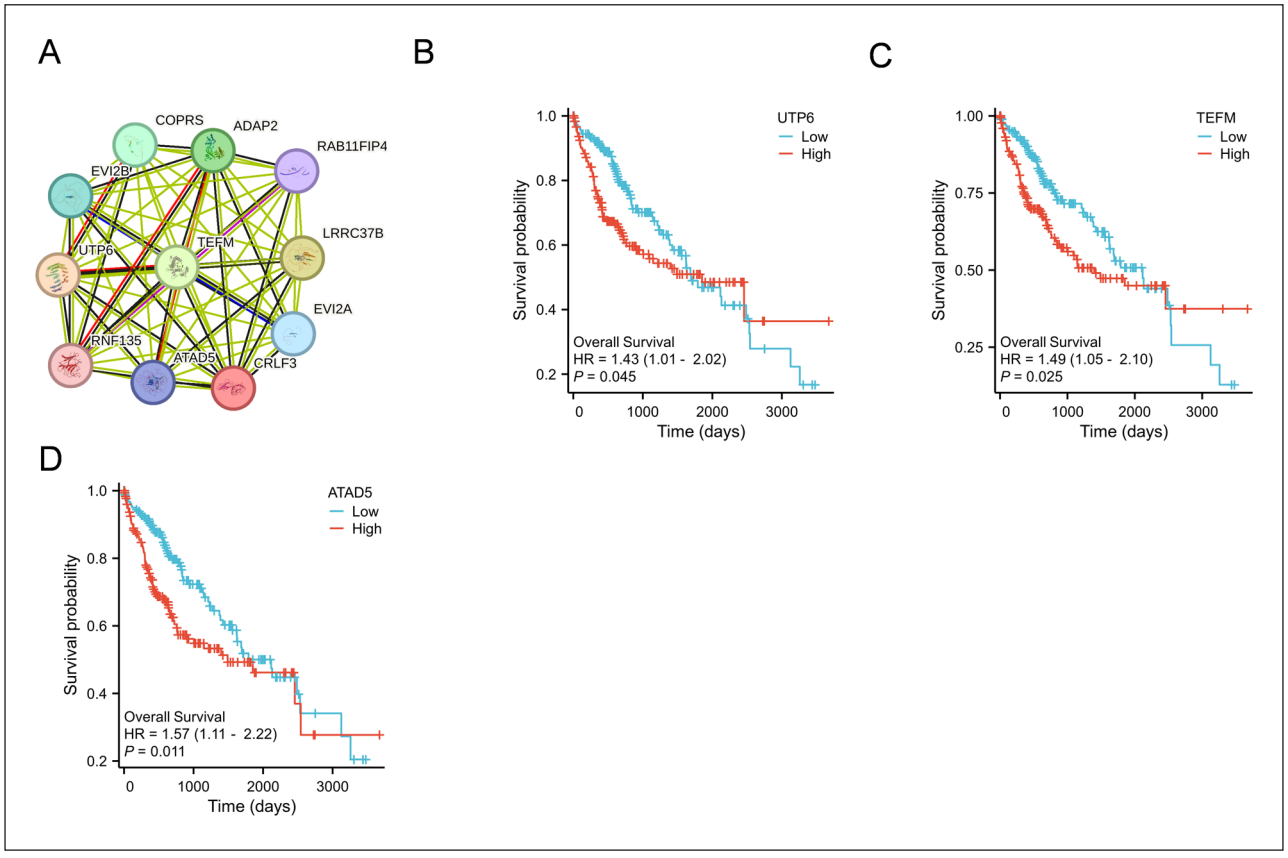

Figure 4. Protein–protein interaction (PPI) network of *CRLF3* and its related proteins constructed using the STRING database. *CRLF3* interaction network. (a) Network mapping of protein–protein interactions was obtained using STRING v10.5. (b) Correlations between *UTP6* and prognosis in LIHC. (c) Correlations between *TEFM* and prognosis in LIHC. (d) Correlations between *ATAD5* and prognosis in LIHC.
CRLF3: cytokine receptor-like factor 3; LIHC: liver hepatocellular carcinoma.

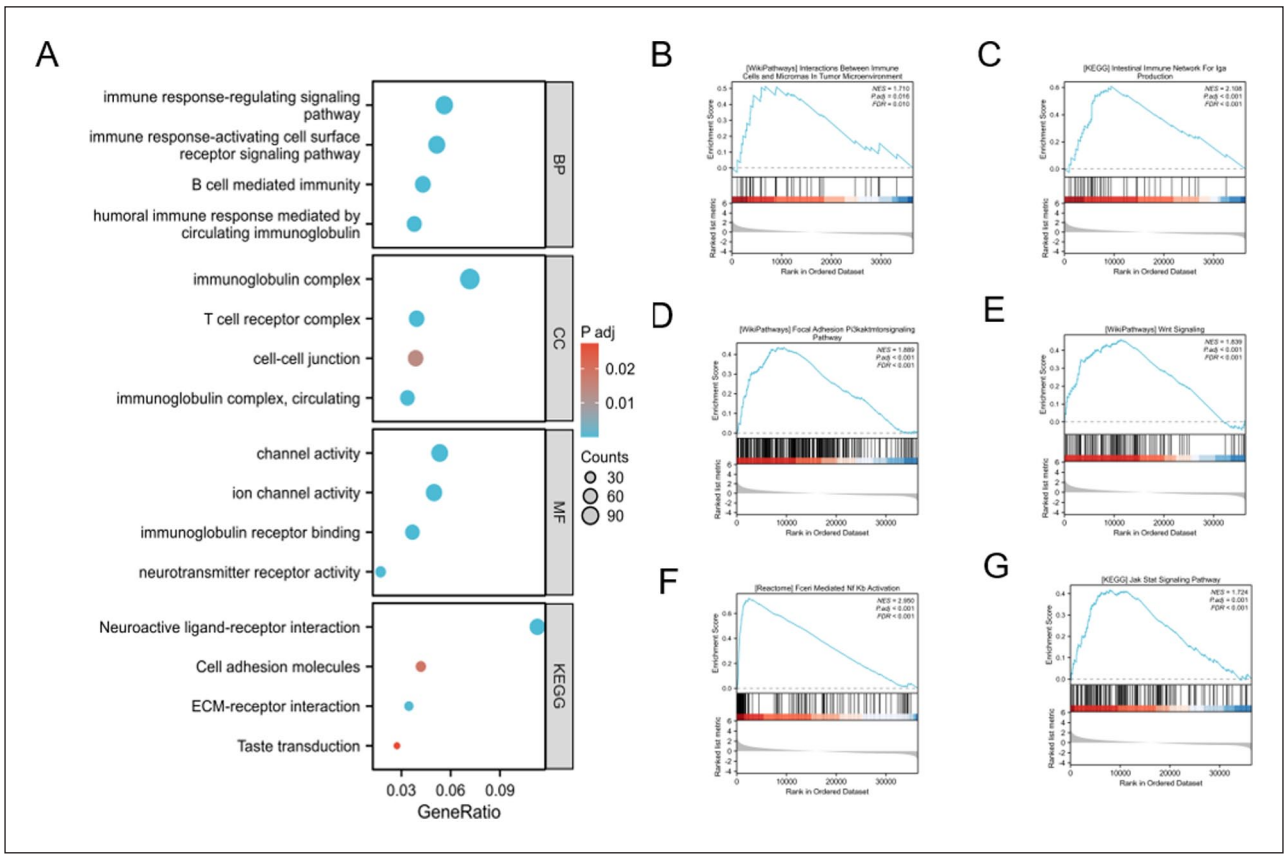

Figure 5. Functional enrichment analysis of *CRLF3* in LIHC. (a) GO functional enrichment and KEGG pathways. (b–g) GSEA revealed the enrichment of five signaling pathways in individuals with LIHC who have high expression of the *CRLF3* gene.
CRLF3: cytokine receptor-like factor 3; GO: gene ontology; KEGG: Kyoto encyclopedia of genes and genomes; LIHC: liver hepatocellular carcinoma.

Discussion

The *CRLF3* gene encodes the protein cytokine receptor-like factor 3 in humans. Phylogenetic studies suggest that *CRLF3* may have originated from a common ancestor of Cnidaria and Bilateria, coinciding with the emergence of the nervous system.[30,31] The *CRLF3* protein is highly conserved across metazoans and features a characteristic cytokine receptor homology domain (CHD). Previous studies have shown that *CRLF3* plays critical roles in various developmental and homeostatic processes, particularly in blood and immune cell function. For instance, *CRLF3* has been reported to be essential for the initiation of hematopoiesis during early embryonic development in zebrafish.[25] Additionally, dysregulation of *CRLF3* expression has been observed in cutaneous squamous cell carcinoma, suggesting a potential role in the disease's pathogenesis, progression, and possible diagnostic applications.[32] However, the prognostic significance and potential biological functions of *CRLF3* in LIHC remain largely unexplored. Therefore, the primary objective of this study is to investigate the expression pattern of *CRLF3* in LIHC, evaluate its prognostic relevance, and explore its potential associated biological pathways using bioinformatics analysis.

In this study, we first examined the expression levels of the *CRLF* family in LIHC, revealing that members of this family were significantly upregulated in tumor tissues compared to normal tissues. Through experimental validation and bioinformatics analysis, we confirmed that *CRLF3* is highly expressed in LIHC tissues compared to normal tissues. We then assessed the *mRNA* and protein expression levels of *CRLF*3 in LIHC tissues and corresponding normal tissues, consistently demonstrating its upregulation. ROC curve analysis suggested that *CRLF3* may have diagnostic potential for LIHC. Univariate and multivariate Cox regression analyses were performed to evaluate the association between *CRLF* family members and OS, which identified *CRLF3* as a candidate of interest. Kaplan–Meier survival analysis showed that LIHC patients with elevated *CRLF3* expression had significantly poorer prognosis. Furthermore, multivariate Cox regression analysis confirmed that high *CRLF3* expression was an independent risk factor for poorer OS in LIHC patients. In summary, our findings suggest that *CRLF3* may serve as a potential diagnostic and prognostic biomarker for LIHC.

To further explore potential molecular associations of *CRLF3*, we identified several genes predicted to interact with

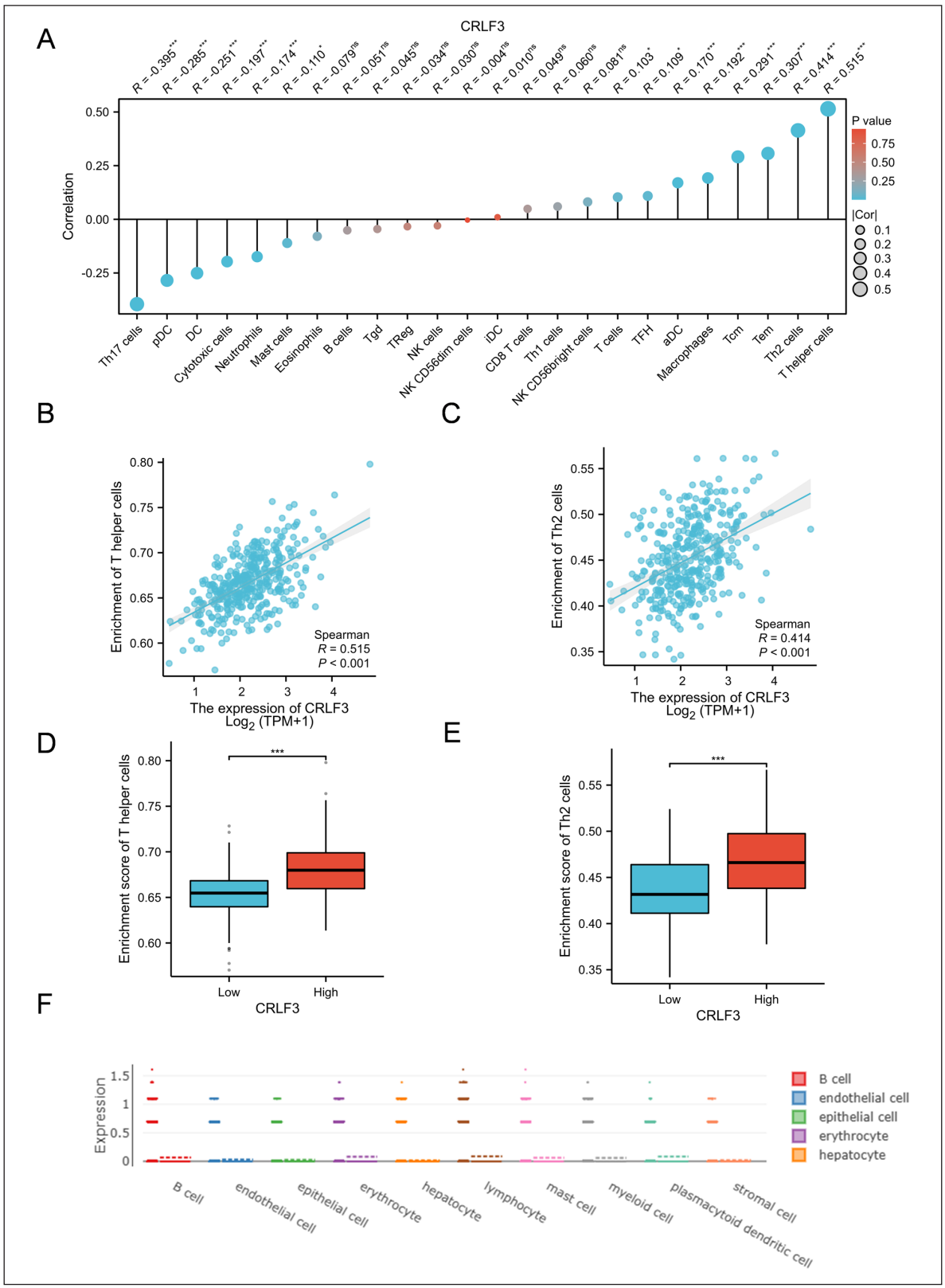

Figure 6. Relationship between *CRLF3* expression and immune infiltration in LIHC tumor microenvironment. (a) correlation between the 24 immunological cells' respective abundances. (b–c) correlation between *CRLF3* expression levels and T helper and Th2 cell infiltration levels. (b) T helper cells. (c) Th2 cells. Spearman's correlation validation of *CRLF3* with T helper cells. (d–e) correlation between levels of Th2 and T helper cell infiltration and *CRLF3* expression. (d) T helper cells. (e) Th2 cells. (f) Validation of *CRLF3* expression in LIHC immune cell subpopulations using single-cell datasets.
CRLF3: cytokine receptor-like factor 3; LIHC: liver hepatocellular carcinoma.

it based on STRING database analysis. Our analysis suggested that *CRLF3* may be associated with *UTP6*, *TEFM*, and *ATAD5* at the level of potential protein–protein interactions. Notably, these genes have been independently implicated in cancer biology by previous studies. For instance, UTP6 has been reported to play regulatory roles in multiple cancer types; its hypermethylation and downregulation are linked to stem cell-like properties, chemoradiotherapy resistance, and prognosis in rectal cancer.[33] Similarly, elevated *TEFM* expression has been shown to promote hepatocellular carcinoma growth and metastasis through activation of the *ROS/ERK* signaling pathway.[34] Genetic and functional defects in *ATAD5* have been shown to increase cancer susceptibility in mammals,[35] and *ATAD5* plays a critical role in various DNA repair pathways.[36] As the human homolog of the yeast protein Elg1, *ATAD5* is involved in the deubiquitination of PCNA.[37] Taken together, these observations suggest a potential functional link between *CRLF3* and cancer-related processes mediated by its predicted interacting partners. However, as these interactions are based on computational predictions, experimental studies are needed to validate whether *CRLF3* indeed functionally interacts with *UTP6*, *TEFM*, and *ATAD5*, and to determine the biological significance of such interactions in LIHC progression.

GSEA analysis revealed that genes correlated with *CRLF3* expression were significantly enriched in multiple cellular processes and signaling pathways, including *PI3K/Akt*, *Wnt*, *FcεRI*-mediated *NF-κB* activation, activation of the intestinal immune network for *IgA* synthesis, interactions between immune cells and microRNAs in the tumor microenvironment, and *JAK/STAT* signaling. These pathways have well-established roles in cancer biology. Dysregulation of the *PI3K/AKT/mTOR* pathway is commonly observed in LIHC, where it regulates metabolic processes involving glucose, lipids, amino acids, pyrimidines, and oxidative reactions in the liver.[38,39] The *Wnt* signaling pathway plays a critical role in cell fate determination, proliferation, and the establishment of cell polarity.[40] Disruption of *WNT/β-catenin* signaling has been implicated in LIHC development, further underscoring its relevance in this malignancy.[41] *FcεRI-mediated NF-κB* activation regulates immune-inflammatory responses and has been shown to influence cancer progression.[42] Interactions between immune cells and *microRNAs* within the tumor microenvironment (TME) contribute to key tumor-related processes, including proliferation, invasion, and immune evasion, and can either promote or suppress tumor development.[43] Moreover, activation of the *JAK/STAT* pathway has been shown to drive the onset and progression of various diseases, including inflammatory disorders, lymphomas, leukemias, and solid tumors.[44,45]

Collectively, these enrichment results suggest that *CRLF3* may be functionally linked to multiple cancer-related pathways, particularly those involved in cell proliferation, metabolism, and immune regulation. However, as these findings are based on computational predictions, experimental studies are needed to validate the precise role of *CRLF3* in these pathways and its functional significance in LIHC progression.

The TME encompasses the local milieu surrounding the tumor, including adjacent blood vessels, immune cells, and other components of the immune system.[46] Previous studies have suggested that immune cells can exert either antitumor or protumor effects through the secretion of cytokines, chemokines, and other factors, playing a critical role in tumor initiation and progression.[47] Recent research has highlighted the significant association between immune cell infiltration and liver carcinogenesis, as well as its potential implications for prognosis and treatment in LIHC.[48,49] In this study, ssGSEA was employed to quantify the infiltration levels of 24 specific immune cell types within the LIHC tumor microenvironment. Our analysis revealed a strong correlation between *CRLF3* expression and T helper cells, particularly Th2 cells. Notably, elevated Th2 cell infiltration has been linked to more advanced tumor stages and poorer therapeutic responses in previous studies.[50,51] These findings suggest a potential association between CRLF3 and Th2 cell-mediated immune responses in the LIHC microenvironment. However, it is important to note that these results are based on computational predictions and correlation analyses. Therefore, further experimental studies are needed to validate the functional relationship between CRLF3 and immune cell infiltration, and to elucidate the underlying mechanisms.

Conclusions

In conclusion, our study demonstrates that *CRLF3* is overexpressed in LIHC tissues and its elevated expression is significantly associated with poor prognosis and survival in LIHC patients. Bioinformatics analysis suggests that *CRLF3* may be functionally linked to multiple signaling pathways, including *PI3K/Akt, Wnt, FcεRI-mediated NF-κB* activation, activation of the intestinal immune network for *IgA* synthesis, interactions between immune cells and microRNAs, and *JAK/STAT* signaling within the tumor microenvironment. Additionally, *CRLF3* expression showed a significant correlation with T helper cell infiltration, particularly Th2 cells, in LIHC. These findings provide a basis for further investigation into the potential mechanisms by which *CRLF3* may contribute to LIHC progression. Despite the comprehensive methodology employed in this study, several limitations should be acknowledged. First, our analysis relied on retrospective data from public databases (TCGA and GEO), which may carry inherent biases despite standardization and batch correction. Second, although *CRLF3* overexpression in LIHC tissues was validated via qPCR and immunohistochemistry, functional experiments directly examining its role in LIHC progression were not performed. Such experimental validation is crucial for establishing causality, but due to constraints, it has not yet been completed. Third, our clinical sample size was moderate and sourced from a single center, potentially limiting the generalizability of our findings.

Future studies involving larger, multicenter cohorts and mechanistic experiments, such as *CRLF3* knockdown or overexpression, are needed to validate and extend these results. Overall, our findings provide a valuable foundation for further investigation into the potential role of *CRLF3* in LIHC progression.

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The project was conceived by XTZ and XYS. Data were analyzed by XXW, who subsequently authored the manuscript. The data were analyzed by ZH, LLH, YW, and CXH. The final manuscript was read and approved by all of the authors.

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Declaration of conflicting interests

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Data availability statement

The article contains the datasets that provide support for the conclusions drawn. The dataset used in this study was created by downloading and collating RNAseq data from the TCGA-LIHC (hepatocellular liver cancer) project STAR process from the TCGA database (https://portal.gdc.cancer.gov) and extracting the data in TPM format.

Supplemental material

Supplemental material for this article is available online.

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