ORIGINAL RESEARCH

The Downregulation of Prognosis- and Immune Infiltration-Related Gene CYFIP2 Serves as a Novel Target in ccRCC

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Background: Increasing evidence indicated that the aberrant expression of the cytoplasmic FMR1-interacting protein (CYFIP) family might possess critical role and potential functions in cancer. But the role of CYFIP2 in clear cell renal cell carcinoma (ccRCC) is still uncharacteristic.

Methods: We investigated the Cancer Genome Atlas Kidney Clear Cell Carcinoma (TCGA-KIRC) database for the expression profile, clinicopathological variables, clinical prognosis information, and promoter methylation levels of CYFIPs in ccRCC. The aberrant CYFIP2 protein expression was validated by the Human Protein Atlas (HPA) and Clinical Proteomic Tumor Analysis Consortium (CPTAC). Quantitative real-time polymerase chain reaction (qRT-PCR) was used to uncover CYFIP2 mRNA levels in 28 pairs of ccRCC cancer tissues. Kaplan–Meier analysis, univariate and multivariate Cox proportional hazard regression were performed to assess CYFIPs' prognosis value. Gene set enrichment analysis (GSEA) was used to determined hallmark functions, gene ontology of CYFIP2. TIMER database was utilized to assess the correlation with immune infiltration in ccRCC.

Results: Results showed CYFIP2 was downregulated in ccRCC, relative to paired normal tissues in TCGA-KIRC database and 28 pairs of clinical samples (P < 0.0001). Similarly, a decreased CYFIP2 protein expression was confirmed by ccRCC tissues. The results showed CYFIP2 was negatively regulated by promoter DNA methylation. Survival analysis results showed CYFIP2 could be an independent biomarker for ccRCC and its reduction predicted a poor overall survival (OS) and disease-free survival (DFS). GSEA showed CYFIP2 was involved in metabolic pathways and epithelial–mesenchymal transition (EMT). Immune infiltration analysis revealed that a list of immune markers was significantly correlated with CYFIP2 expression especially with CD4+ cells and CD8+ cells in ccRCC.

Conclusion: These results show that CYFIP2 was downregulated in ccRCC patients and predicted an unfavorable prognosis. CYFIP2 might be a potential novel prognostic molecule, and related to immune infiltration, the metabolism, as well as EMT process in ccRCC. CYFIP2 could act as tumor suppressor gene in ccRCC and positive modulation of CYFIP2 might lead to development of a novel strategy for ccRCC treatment.

Keywords: CYFIP2, ccRCC, methylation, prognosis, immune infiltration

Introduction

Current research showed clear cell renal cell carcinoma (ccRCC) was associated with most kidney cancer-related deaths (approximately more than 75%).[1] Indeed, most deaths were due to tumor progression, migration and invasion, or postoperative recurrence. Cancer metastasis and invasion determined the fate of tumor progression and influence of ccRCC.[2–4] Although target of tyrosine kinase inhibitor

(TKI) drugs had shown encouraging efficacy on metastatic ccRCC,[5] patients of ccRCC still had a poor prognosis due to the TKI resistance.[6] Therefore, there was a great clinical significance to identify novel prognosis indicators and underlying molecular mechanisms to improve prognosis prediction and advancement in ccRCC therapy.

The cytoplasmic FMR1-interacting protein (CYFIP) family, CYFIP1 and CYFIP2 (also known as SRA-1 and PIR121, respectively), were initially identified as binding partners of the fragile X mental retardation protein (FMRP).[7] The 145kDa protein they encode is involved in the regulation of messenger RNA translation and actin dynamics in the nervous system. Their destruction can cause serious physiological consequences, especially brain disorder. Thus, CYFIPs originally widely regard as brain disorder-associated proteins.[8] However, unlike the clear association between CYFIP1 and brain diseases, genetic variants in CYFIP2 have not been clearly associated with brain disorders until recently.[9] Specifically, mutations in CYFIP2 have been found in Alzheimer's disease,[10] early-onset epileptic encephalopathy,[11] intellectual disability,[12] and developmental delay. Result reported that CYFIP2-null mice die soon after birth.[13] Patients with CYFIP2 variants appear symptoms of microcephaly and seizures at 3 to 6 months old.[11]

The aberrant expression of CYFIPs and its role in tumors has currently attracted increasing attention. CYFIP1 was a subunit of the WAVE complex. Studies have shown that CYFIP1 was commonly deleted and associated with poor prognosis during invasion of epithelial tumors.[14] CYFIP1 was a new candidate tumor suppressor gene in nasopharyngeal carcinoma[15] and was controlled by NOTCH1 signal in squamous cell carcinoma of the skin.[16] Nevertheless, CYFIP2 has yet to be thoroughly explored, particularly its association with human cancer has yet to be conducted. To date, CYFIP2 mutations have also been identified in endometrial cancer patients.[17] Study showed the pairs of gene IL2R and CYFIP2 are involved in the purine ribonucleoside diphosphate metabolic process in colorectal cancer.[18] Moreover, study showed CYFIP2 could be a potential biomarker to distinguish basal cell carcinoma and healthy individuals. However, the expression and the potential function of CYFIP2 in ccRCC have not been studied yet.

Herein, therefore, we focused on the expression of CYFIP2 and its potential value in human ccRCC. Our results revealed that CYFIP2 was downregulated in ccRCC, and patients with low CYFIP2 expression

indicated a poor prognosis outcome and higher clinical characteristics. Moreover, low CYFIP2 expression was might result in its high DNA promoter methylation. Finally, we found that aberrant expression of CYFIP2 exhibiting a significant correlation in immune cell infiltration of ccRCC. Combining these findings, CYFIP2 might be a novel biomarker and therapy targeted for ccRCC.

Materials and Methods
Public Databases
The mRNA expression profiles of CYFIPs were downloaded from the Cancer Genome Atlas Kidney Clear Cell Carcinoma (TCGA-KIRC) database (https://xenabrowser. net/heatmap/#). A total of 533 ccRCC cases, including 72 paired cases, and corresponding clinical information were also obtained from TCGA-KIRC database, and used for subsequent pathological correlation analysis and survival analysis. Clinical data comprising various clinicopathological variables, prognostic outcomes, as well as overall survival (OS) and disease-free survival (DFS) time of ccRCC patients. The promoter methylation of CYFIPs and beta values of 10 CpG sites in 450k was also obtained from TCGA-KIRC database.

The immunohistochemistry assays of CYFIP2 in ccRCC and normal tissues were obtained from the Human Protein Atlas (HPA) (https://www.proteinatlas. org/). And the protein expression levels, based on sample types, grade, and TNM stage were obtained from Clinical Proteomic Tumor Analysis Consortium (CPTAC) (https:// proteomics.cancer.gov/data-portal).

Immune Infiltration Analysis
To perform tumor immune research, TIMER (https://cis trome.shinyapps.io/timer/) was used to explore and visualize tumor immunologic and genomics data. The correlation between CYFIP2 with key prognosis-related immune cell markers was analysed via the "Correlation" module of TIMER. These gene markers comprising CD8 + T cells, CD4+ T cells, monocytes, B-cells, regulatory cells (Tregs), dendritic cells, natural killer (NK) cells. The immune gene markers were searched by the website of CellMarker (http:// biocc.hrbmu.edu.cn/CellMarker/). The Spearman method was used to identify the correlation coefficient and P-values.

Gene Set Enrichment Analysis (GSEA)
As previously described, GSEA (http://www.broadinsti tute.org/gsea), based on TCGA-KIRC, was utilized to

evaluated CYFIP2's possible pathogenesis in ccRCC.[19] GSEA was carried out between low or high CYFIP2 expression datasets. Gene set permutations were performed 1000 times for each analysis. The normalized enrichment score (NES), nominal p-value, and false discovery rate (FDR) q-value indicated the significance of the relation between gene sets and pathways.[20]

Kaplan–Meier Analysis

The Kaplan–Meier analysis was used to assess the relationship between CYFIPs expression with patients' prognosis. All ccRCC patients were first divided into "high" and "low" groups according the median value of CYFIPs expression, and then survival analysis was performed. Survival of overall (OS) and disease-free (DFS) in TCGA-KIRC were analyzed and drawn by GraphPad 8.0. The hazard ratio (HR), 95% confidence interval (CI) and log-rank P-value were also computed.

RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

This experimental study was approved by The Ethics Committee of Huazhong University of Science and Technology (S2086) and written informed consent was obtained from all patients as previously described.[21] Twenty-eight paired renal cancer tissues were obtained from Wuhan Union Hospital and have been performed with the principles stated in the Declaration of Helsinki. After total RNA extraction and reverse-transcription, real-time PCR analysis was performed using SYBR Green mix (Thermo, Massachusetts, USA). GAPDH was used as normalized gene. Gene primers of CYFIP2 and GAPDH were obtained from Sangon Biotech (Shanghai):

CYFIP2
Forward 5′-ATCCAAGCCTCTCAACATTGCCTAC-3′
Reverse 5′-GTTCCTCCATGACCACAGCGATG-3′
GAPDH
Forward 5′-GAGTCAACGGATTTGGTCGT-3′
Reverse 5′-GACAAGCTTCCCGTTCTCAG-3

Statistical Analysis

All statistical analyses were performed using GraphPad Prism 8.0 and SPSS Statistics 22.0 (IBM SPSS, Chicago, IL). Data sets were described with median and SEM. Data of unpaired cases were performed with a one-way analysis of variance (ANOVA) or t-test, while paired cases were analyzed with paired sample t-test. The correlation of variables was analyzed by Pearson correlation analysis. Univariate and multivariate Cox proportional hazard regression and Pearson chi-square test were used to assess CYFIPs' prognosis value. P value < 0.05 was defined as statistical significance.[22] *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

Results
The Expression of CYFIP Family in ccRCC

CYFIP family mRNA expression profile in TCGA-KIRC database was showed in Figure 1A with heat map. CYFIP1 and CYFIP2 expression in total ccRCC tissues (n = 533) and normal tissues (n = 72) was shown in Figure 1B and D. Moreover, CYFIP1 and CYFIP2 expression in paired ccRCC tissues and matched non-cancerous normal tissues (n = 72) was shown in Figure 1C and E. The results showed that CYFIP2 but not CYFIP1 was markedly downregulated in cancer tissues (P < 0.05).

Prognostic Role of CYFIP Family in ccRCC

Kaplan–Meier analysis, univariate and multivariate Cox proportional hazard regression analyses were used to evaluate the prognostic role of CYFIP family in ccRCC. The results illustrated that ccRCC patients with low CYFIP1 or CYFIP2 expression had a shorter overall survival (OS) (Figure 2A), and shorter disease-free survival (DFS) (Figure 2B). Then, the prognostic role of CYFIP1 or CYFIP2, age, sex, TNM stage and grade with OS and DFS of ccRCC was assessed by univariate and multivariate analysis (Figure 2C and D). The multivariate analysis results confirmed that CYFIP2 was an independent risk factor of OS and DFS for ccRCC, OS: CYFIP2 (HR, 0.37; P = 0.000); DFS: CYFIP2 (HR, 0.485; P = 0.000). Then, we focused on this significant prognosis- or clinical outcome-related gene for further research.

Clinical Characteristics with CYFIP2 in ccRCC

Subsequently, we evaluated the correlation between CYFIP2 and the clinical characteristics of ccRCC. The results showed CYFIP2 expression was significantly lower in patients of dead (Figure 3A), patients of recurred (Figure 3B), patients with distant metastasis (Figure 3C), patients with higher T stage

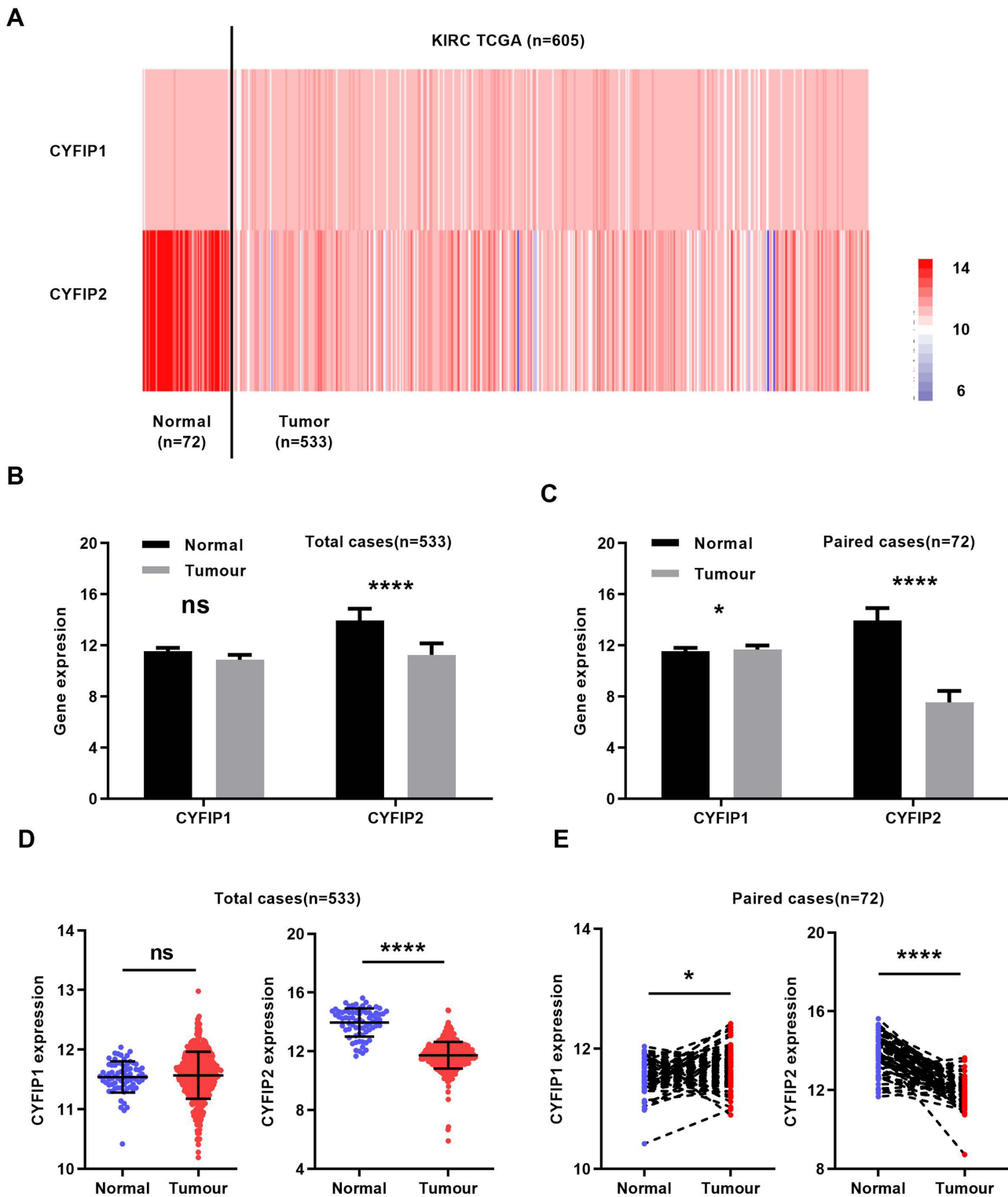**Figure 1** CYFIP family expression in ccRCC. (**A**) The CYFIP family expression in TCGA-KIRC datasets with heat map (n = 605). (**B–D**) CYFIP family expression in total and paired cases in TCGA-KIRC. Blue: low expression, Red: high expression; white: medium expression. TCGA-KIRC, The Cancer Genome Atlas kidney renal clear cell carcinoma. *P < 0.05, ****P < 0.0001, ns, not significant.

(Figure 3D and E), patients with higher grade (Figure 3F and G), and patients with higher TNM stage (Figure 3H and I), but not lymph node metastasis (Supplementary Figure 1A), female (Supplementary Figure 1B), older patients (Supplementary Figure 1C).
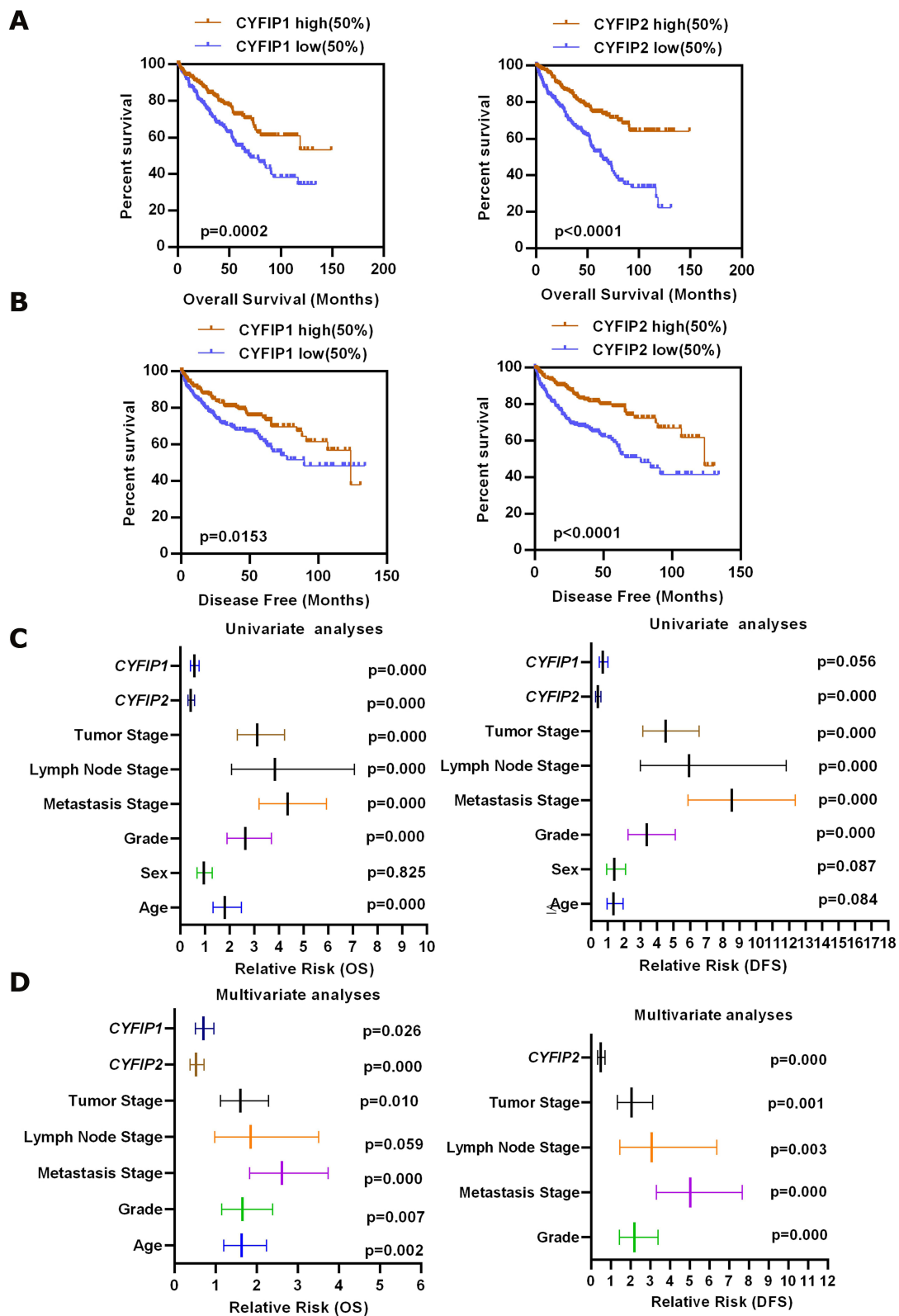
Figure 2 Prognostic role of CYFIP family in ccRCC. (**A**) High CYFIP1 or CYFIP2 expressers had a good OS. (**B**) High CYFIP1 or CYFIP2 expressers had a good DFS. (**C**) Univariate analysis of OS and DFS with CYFIP1 or CYFIP2. (**D**) Multivariate analysis of OS and DFS with CYFIP1 or CYFIP2. OS, overall survival; DFS, disease-free survival.

Confirm of the Expression Levels of CYFIP2 in RCC

Immunohistochemistry images (Figure 4A) and CPTAC database (Figure 4B) were used to explore the protein expression of CYFIP2. Results showed that CYFIP2 protein was downregulated in ccRCC cancer tissues relative normal tissues. Moreover, the aberrant protein expression of CYFIP2 was also found in ccRCC patients with higher Grade (Figure 4C) and TNM stage (Figure 4D). At last, we also verified CYFIP2 mRNA levels by qRT-PCR analysis on 28 pairs of clinical samples. Similarly, a decreased CYFIP2 expression in ccRCC was detected, consistent with the previous results (Figure 4E).
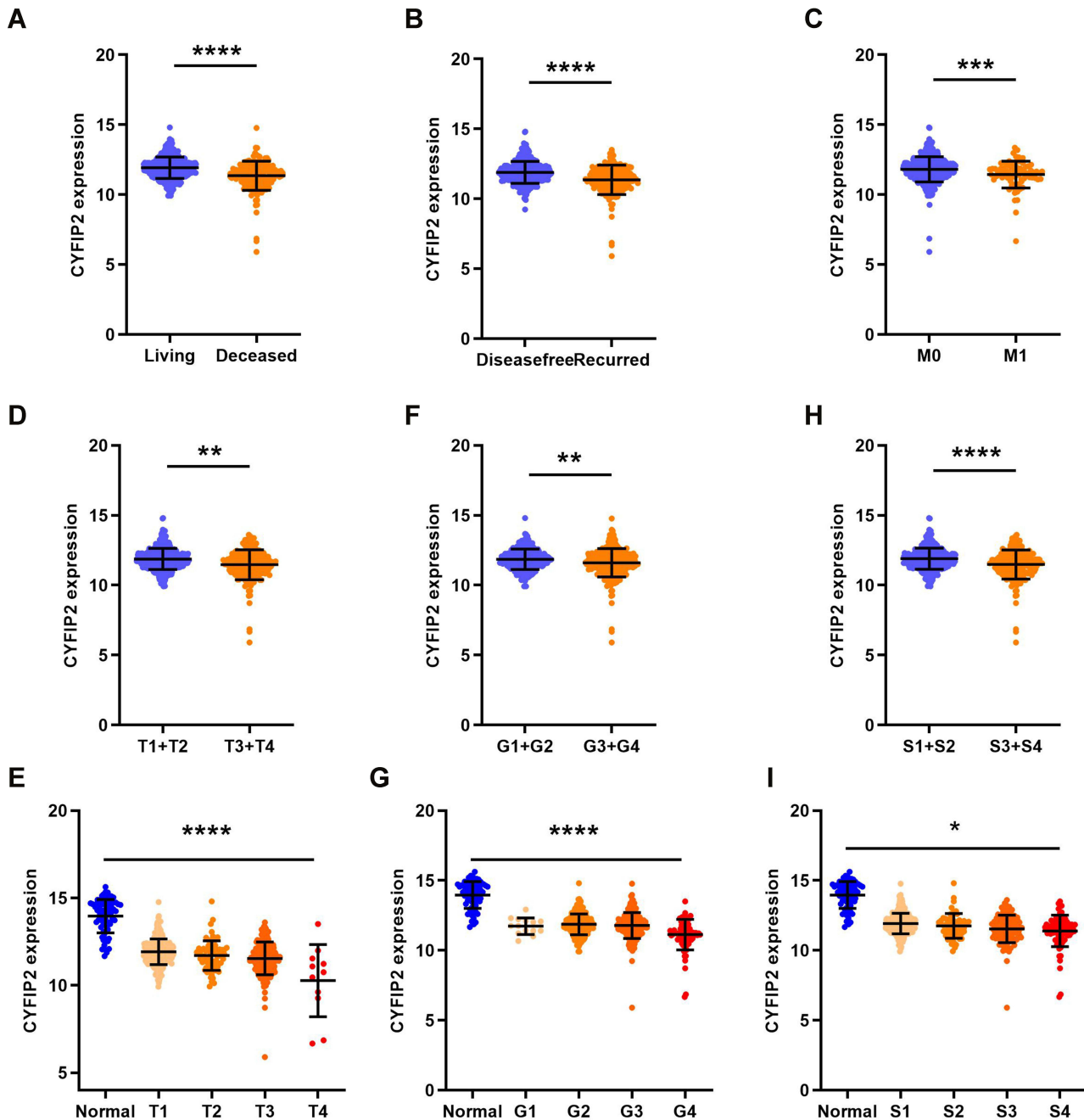
Figure 3 CYFIP2 expression correlated with clinicopathological parameters in TCGA-KIRC. (**A**) Patients survival status, (**B**) Patients disease free status, (**C**) patients with M stage, (**D**) and (**E**) patients with T stage, (**F**) and (**G**) patients with four grade, (**H**) and (**I**) patients with TNM stage. S: TNM stage. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.
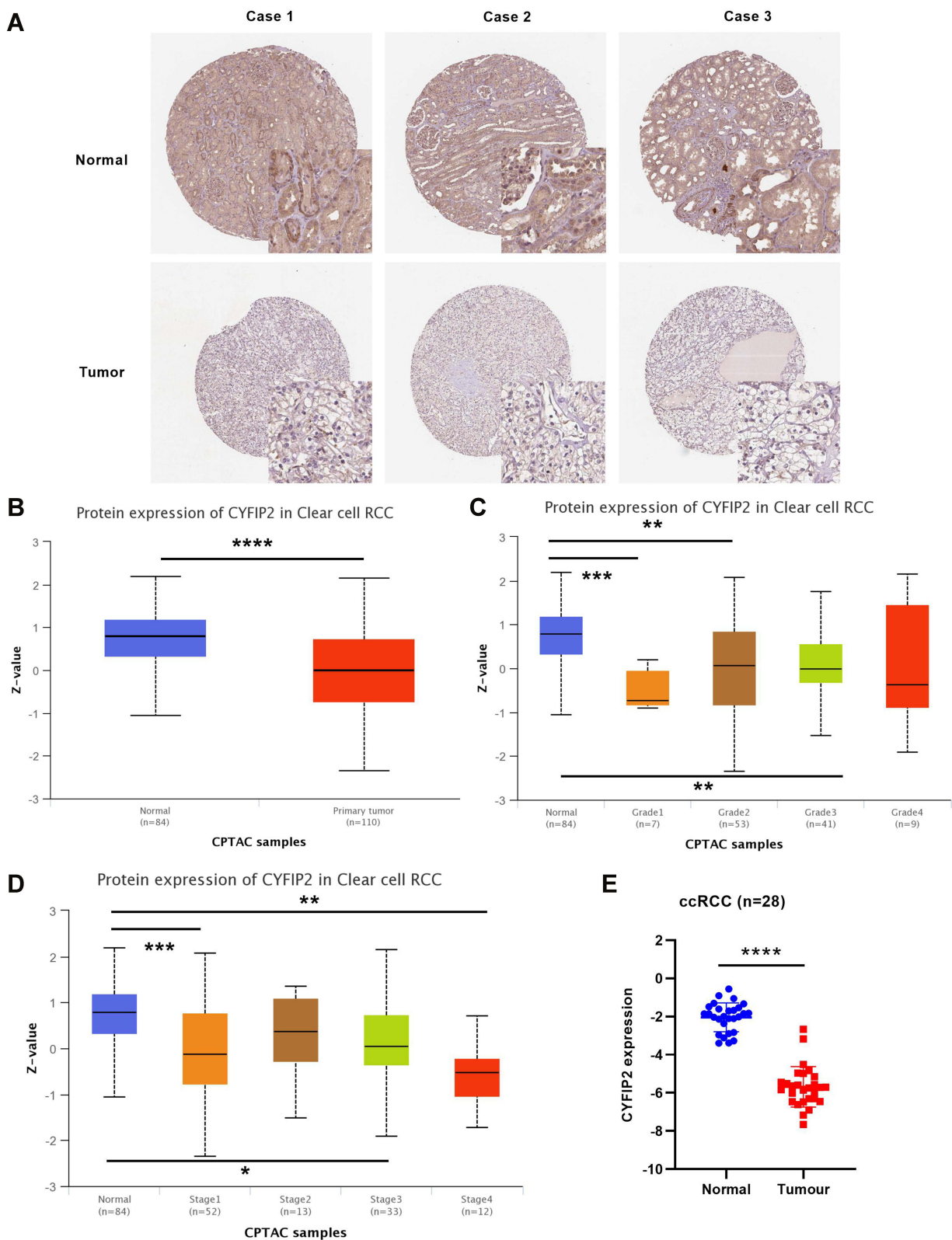
Figure 4 The expression levels of CYFIP2 in ccRCC. (**A**) Immunohistochemistry of CYFIP2. (**B**) The CYFIP2 protein expression in CPTAC ccRCC samples. (**C**) and (**D**) Low protein expression of CYFIP2 in ccRCC patients with subgroup of Grade and TNM stage. (**E**) The CYFIP2 mRNA was downregulated in ccRCC. CPTAC, Clinical Proteomic Tumor Analysis Consortium. **$P < 0.01$ and ***$P < 0.001$, ****$P < 0.0001$.

CYFIP2 Methylation in Clinical and Prognostic of ccRCC

To investigate the underlying decreased mechanism of CYFIP2 in ccRCC, we analyzed its promoter methylation level and beta values of CpG sites in 450k. Results showed DNA methylation might contribute to the downregulation of CYFIP2 in ccRCC (Figure 5A and B). And several CpG sites exhibited strong negative correlation with CYFIP2 mRNA (Figure 5C).

Signaling Pathways of CYFIP2 in ccRCC

GSEA results showed that genes positively associated with CYFIP2 were enriched on hallmark of fatty acid (Figure 6A) and bile acid metabolism (Figure 6B). Genes negatively correlation with CYFIP2 were enriched on hallmark of Epithelial–Mesenchymal Transition (EMT) (Figure 6C), the G2M checkpoint (Figure 6D) and angiogenesis (Figure 6E). The Kyoto Encyclopedia of Genes and Genomes (KEGG) function showed that the gene positively related to citrate tricarboxylic acid cycle (TCA cycle) (Figure 6F), peroxisome (Figure 6G), and peroxisome proliferators-activated receptor (PPAR) signaling pathway (Figure 6H), valine leucine and isoleucine degradation (Supplementary Figure 2A), propanoate metabolism (Supplementary Figure 2B), drug metabolism (Supplementary Figure 2C and D). Gene ontology (GO) analysis showed CYFIP2 was mainly enriched in amino acid catabolic process and ion transmembrane transporter (Supplementary Figure 2E and F).

Relationships of CYFIP2 with Immune Cells

Finally, we explored the interaction of CYFIP2 with tumor-immune cells. The results illustrated that CYFIP2 expression was correlated with several immune markers, including CD8 + T cells (Figure 7A), CD4 + T cells (Figure 7B), natural killer (NK) cells (Figure 7C), monocyte cells (Figure 7D), dendritic cells (Figure 7E), B lymphocyte cells (Figure 7F), T cells regulatory (Tregs) (Figure 7G), and cancer associated fibroblasts (Figure 7H) in TIMER database.

Discussion

The importance of CYFIP2 expression and potential function in cancer is increasing. Inhibition of CYFIP2 promoted cell proliferation and chemoresistance by activation of the Akt signal in gastric cancer.[23] CYFIP2 could act as a p53-inducible gene and promoted cell apoptosis in colorectal adenocarcinoma.[24] Activation cell death programs could suppress cell proliferation of tumor cells.[25,26] In addition, there are no research of CYFIP2 in other oncology fields. Our analysis of publicly available databases demonstrated that CYFIP2 expression was decreased in ccRCC. More importantly, currently, this is the first time CYFIP2 has been detected to be lowly expressed and associated with poorer clinicopathological parameters of ccRCC patients, by analyzing the TCGA database and clinical samples. Furthermore, our results first revealed that CYFIP2 reduction was strongly negatively regulated by its DNA methylation. Increasing evidence showed DNA methylation could cause changes in gene expression and function.[27–29] Promoter methylation of PTEN suppressed mitochondrial oxidative phosphorylation (OXPHOS) but improved glycolysis in esophageal cancer.[30] Melatonin and/or SIRT1 administration blocked the methylation of Aplasia Ras homolog one (ARHI) to increase ARH1 mRNA expression, and can reestablish sensitivity to paclitaxel in breast cancer.[31] In the current study, we discovered 10 CpG islands in CYFIP2 were negatively linked to CYFIP2 mRNA expression, with $P < 0.05$. Thus, we hold that DNA methylation might contribute to the downregulation of CYFIP2 in ccRCC. Overall, our study demonstrated that CYFIP2 gene might be an unfavorable prognostic marker of ccRCC patients.

In addition, the current work revealed the relationship between CYFIP2 and metabolism for the first time. Metabolic reprogramming and disorders were the hallmarks of cancer. These hallmarks provide possible research directions for the diagnosis, prognosis, and treatment of cancer.[32,33] Metabolic reprogramming of tumors can produce enough substrates and energy to promote cell proliferation, migration, and other malignant characteristics of tumors. The metabolic intermediates, fumarate and 2-hydroxyglutarate pathological accumulation can promote tumor occurrence and development. Fatty acids and glucose will undergo metabolic reprogramming during the development of ccRCC.[34,35] Nowadays, ccRCC is called as a "metabolic disease" as a variety of biological energy functions and pathways have great changes compared with normal cells.[33] The reprogramming of lipid, glucose, and amino acid metabolism greatly affects the development of renal cancer cells.[36] It may become one of the directions for the treatment of renal cancer.[37–39] In the current study, Gene Ontology and GSEA analysis results showed that CYFIP2 was involved in metabolic pathways, fatty acid degradation, amino acid degradation.
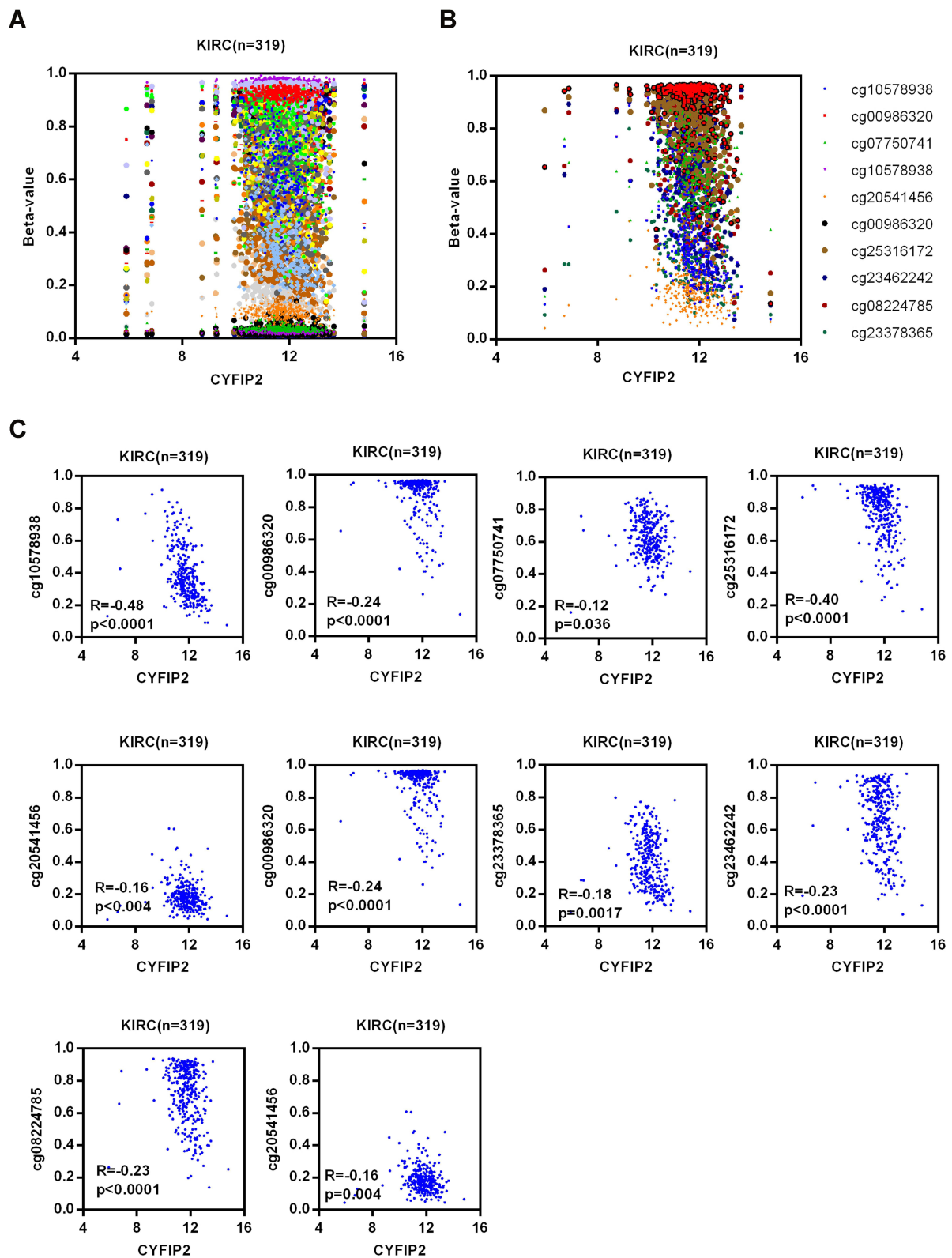
Figure 5 The methylation of CYFIP2 in ccRCC. (**A**) and (**B**) Methylation of CYFIP2 in renal cancer. (**C**) CYFIP2 was regulated by CYFIP2 DNA methylation.
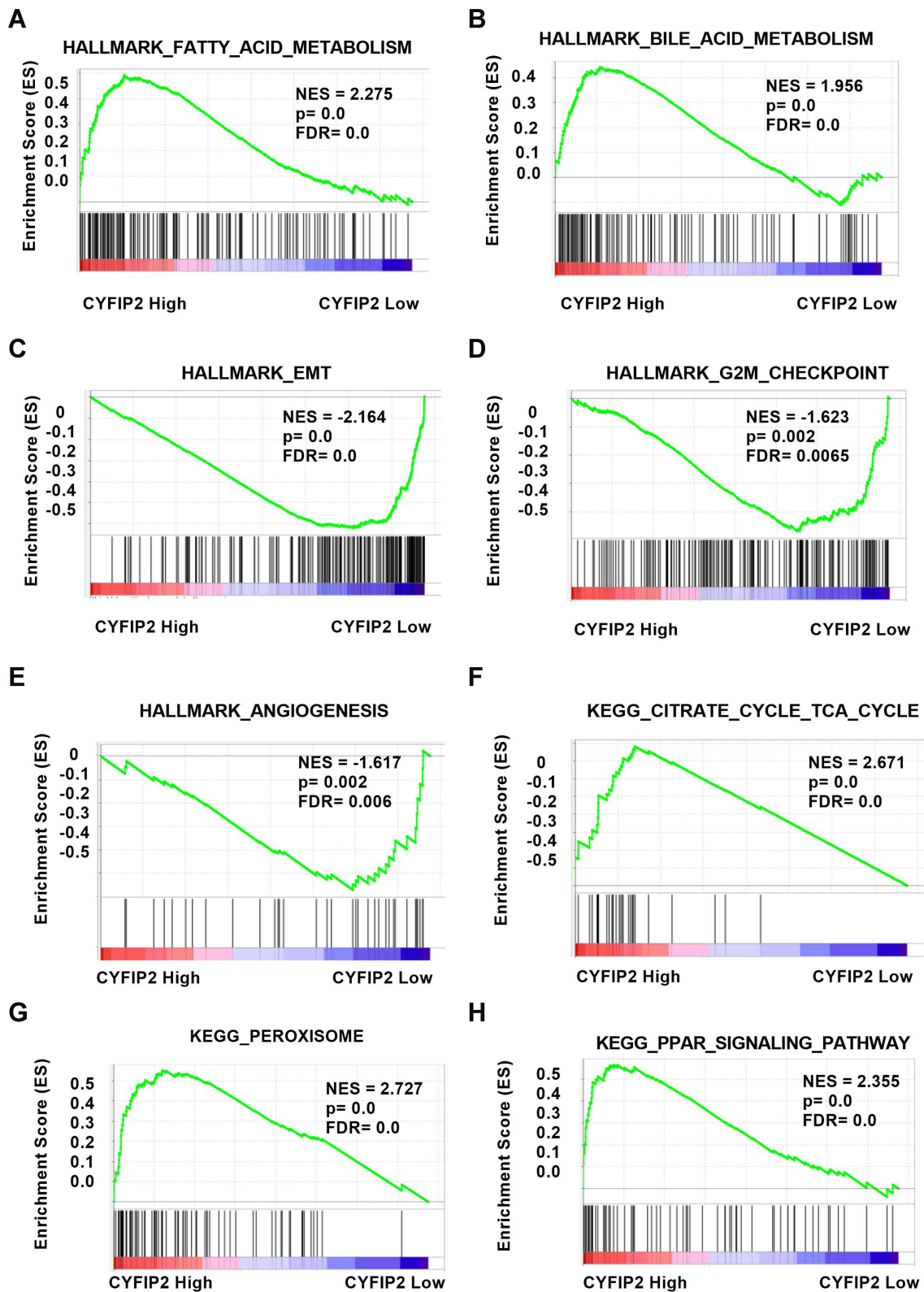
Figure 6 Pathogenesis of CYFIP2 in TCGA-KIRC with gene set enrichment analysis (GSEA). (**A** and **B**) Genes positively associated with CYFIP2 were enriched on hallmark of fatty acid and bile acid metabolism. (**C**–**E**) Genes negatively correlation with CYFIP2 were enriched on hallmark of EMT, the G2M checkpoint, angiogenesis. (**F**–**H**) Gene positively related to CYFIP2 were mainly enriched citrate TCA cycle, peroxisome, PPAR signaling pathway. GSEA, gene set enrichment analysis.
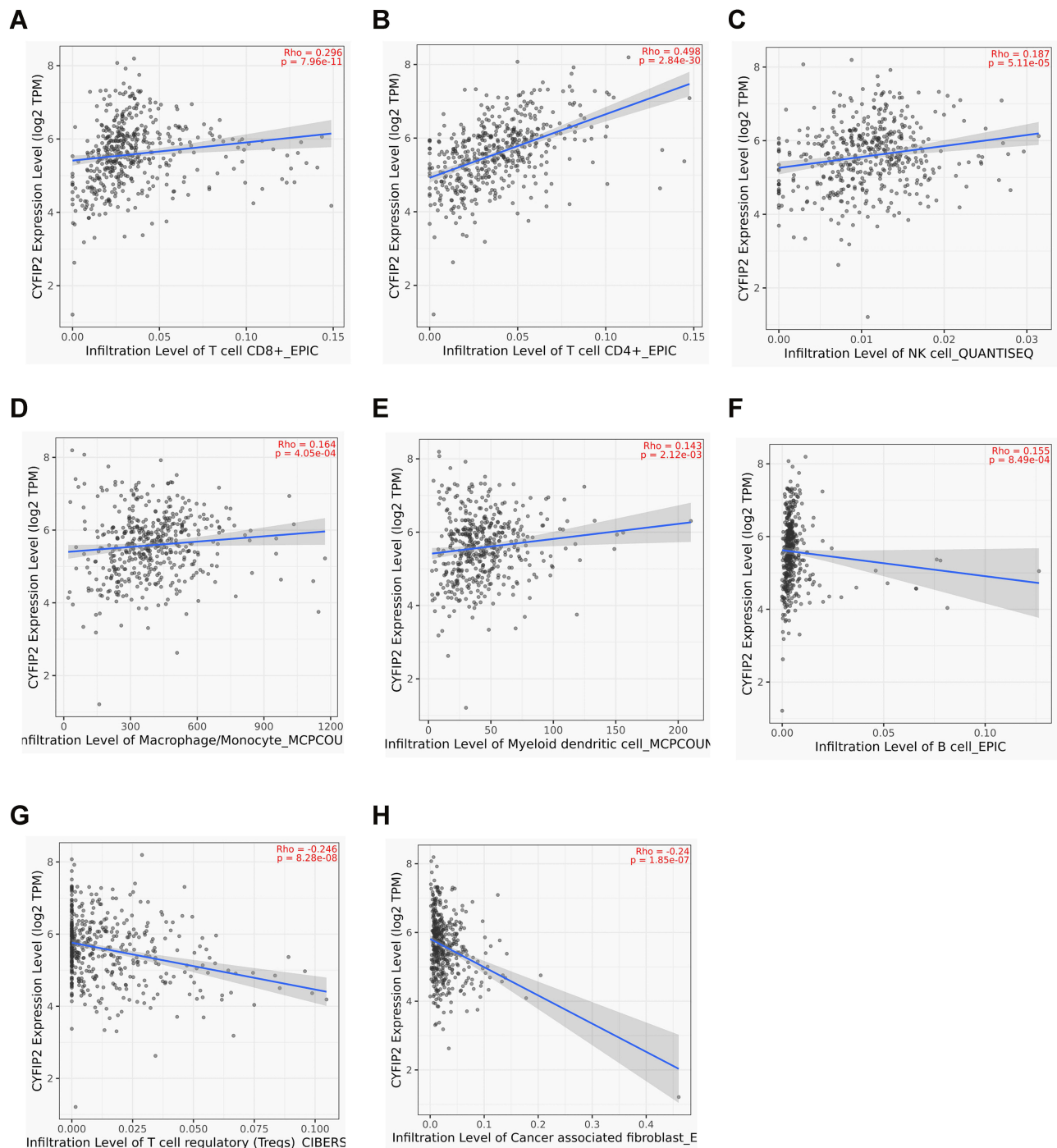
Figure 7 The correlation of CYFIP2 and gene markers of immune cells. (**A**) CD8+ T cells, (**B**) CD4+ T cells, (**C**) NK cells, (**D**) dendritic cells, (**E**) dendritic cells. (**F**) B cells, (**G**) T cell regulatory (Tregs), and (**H**) cancer associated fibroblasts.

The major biological processes were ion transmembrane transport, drug metabolism, and amino acid transport. And the results showed that genes positively associated with CYFIP2 were enriched on hallmark of fatty acid and bile acid metabolism. Additionally, emerging evidence suggests that the plasticity of the epithelial–mesenchymal transition (EMT) phenotype of tumor cells had an important clinical significance for the progress of cancer and chemotherapy resistance.[40,41] In this study, we also observed genes negatively correlation with CYFIP2 were enriched on hallmark of EMT and angiogenesis. Taking together, we hypothesized that CYFIP2 might play a regulatory role in metabolic reprogramming and the EMT pathway in ccRCC.

Current research showed that inhibitors of immune checkpoint were used to treatment advanced cancers.[42,43] LAG3 is expressed on activated immune cells, but the promoter methylation of LAG3 could be an epigenetic biomarker correlating, immune cell infiltration OS and DFS in melanoma.[44] Previous study showed CYFIP2 was highly abundant in CD4+ cells and regulated T cell adhesion in multiple sclerosis patients.[45] In the current study, we evaluated the correlation between CYFIP2 and immune markers in ccRCC for the first time by using the TIMER database. Our analyses revealed that a list of immune markers was significantly correlated with CYFIP2 expression especially with CD4+ cells and CD8 + cells in ccRCC.

In summary, this study found that CYFIP2 was down-regulated and the downregulation of CYFIP2 might be due to DNA methylation. CYFIP2 could be an independent predictor for ccRCC. Results demonstrated that CYFIP2 might involve in the metabolic reprogramming, the EMT pathway, and immune infiltration process in ccRCC. CYFIP2 might be a potential novel prognostic molecule and a drug target for ccRCC. However, the aberrant expression of CYFIP2 and its down-regulation mechanism still need to be verified by more clinical specimens. And the specific mechanism of action between CYFIP2 and the carcinogenesis pathway in ccRCC, as well as in vivo and in vitro studies, were not well explored and therefore form the basis of our further research.

Ethics

This investigation was approved by the Ethics Committee of Huazhong University of Science and Technology (S2086).

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declared no competing interests.

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