


Downregulation of Crystallin Lambda I is a New Independent Prognostic Marker in Clear Cell Renal Cell Carcinoma

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Background: Clear cell renal cell carcinoma (ccRCC), the most prevalent kidney cancer subtype, has a high mortality rate. Crystallin lambda 1 (CRYL1) encodes an enzyme that catalyzes the dehydrogenation of L-gulonate into dehydro-L-gulonate in uronate cycle. CRYL1 dysregulation has been linked to the progression of several cancers. This research aimed to evaluate the prognostic significance of CRYL1 expression in ccRCC prognosis.

Methods: Clinical data and gene expression profiles on ccRCC were retrieved from the University of California Santa Cruz Xena platform. Differences (variations) in the expression profiles of CRYL1 in ccRCC and healthy tissues were found using RNA-sequencing data, and these findings were validated using qPCR with real-world samples. CRYL1 expression levels were also linked to clinicopathological characteristics, survival, and immune microenvironments. The potential pathway via which CRYL1 expression levels impact the prognosis of patients with ccRCC was investigated using gene set enrichment analysis (GSEA).

Results: In ccRCC tissues, CRYL1 expression levels were lower compared to healthy renal tissues in TCGA cohort ($n = 535$, $P < 0.001$), which was validated in another real-world cohort ($n = 14$, $P < 0.001$). Lower CRYL1 expression levels were linked to unfavorable clinicopathological characteristics and prognoses ($P < 0.001$). According to multivariate Cox regression analysis ($P < 0.001$), CRYL1 expression levels in patients with ccRCC could serve as an independent prognostic indicator. Furthermore, a strong link between CRYL1 expression levels and immune microenvironment was observed ($P < 0.001$). Finally, GSEA revealed that CRYL1 expression levels ($P < 0.001$) were associated with fatty acid metabolism, G2M checkpoint delays, and epithelial-mesenchymal transitions in ccRCC.

Conclusion: Our study found that lower levels of CRYL1 expression were linked to unfavorable clinicopathological characteristics and worse prognoses, and CRYL1 could serve as a new target for the treatment of ccRCC, which is useful for personalized medicine.

Keywords: clear cell renal cell carcinoma, CRYL1, prognosis, biomarker

Introduction

Renal cell carcinoma (RCC) is among the ten most prevalent malignancies in the world.¹ Clear cell renal cell carcinoma (ccRCC) is an RCC subtype that accounts for approximately 75% of all RCC cases; it is highly malignant and has a high morbidity and mortality rate.² Although surgery remains the treatment of choice, immunotherapy and targeted molecular therapy have significantly improved the prognosis of patients with ccRCC. However, survival rates for patients with metastatic ccRCC remain unsatisfactory.³ Therefore, identifying new and accurate biomarkers is crucial for tracking the prognosis of patients with ccRCC.

Crystallin is a functional term that was first used to describe the abundance of soluble proteins found in clear vertebrate eye lenses.⁴ They are expressed in non-ocular tissue, essential in normal physiology and certain diseases such as cancer, in addition to functioning as structural proteins in the lens. Crystallins are recruited from stress-protective proteins and metabolic enzymes via a gene-sharing mechanism, and they are classified into two subfamilies: stress-crystallins and enzyme-crystallins. The former are small heat shock proteins vital to cancer progression and tumor survival. For instance, crystallin AB is a well-known heat shock protein that promotes invasion and metastasis in many

cancers and is being studied as a therapeutic target.⁵ On the other hand, enzyme-crystallins are involved in metabolic mechanisms essential for normal physiology or cancer progression. For instance, μ -Crystallin functions as a ketamine reductase⁶ and can arrest the progression of prostate cancer. It is also a biomarker for early recurrence and poor prognosis in prostate cancer.⁷

Crystallin lambda 1 (CRYL1) is another member of the enzyme-crystallins found on chromosome 3q12. It encodes an enzyme involved in the catalysis of a reaction in which L-gulonate is dehydrogenated into dehydro-L-gulonate in the uronate pathway, an alternate glucose metabolism pathway.⁸ CRYL1 is a much less well-known member of the crystallin family than other members. Previous studies have linked CRYL1 to Alzheimer's disease.^{8,9} The role of CRYL1 in cancer has only been reported in liver cancer. It was found that CRYL1 expression is significantly higher in liver cancer tissues than in normal tissues.¹⁰ Furthermore, lower CRYL1 expression levels are associated with larger tumor size, higher tumor stage, and worse prognosis; moreover, CRYL1 downregulation appears to benefit liver cancer cells' ability to proliferate by shortening the G2-M phase.¹¹ CRYL1 was overexpressed in normal kidney tissue, but the CRYL1 expression profiles and their value as prognostic indicators in ccRCC patients remain unknown. Given the importance of crystallins in cancers, this study investigated variations in CRYL1 expression levels among patients with ccRCC and their potential as a prognostic indicator.

Materials and Methods

Data Extraction

We obtained RNA-sequencing and clinicopathological data for ccRCC using the University of California Santa Cruz Xena database platform (<https://xena.ucsc.edu/>).¹² The cohort included 535 ccRCC tumors and 72 normal samples. The data of 518 ccRCC patients with a follow-up period longer than one month were collected after comparing survival and gene expression data. Tumor Immune Estimation Resource (TIMER) (<https://cistrome.shinyapps.io/timer/>) and Gene Expression Profiling Interactive Analysis 2 (GEPIA2) (<http://gepia2.cancer-pku.cn/>) were additionally used as online external validation sources.^{13,14}

Tissue Specimens

We collected 14 pairs of tumor and normal renal tissue samples from ccRCC patients who had undergone partial or complete nephrectomies at Meizhou People's Hospital. Written informed consent was obtained from every patient. The mean patient age was 58 years. Histologically, there were eight (57.1%) well-differentiated (G1 or G2) and six (42.9%) poorly differentiated (G3 or G4) tumors. Nine (64.3%) were stage I/II tumors, while the remaining five (35.7%) were stage III/IV cancers.

Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

Using an RNA extraction kit (Haigene Biotech Co., Ltd., Harbin, China), total RNA was extracted from tissues. We subsequently synthesized complementary DNA using the PrimeScript RT reagent kit (Takara Bio Inc., Dalian, China) as per the manufacturer's instructions. The SYBR Green PCR kit (Takara Bio, Inc., Dalian, China) was used to perform PCR reactions on the ABI 7500 fluorescent quantitative PCR system (Applied Biosystems Inc. USA). We used the following primer sequences: CRYL1 (forward: GTGGTGATCGTTGGCAGTG; reverse: TTCCTTATCTGCTGTTGCTCAAT), GAPDH (forward: GTCAAGGCTGAGAACGGGAA; reverse: AAATGAGCCCCAGCCTTCTC). CRYL1 expression levels in the sample tissues were determined using the $2^{-\Delta\Delta C_t}$ method.

Correlation Between CRYL1 Expression and the Immune Microenvironment

We used the R package ESTIMATE (Estimation of STromal and Immune cells in MAlignant Tumors) to assess the fraction of immune and stromal cells in each ccRCC sample in The Cancer Genome Atlas (TCGA) dataset.¹⁵ After matching the immune and stromal scores to gene expression data, we analyzed their correlations. In addition, we investigated the correlation between the CRYL1 expression levels and immune cell infiltration using the TISIDB

platform (<http://cis.hku.hk/TISIDB/index.php>), an integrated repository portal for interactions related to the tumor-immune system.¹⁶

Gene Set Enrichment Analysis (GSEA)

Using GSEA, we determined the potential biological role of CRYL1 in ccRCC. After identifying genes co-expressed with CRYL1, we used GSEA to identify all substantially enriched signaling pathways associated with these genes. Moreover, the sample-wise gene set activities of different signaling pathways were measured using gene set variation analysis (the method labeled “single-sample GSEA”), and the associations between CRYL1 expression levels and the enriched signaling pathways were investigated.

Statistical Analysis

The R software 3.6.1 and Strawberry Perl 5.30.1.1 were used for data processing and statistical analysis. Differences in the CRYL1 expression levels between or among groups of tissue samples were identified using *t*-tests or analyses of variance. Kaplan-Meier curves and Log rank tests were used for survival analyses. Cox regression models were used for univariate and multivariate survival analyses. Spearman’s rank correlation test was used for correlation analyses. $P < 0.05$ indicated a significant difference.

Results

CRYL1 Expression Levels Were Significantly Negatively Correlated with Clinicopathological Features in ccRCC

We first detected variations in CRYL1 expression levels between ccRCC tumor tissue and healthy renal tissue samples in the TCGA cohort; our analyses revealed that CRYL1 expression levels were substantially ($P < 0.001$) lower in ccRCC tissue than in healthy renal tissue samples (Figure 1A). To validate the above findings, we used qPCR to examine CRYL1 expression in 14 pairs of ccRCC and healthy renal tissue samples. CRYL1 expression levels were lower in 12 of the 14 paired tissue samples than in normal renal tissue samples (Figures 1B and C). Furthermore, we investigated CRYL1 expression profiles in a pan-cancer dataset from the TIMER database platform. In addition to ccRCC, we also discovered that CRYL1 expression levels were below normal in several other cancers, including bladder urothelial carcinoma, breast invasive carcinoma, and kidney chromophobe carcinoma (Figure 1D). Furthermore, our subsequent analysis shows that CRYL1 expression levels were lower in affected males than in affected females ($P < 0.001$), in patients with different tumor status ($P < 0.001$), in tumors of higher histological grades than those of lower grades ($P < 0.001$), in larger versus smaller tumors ($P < 0.001$), lymph node metastasis ($P < 0.0093$), distant metastasis ($P < 0.032$), and advanced stage ($P < 0.001$), but not in patients with different age ($P < 0.29$) (Figures 1E–L).

Prognostic Value of CRYL1 in Patients with ccRCC

The median values of CRYL1 expression were used to divide the 518 ccRCC patients with longer than 1 month of follow-up into high and low groups. The Kaplan-Meier survival analysis demonstrated a negative correlation between CRYL1 expression level and overall survival (OS; $P < 0.001$) and disease-free survival rates (DFS; $P < 0.001$) (Figure 2A). Similar patterns were observed when online data from the GEPIA2 database was analyzed (Figure 2B). Furthermore, we found that CRYL1 could be used as a prognostic indicator for numerous malignancies, including liver cancer, renal papillary cell carcinoma, and lung adenocarcinoma (Figure 2C). Cox regression analyses were used to determine if CRYL1 expression was an independent prognostic factor for patients with ccRCC. Patients in the high-CRYL1 expression and low-CRYL1 expression groups had significantly different OS and DFS in both univariate (hazard ratio (HR) = 0.590, $P < 0.001$ for OS; HR = 0.502, $P < 0.001$ for DFS) and multivariate analyses (HR = 0.630, $P < 0.001$ for OS; HR = 0.562, $P < 0.001$ for DFS); this suggests that CRYL1 expression levels have an independent prognostic value for ccRCC patients (Figures 2D–G). Furthermore, our analyses indicated that the histological grade and stage of the tumor were also independent prognostic factors for ccRCC patients (Figures 2E and G). In a stratified analysis, we found that lower CRYL1 expression levels were significantly linked to the poorer OS in patients with grade 3/4 cancer ($P <$

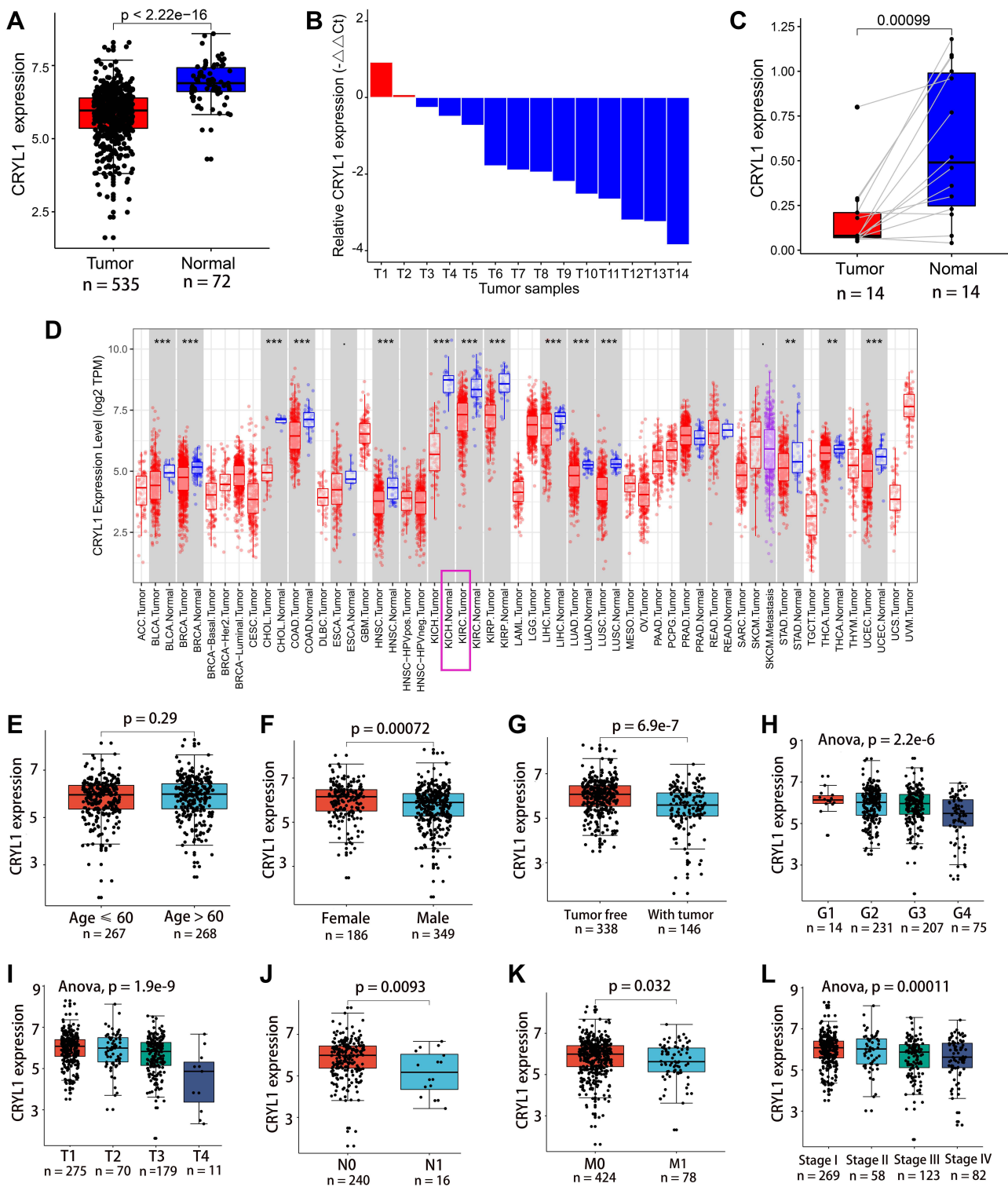


Figure 1 CRYL1 expression is significantly lower in ccRCC tissue samples than in normal tissue samples. **(A)** Differential expression of CRYL1 in the TCGA cohort. **(B)** CRYL1 expression was downregulated in 12 of 14 ccRCC samples. **(C)** CRYL1 levels were significantly lower in ccRCC tissue samples in 12 of the 14 paired tissue samples. **(D)** CRYL1 expression levels in the pan-cancer dataset on the TIMER database (<https://cistrome.shinyapps.io/timer/>). **(E)** No significant differences in CRYL1 expression levels in ccRCC tissue samples between young and old patients. **(F–L)** CRYL1 expression levels are significantly different in patients with different genders, tumor status, pathological grades, tumor sizes, presence/absence of lymph node metastasis, presence/absence of metastasis, and tumor stage. **Notes:** ** $P < 0.01$; *** $P < 0.001$.

Abbreviations: ccRCC, clear cell renal cell carcinoma; CRYL1, crystallin lambda 1.

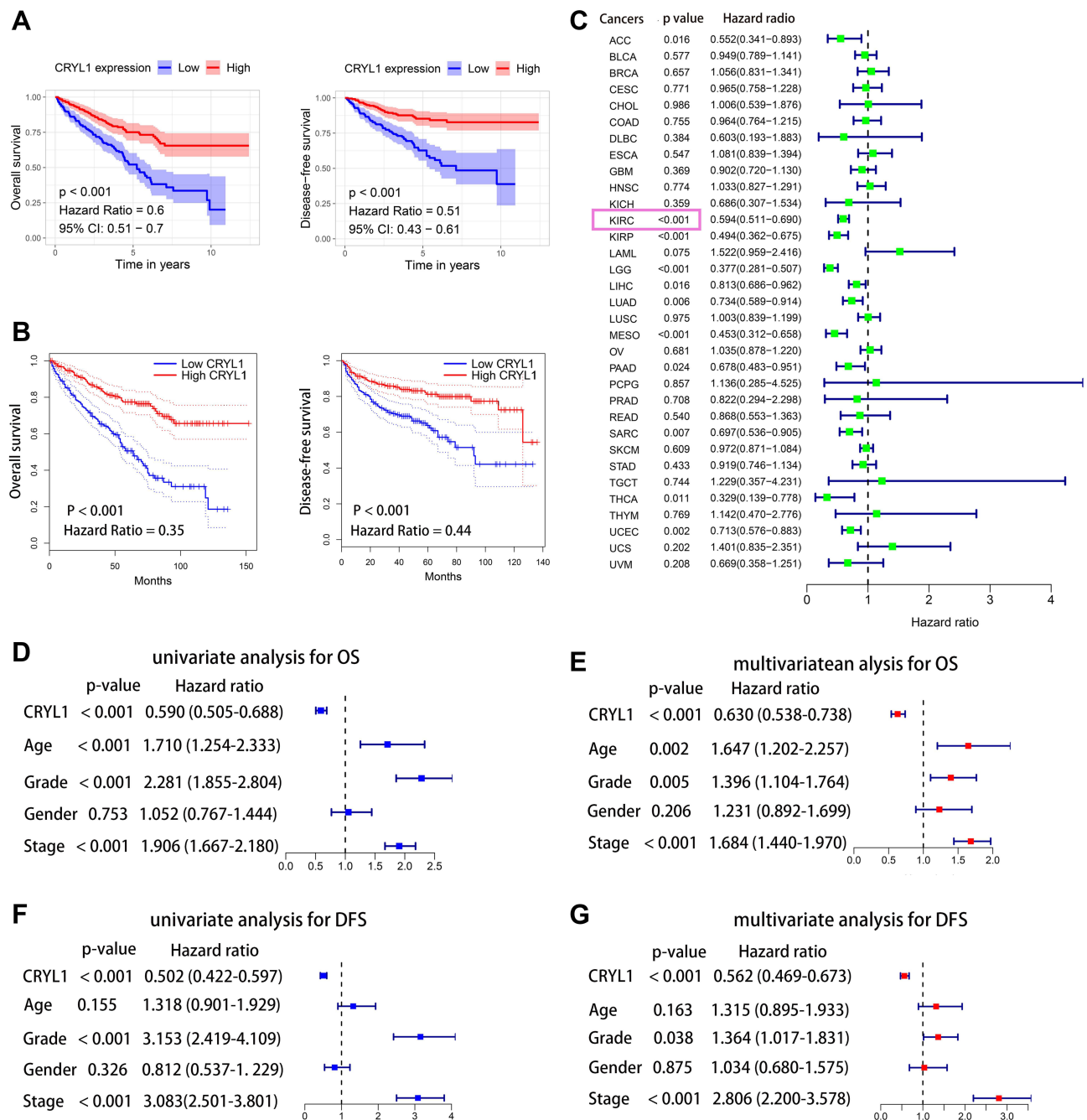


Figure 2 Prognostic value of CRYL1 in clear cell renal cell carcinoma. **(A)** CRYL1 expression levels were significantly negatively correlated with OS and DFS. **(B)** Validating the correlation between CRYL1 expression levels and OS and DFS on the GEPIA2 platform (<http://gepia2.cancer-pku.cn/>). **(C)** Prognostic value of CRYL1 expression in the pan-cancer dataset. **(D and E)** Univariate and multivariate Cox regression analyses of CRYL1 expression levels for OS. **(F and G)** Univariate and multivariate Cox regression analyses of CRYL1 expression levels for DFS. **Abbreviations:** CI, confidence interval; CRYL1, crystallin lambda 1; DFS, disease-free survival; OS, overall survival.

0.001), stage I/II cancer ($P < 0.001$), or stage III/IV cancer ($P < 0.001$). However, this association did not hold for patients with grade 1/2 cancer ($P < 0.097$) (Figures 3A and B). DFS had similar results (Figures 3C and D).

CRYL1 Expression Correlates with the Immune Microenvironment

Since immunotherapy has shown great promise in the treatment of ccRCC, we investigated the relationship between CRYL1 expression levels and tumor immune microenvironment. The findings of this study highlighted that CRYL1

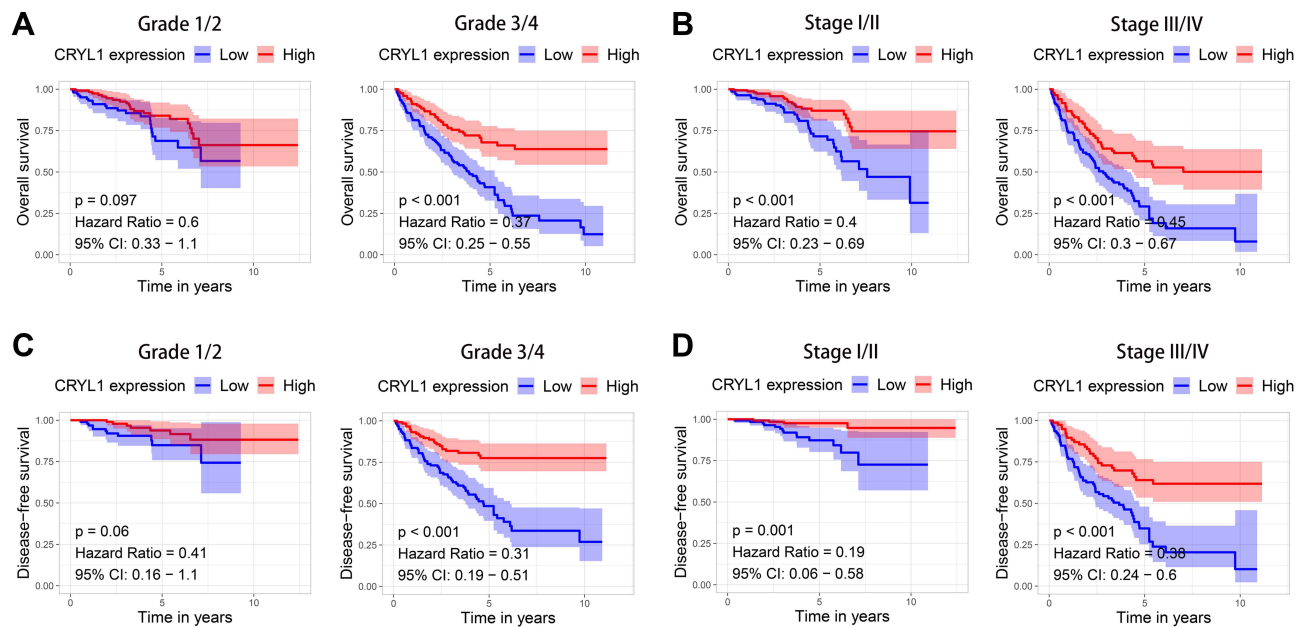


Figure 3 Stratified survival analysis of CRYL1 for overall survival (OS) and disease-free survival (DFS) according to histological grade and stage of ccRCC tumors. (A) Grade 1 or 2 and grade 3 or 4 for OS. (B) Stage I/II and stage III/IV for OS. (C) Grade 1 or 2 and grade 3 or 4 for DFS. (D) Stage I/II and stage III/IV for DFS.

Abbreviations: CI, confidence interval; CRYL1, crystallin lambda 1.

expression levels were remarkably negatively linked to immune and stromal scores, indicating that high CRYL1 expression levels were associated with lower levels of immune infiltration (Figure 4A). Moreover, an analysis of the TISIDB data demonstrated that CRYL1 expression levels were significantly negatively linked to the numbers of cells in immune cell infiltrates known for their tumor-promoting roles, including macrophages, myeloid-derived suppressor cells (MDSCs), and regulatory T cells (Tregs) (Figure 4B). These findings suggest that higher levels of CRYL1 expression are associated with ccRCC suppression via immune microenvironment modulation.

The GSEA

GSEA has been widely used for functional enrichment analysis.¹⁷ The GSEA results of this study indicated that high levels of CRYL1 expression were significantly linked to several important metabolic processes and signaling pathways, including fatty acid metabolism, epithelial-mesenchymal transition (EMT), and the G2M checkpoint (Figures 5A and B). The correlation analysis also revealed that CRYL1 expression levels in ccRCC tissue samples were significantly positively linked to hallmarks of fatty acid metabolism ($r = 0.712$, $P < 0.001$) and negatively linked to the hallmarks of the G2M checkpoint ($r = -0.22$, $P < 0.001$) and EMT ($r = -0.393$, $P < 0.001$) (Figures 5C–E). Interestingly, these relationships were also seen in many other cancer types (Figures 5C–E).

Discussion

Increasing evidence suggests that the crystallin family plays a vital role in developing and progressing of many types of cancers.^{5,7,18} CRYL1, a member of the enzyme-crystallin family, was less reported in cancers except for liver cancer. It has been reported that CRYL1 expression levels are lower in liver cancer tissues, and this decrease is linked to poor prognosis.¹¹ Although another study found CRYL1 to be highly expressed in normal kidney tissue, the expression and role of CRYL1 in ccRCC remain unknown.¹⁰ Our study explored this issue.

First, we investigated ccRCC high-throughput RNA-sequencing data from the TCGA cohort. Consistent with previous findings in liver cancer, our current study shows that CRYL1 expression levels are significantly lower in ccRCC tissue samples compared to healthy renal tissue samples and that this decrease in expression levels is associated with poor OS and DFS in ccRCC patients. We have validated these findings using data from 14 paired samples (ccRCC tissue and normal renal tissue samples) from Meizhou People's Hospital patients. Furthermore, our findings show that

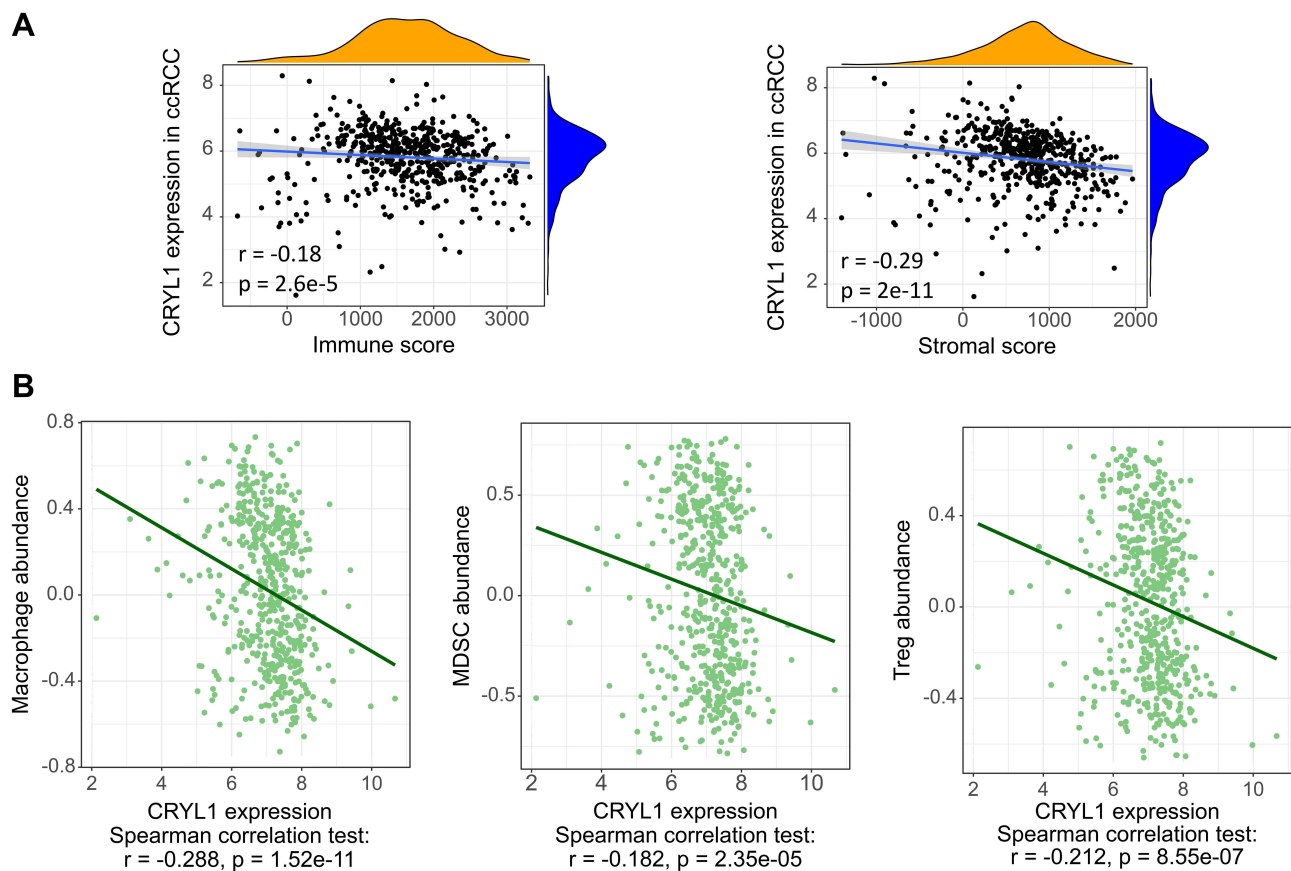


Figure 4 The correlation between CRYL1 expression levels and the immune microenvironment. **(A)** Significant negative correlations exist between CRYL1 expression levels and immune and stromal scores. **(B)** Correlation between CRYL1 expression levels and the numbers of immune cells.

Abbreviations: ccRCC, clear cell renal cell carcinoma; CRYL1, crystallin lambda I; MDSC, myeloid-derived suppressor cells; Treg, regulatory T cells.

lower levels of CRYL1 expression are associated with negative clinicopathological characteristics in ccRCC, including a higher histological tumor grade, larger tumor size, and the presence of metastatic potential. Multivariate Cox regression analyses demonstrated that CRYL1 expression, histological tumor grade, and tumor stage were independent prognostic factors for OS and DFS in patients with ccRCC.

Following that, when we conducted stratified analyses based on independent factors such as histologic grade and tumor stage, we found that patients with ccRCC who had lower levels of CRYL1 expression had considerably worse OS and DFS rates. However, for patients with lower-grade cancers (grade 1 or 2), the relationship between CRYL1 expression levels and OS and DFS was not statistically significant; this could be due to our small sample sizes (patients with grade 1 or 2 cancer were rare) or to the lower malignancy of such tumors. These findings suggest that CRYL1 could be a novel prognostic marker for ccRCC.

The GSEA results of this study show that CRYL1 is involved in several signaling pathways, including fatty acid metabolism, the G2M checkpoint, and EMT. Moreover, the relationships were observed in many other cancer types. CRYL1 is an enzyme-crystallin with FAD and NAD domains that can catalyze the β -oxidation of fatty acids,¹⁹ consistent with our GSEA results. Lower levels of CRYL1 expression may reduce fatty acid oxidation and increase the supply of free fatty acids. Reprogramming of fatty acid metabolism is a hallmark of most cancer cells because it allows the rapidly proliferating cancer cells to obtain more free fatty acids for their massive energy requirements, membrane synthesis, and signaling molecules.^{20,21} According to a study, increased CRYL1 expression levels inhibit liver cancer cell growth and proliferation by delaying the G2-M transition of the cell cycle.¹¹ Furthermore, another member of the crystallin family, $\beta\gamma$ -crystallin, can inhibit melanoma cell proliferation by inducing G2-M transition delays.²² Consistently our GSEA

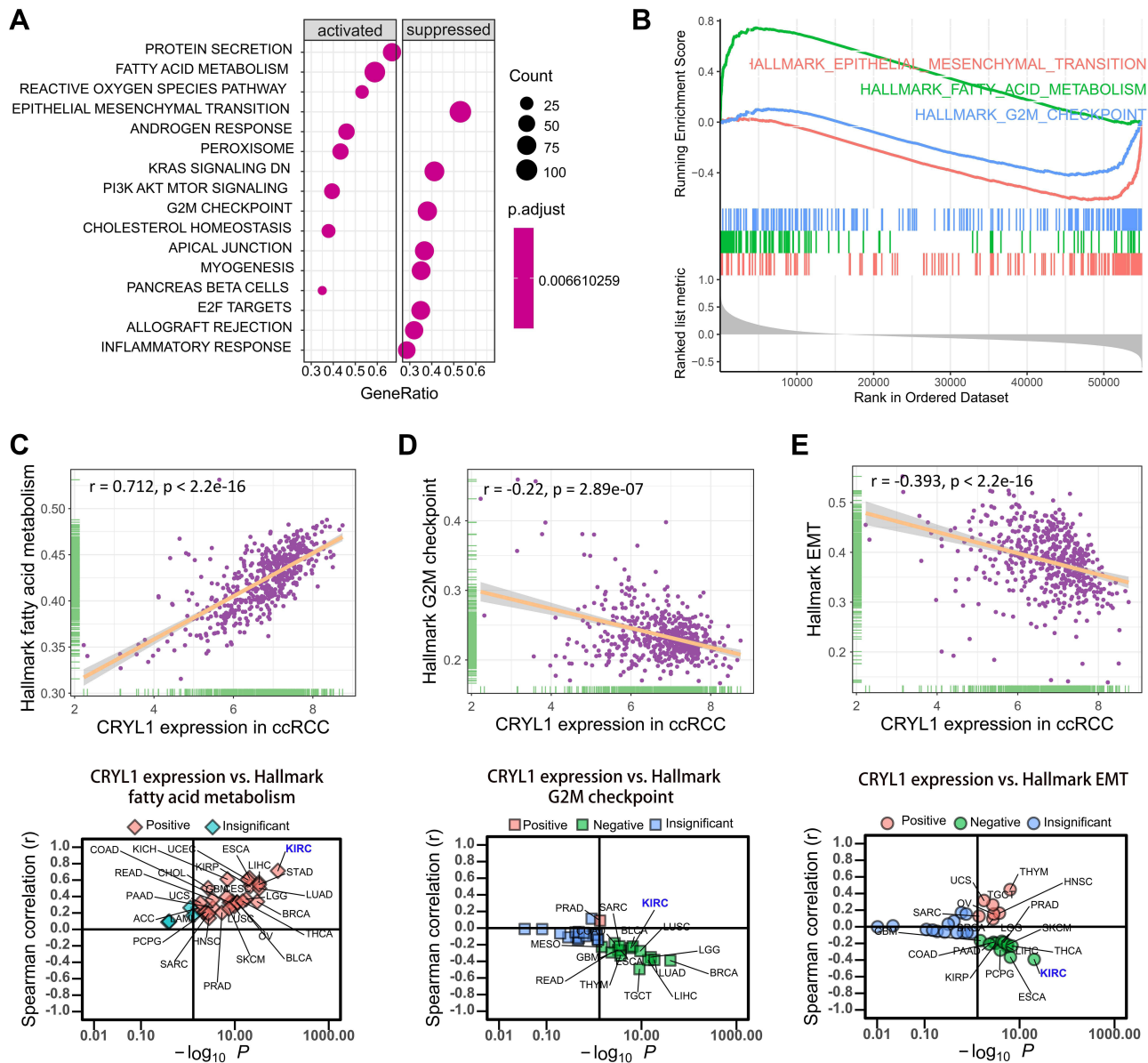


Figure 5 Results of gene set enrichment analysis (GSEA). **(A)** The activated and suppressed signaling pathway hallmarks in ccRCC with increased CRYL1 expression. **(B)** Enrichment plot showing three selected representative pathways. **(C)** Significant correlations between CRYL1 expression levels and hallmark fatty acid metabolism in the ccRCC and pan-cancer datasets. **(D)** Significant correlations between CRYL1 expression levels and the hallmark G2M checkpoint in the ccRCC and pan-cancer datasets. **(E)** Significant correlations between CRYL1 expression levels and hallmark EMT in ccRCC and pan-cancer datasets.

Abbreviations: ccRCC, clear cell renal cell carcinoma; CRYL1, crystallin lambda I; EMT, epithelial-mesenchymal transition.

results revealed a close relationship between CRYL1 and the G2M checkpoint. All these findings suggest that CRYL1 may play a vital role in the cancer cell cycle.

The tumor microenvironment refers to the microenvironment in which tumor cells reside, which includes immune cells, stromal cells, extracellular matrix, and other biomolecules.²³ Immune and stromal cell fractions can be assessed using immune and stromal cell scores, respectively. More and more studies have shown that immune and stromal cells play a crucial role in cancer development and immunotherapy.^{24,25} Our findings also suggest that CRYL1 expression may impact the tumor microenvironment in ccRCC. CRYL1 expression levels were found to be significantly inversely correlated with immune scores, stromal scores, and the presence of tumor-promoting immune cells such as MDSCs, macrophages, and Tregs. Therefore, we hypothesized that lower levels of CRYL1 expression might promote immune cell infiltration and tumor progression in ccRCC. This is consistent with the findings of other studies, as immune infiltration

and changes in fatty acid metabolism are also closely related to the onset of EMT,^{24,26,27} which is a critical feature of ccRCC progression.²⁸ Given the close relationship between CRYL1 expression, reprogramming of fatty acid metabolism, immune infiltration, and EMT, our research suggests that changes in CRYL1 expression levels may influence the onset of EMT by promoting immune infiltration and changing fatty acid metabolism, all of which affect the survival of patients with ccRCC.

Although our findings suggest that CRYL1 could be a useful biomarker for ccRCC progression, it does have several limitations. For instance, all our survival analysis data were obtained from online databases; these findings must be confirmed in other cohorts. In addition, *in vitro*, and *in vivo* analyses are required to understand how changes in CRYL1 expression levels affect ccRCC cells.

Conclusions

In conclusion, we comprehensively investigated the expression, clinical value, and potential biological function of CRYL1 in ccRCC using data from multiple public databases, clinical tissue specimens, and the GSEA method. Our findings revealed that CRYL1 expression levels are significantly lower in ccRCC tissues. This decrease in expression levels has been linked to unfavorable clinicopathological characteristics and a poor prognosis in patients with ccRCC. Therefore, we believe that CRYL1 could be a novel prognostic marker and a potential target for the treatment of ccRCC.

Data Sharing Statement

This article includes all the data presented to support the findings of this study. Further inquiries can be directed to the corresponding author.

Acknowledgments

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Ethics Approval and Informed Consent

This study was approved by the ethics committee of Meizhou People's Hospital (approval number 2020-CY-06) and followed the Declaration of Helsinki. Written informed consent was obtained from all participants.

Disclosure

The authors have no competing interests to declare in this work.

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