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ORIGINAL RESEARCH

# Prognostic Values of E2F1/2 Transcriptional Expressions in Chromophobe Renal Cell Carcinoma Patients: Evidence from Bioinformatics Analysis



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**Background:** Numerous studies on the E2F transcription factors have led to increasing insights that E2Fs could be an important driver of the formation and progression of many human cancers. Little is known about the function of distinct E2Fs in chromophobe renal cell carcinoma (chRCC).

**Methods:** We utilized the UALCAN, GEPIA, Cancer Genome Atlas (TCGA) database, cBioPortal, Metascape, STRING, Cytoscape, GeneMANIA, TIMER, TISIDB, GSCALite, and MEXPRESS databases to investigate the transcription level, genetic alteration, methylation, and biological function of E2Fs in chRCC patients, and its association with the occurrence, progress, prognosis, and immune cell infiltration in patients with chRCC.

**Results:** We found that E2F1/2/4/7/8 were more expressed in chRCC tissues than in normal tissues, while the expression of E2F5/6 was lower in the former than in the latter, and the expression levels of E2F1/2/4/5/6//7/8 were also associated with the histological parameters of chRCC, including T-stage and N-stage. Higher expression of E2F1/2/7/8 was found to be significantly correlated with worse overall survival (OS) in chRCC patients. Cox regression and time-dependent ROC analysis further suggested that E2F1/2 could be the potential independent biomarkers for chRCC prognosis. Besides, a moderate mutation rate of E2Fs (34%) was noticed in chRCC, and the genetic mutations in E2Fs were associated with poor survival of chRCC patients. We noticed that the expression of E2Fs was statistically correlated with the immune cell infiltration in chRCC. Moreover, we also found that the expression of E2F1 was significantly correlated with tumor-infiltrating lymphocytes and immunomodulators, E2F7 expression was associated with MHC molecules, and the expression of E2F1/8 was correlated to their methylation levels.

**Conclusion:** Our results provide novel insights for selecting the prognostic biomarkers for chRCC and suggest that E2F1/2 could act as potential prognostic biomarkers for the survival of chRCC patients. However, more in-depth experiments are required to identify the underlying mechanisms and verify the clinical value of E2F1/2 in the prognosis of chRCC.

**Keywords:** chromophobe renal cell carcinoma, E2Fs, prognosis, UALCAN, GEPIA

## Introduction

Renal cell carcinoma (RCC) is one of the most seen neoplasms of the kidney, accounting for 2–3% of human malignancies.[1] In the USA, the approximate number of diagnosed and death cases of RCC in 2020 was 73,000 and 14,000, respectively.[2] According to the definition of 2016 World Health Organization classification, RCC consists of several histological subtypes, including clear cell RCC (ccRCC, accounts for 70–75%), papillary RCC (pRCC 1 and 2, accounts for 10–15%),

chromophobe RCC (chRCC, accounts for 3–5%), medullary, translocation, collecting duct, and other rare subtypes[3] (accounts for less than 1% for each). The overall survival (OS) and relapse-free survival (RFS) of ccRCC have been significantly improved over the past several years due to the targeted therapies.[4] However, the optimal treatment for other types of RCC, which are often termed "non-ccRCC", remains unclear because of the relatively low prevalence and rare clinical trials of these malignancies.[5] pRCC is the most common subtypes of non-ccRCC, and the mechanisms of the carcinogenesis, prognosis, genomic and epigenomic features of pRCC have been more studied compared with other types of non-ccRCC.[6,7] chRCC ranks the second most prevalent form of non-ccRCC.[8] It is generally believed that chRCC patients have better clinical outcomes than patients with ccRCC, since the former present with a lower stage and grade.[9,10] Nevertheless, the studies for the carcinogenesis and prognosis of chRCC are relatively limited, and the data regarding the independent prognostic values of this cancer is conflict.[9,11] The commonly believed candidate mutated gene in chRCC is TP53, which is frequently implicated in cancers, and participated in the occurrence and progress of chRCC by regulating cell cycle arrest, cell differentiation, and apoptosis.[12] Currently, no other effective potential biomarkers have been reported yet. Therefore, understanding the inherent pathogenesis and etiology of chRCC, as well as identifying novel and effective biomarkers of chRCC would show light in assessing the malignancy and enhancing the individualized therapeutic potential for this carcinoma.

E2F transcription factors (E2Fs) are a group of proteins comprising eight distinct members (E2F1-E2F8), which participated in the control of the cell cycle, DNA synthesis, cell differentiation, and cell death.[13,14] It is reported that the transcriptional regulation of E2Fs depends on the activating and repressing functions.[15] Therefore, the mammalian proteins E2F1 to E2F8 can be divided into two subfamilies: transcription activators E2F1-3, and transcription repressors E2F4-8.[16] Mounting evidence had proved that deregulation of the E2Fs genes is significantly involved in the occurrence of several human carcinomas, including gastric cancer, lung cancer, and breast cancer.[17–19] In Kidney cancer, the role of E2Fs had been reported: E2F1-3 was crucially involved in the progression of ccRCC and could serve as valuable diagnostic markers for ccRCC.[20,21] However, little is known about the role of E2Fs in the development and prognosis of chRCC. Herein, in the current study, we filled in the blank by taking the expression and mutation data of various E2Fs factors and their relations with clinical parameters in patients with chRCC into analysis, aimed to identify the potential prognostic values of E2F transcription factors in this neoplasm.

# Materials and Methods

## Gene Expression Analysis

We first used the R package of "ggplot2" in R studio to explore the difference of E2Fs expression between chRCC tissues and normal samples in The Cancer Genome Atlas (TCGA) database.[22] Then, the UALCAN portal (http://ualcan.path.uab.edu/analysis.html),[23] an interactive web tool, which can be applied to analyze tumor transcriptome data based on level 3 RNA-seq and clinical data (eg, patient survival information) of various cancer types from the TCGA database, is applied to examine the differential expression of E2Fs between chRCC tissues and normal samples. We also explored the mRNA expression of E2Fs family members in normal and tumor specimens of chRCC in different stages using the UALCAN web resource. Besides, differences of E2Fs expression among various pathological stages of chRCC patients were compared using the GEPIA database, which is a newly developed analysis tool that included the data of 9736 tumors and 8587 normal samples from both the TCGA and GTEx (Genotype Tissue Expression) programs.[24] In addition, the correlation between expression of the E2Fs and other histological parameters, including the pathologic stage, T-stage, and N-stage were explored using the Kruskal–Wallis Test with the RNAseq and clinical data retrieved from TCGA. The cutoff p-value was set as 0.05.

## Survival Prognosis Analysis

Based on the expression status of the E2Fs family, we also performed a survival analysis using the GEPIA database, and the Kaplan-Meier curves were plotted. By entering the gene name in the "Survival Analysis" module of GEPIA, "Overall Survival (OS)" and "Disease-Free Survival (DFS)" data of E2Fs expression in chRCC patients can be obtained. The group cutoff was set as "Quartile". Moreover, the effect of E2Fs expression on chRCC patient survival was examined by the UALCAN database.

To further evaluate the potential independent prognostic value of E2Fs in chRCC patients, clinicopathological data, including gender, age, race, OS time and status, T/N/M stage, and pathologic stage, as well as mRNA

expression of E2Fs of 65 chRCC patients were retrieved from TCGA (Table S1) for further analysis. Using SPSS version 24.0, we explored the association of mRNA expression of E2Fs with 65 chRCC patient's survival status with the Cox regression analysis. First, we used the univariate Cox regression to evaluate the influences of clinicopathological parameters and mRNA expression of E2Fs on the survival of chRCC patients. Then, parameters with $p<0.05$ were retained for further multivariate analysis, which adjusted for other criteria (eg, gender, age, pathological stage). Statistical significance was set as $p<0.05$. Besides, R packages of "ggrisk", "survival", "survminer", and "time ROC" were utilized for survival analysis and to generate figures.

## Genetic Alteration Analysis

In the current study, genomics profiles data, including genetic mutations and putative copy-number alterations, were retrieved from cBioPortal,[25] a comprehensive online web resource that is held and supported by Memorial Sloan Kettering Cancer Center based on 65 chRCC samples from the TCGA database. mRNA expression z-Scores (RNAseq V2 RSEM) were obtained with a z-score threshold of ±1.8. Using Kaplan-Meier plots, the genetic mutations in 8 E2Fs family members and their association with OS, Disease-Specific Survival (DSS), DFS, and Progression-Free Survival (FP) of chRCC patients were demonstrated, and the statically significant difference was set as p-value <0.05. Co-expression of E2Fs in chRCC was calculated adhered to the instructions of cBioPortal.

## E2Fs-Related Gene Enrichment Analysis

Using the R package of "stat", we obtained the top 20 genes which were most correlated to E2F1 expression in chRCC from the TCGA database. Using the same way, other 140 genes that are most associated with E2F2-8 were obtained. After combining E2F1-8 themselves and removing the duplicated genes, we finally get 153 genes that were utilized for further enrichment analysis. In the current study, the interaction of E2Fs and their closely correlated genes were constructed by STRING (http://string-db.org/)[26] and visualized by Cytoscape.[27] The plug-in Molecular Complex Detection (MCODE) of Cytoscape was applied to identify the densely connected module. The parameters of MCODE were set as follows: degree cut-off=34, k-score=2, node score cut-off =0.2, and Max depth=100. Obviously, the higher the degree of connectivity of the node, the more important it is in maintaining the stability of the entire network. In the current study, the top ten genes with the highest degree of connectivity were deemed as hub genes.

We also used the Metascape (http://metascape.org)[28] portal to perform the Gene Oncology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis. The former contains three aspects that can predict the functional roles of genes closely related to E2Fs, including molecular functions (MF), cellular components (CC), and biological processes (BP), while the latter can delineate the pathways of the genes related to E2Fs. Moreover, the GeneMANIA[29] database was utilized to construct the interaction network of E2Fs.

## Immune-Related Analysis in TIMER and TISIDB Database

By logging into the TIMER database, a comprehensive web server that contains 10,897 samples from 32 human tumors,[30] the relationship between typical infiltrating immune cells (B cells, CD4+ T cells, CD8+ T cells, neutrophils, macrophages, and dendritic cells) and E2Fs expression in chRCC was explored. The correlation was presented as a heatmap and the details were shown in scatterplots, the partial Spearman correlation that is purity-corrected was provided. Besides, the Kaplan-Meier plots for E2Fs expression and immune infiltrates were generated using the "Survival" module of the TIMER database to visualize the survival differences.

To further investigate the association between E2Fs expression and immune cells, the typical gene markers of specific immune cells from the R&D Systems website (https://www.rndsystems.com/cn/resources/cell-markers/immune-cells)[31] were chosen. We then used the "Correlation" module of the TIMER database to perform the Spearman correlation analysis between E2Fs expression and the selected gene markers.

We then used the TISIDB (http://cis.hku.hk/TISIDB/index.php)[32] database, an integrated website for tumor-immune system interactions, to explore the relationship between the abundance of 28 tumor-infiltrating lymphocytes (TILs), three kinds of immunomodulators (immunoinhibitor, immunostimulator, and MHC molecule), and E2Fs expression in chRCC. The correlation values were recorded and presented as heatmaps.

## Methylation Analysis

Using GSCALite (http://bioinfo.life.hust.edu.cn/web/GSCALite/),[33] a web-based platform with data merged

by TCGA barcode for Gene Set Cancer Analysis, we examined the relationship between paired E2Fs expression and DNA methylation. The Person's correlation was performed, and the p-value was corrected by FDR. Besides, MEXPRESS (https://mexpress.be/)[34] database was also applied to explore the association between DNA methylation and expression levels of the E2Fs family. The adjusted p-value (Benjamini-Hochberg) and the Pearson correlation coefficient value (R) of each probe were provided.

# Results

## Aberrant Expressions of E2Fs in chRCC Patients

We first compared the transcriptional levels of E2Fs in chRCC and adjacent normal tissues to explore their potential therapeutic and prognostic values. As shown in Figure 1A, the transcriptional levels of E2F1/2/3/4/8 ($p<0.05$ for all) in chRCC tissues were significantly higher compared with that in normal samples. While the higher expression of E2F5 and E2F6 was noticed in the normal samples (Figure 1A, $P<0.05$ for both). We also measured the mRNA expression patterns of 8 E2Fs family members with UALCAN. As shown in Figure 1B, the transcriptional levels of E2F1/2/4/7/8 in chRCC tissues were significantly increased while the transcriptional levels of E2F5, and E2F6 were decreased ($p<0.05$ for all).

## Association of mRNA Expression Levels of E2Fs Family Members with the Clinicopathological Parameters of chRCC Patients

After exploring the relationship between mRNA expression of E2Fs in chRCC tissues and normal samples, we next analyzed the differences of mRNA expression levels of E2Fs between normal samples and tissues with individual chRCC stages by the UALCAN database. As shown in Figure 2A, we found E2F1/2/4/8 were more expressed in chRCC patients with different pathologic stages than the normal samples, while the higher expression of E2F5 and E2F6 was observed in the normal groups ($p<0.05$ for all). In addition, we also used the GEPIA database to investigate the relationship between the expression of E2Fs and tumor stage in ccRCC patients. As presented in Figure 2B, the expression levels of E2F1/2/6/7 were statistically associated with the tumor stage of ccRCC patients ($p<0.05$ for all). Moreover, we also noticed a higher expression of E2F1/2/8 in chRCC patients with different T/N stages compared with that in the normal samples, while E2F5 and E2F6 were more expressed in the normal samples than in chRCC patients with different T/N stages (Figure S1A, B, $p<0.05$ for all). Meanwhile, higher expression of E2F2 and E2F7 were observed in patients with N1&N2 stages than with the N0 stage ($p<0.05$ for both) (Figure S1B). In short, these data suggest that the expression of E2Fs may associate with the progression of chRCC.

## Prognostic Value of mRNA Expression of E2Fs in Patients with chRCC

Utilizing the GEPIA and UALCAN databases, we also explored the value of mRNA expression of E2Fs in the prognosis of chRCC by analyzing the correlation between differentially expressed E2Fs and clinical outcome of chRCC patients. As shown in Figure 3A, poorer OS of chRCC patients was significantly associated with the increase of E2F1 ($p=0.0047$), E2F2 ($p=0.011$), and E2F7 ($p=0.021$), while the worse DFS of chRCC patients was significantly associated with the increase of E2F1 ($p=0.028$), E2F2 ($p=0.039$), and E2F8 ($p=0.04$). Evidence from the UALCAN database also proved that the survival probability of chRCC patients was significantly associated with the expression levels of E2F1 ($p=0.00017$), E2F2 ($p<0.0001$), and E2F7 ($p=0.023$) (Figure 3B).

## Independent Prognostic Value of mRNA Expression of E2Fs in Terms of OS in Patients with chRCC

To assess the independent prognostic value of E2Fs family members for patients with chRCC, we further downloaded the clinical and mRNA expression data of E2Fs from the TCGA database for Cox regression analysis. In the univariate analysis, we discovered that the risk of death was statistically greater in patients with higher T stage (HR=10.298, 95% CI: 2.203–48.140, $p=0.003$) and pathologic stage (HR=7.702, 95% CI: 2.652–22.367, p=1.75e-4) than those with lower stages. Besides, older age (>70 years old) (HR=6.022, 95% CI: 1.501–24.168, $p=0.011$) was related to the poorer OS of chRCC patients. Moreover, we found higher mRNA expression levels of E2F1 (HR=7.600, 95% CI: 1.899–30.423, $p=0.004$), E2F2 (HR=13.446, 95% CI: 2.777–65.109, $p=0.001$), E2F7 (HR=4.204, 95% CI: 1.122–15.759, $p=0.033$), and E2F8 (HR=3.851, 95% CI: 1.032–14.364, $p=0.045$) were associated with worse survival of chRCC patients (Table S2). Multivariate analysis also

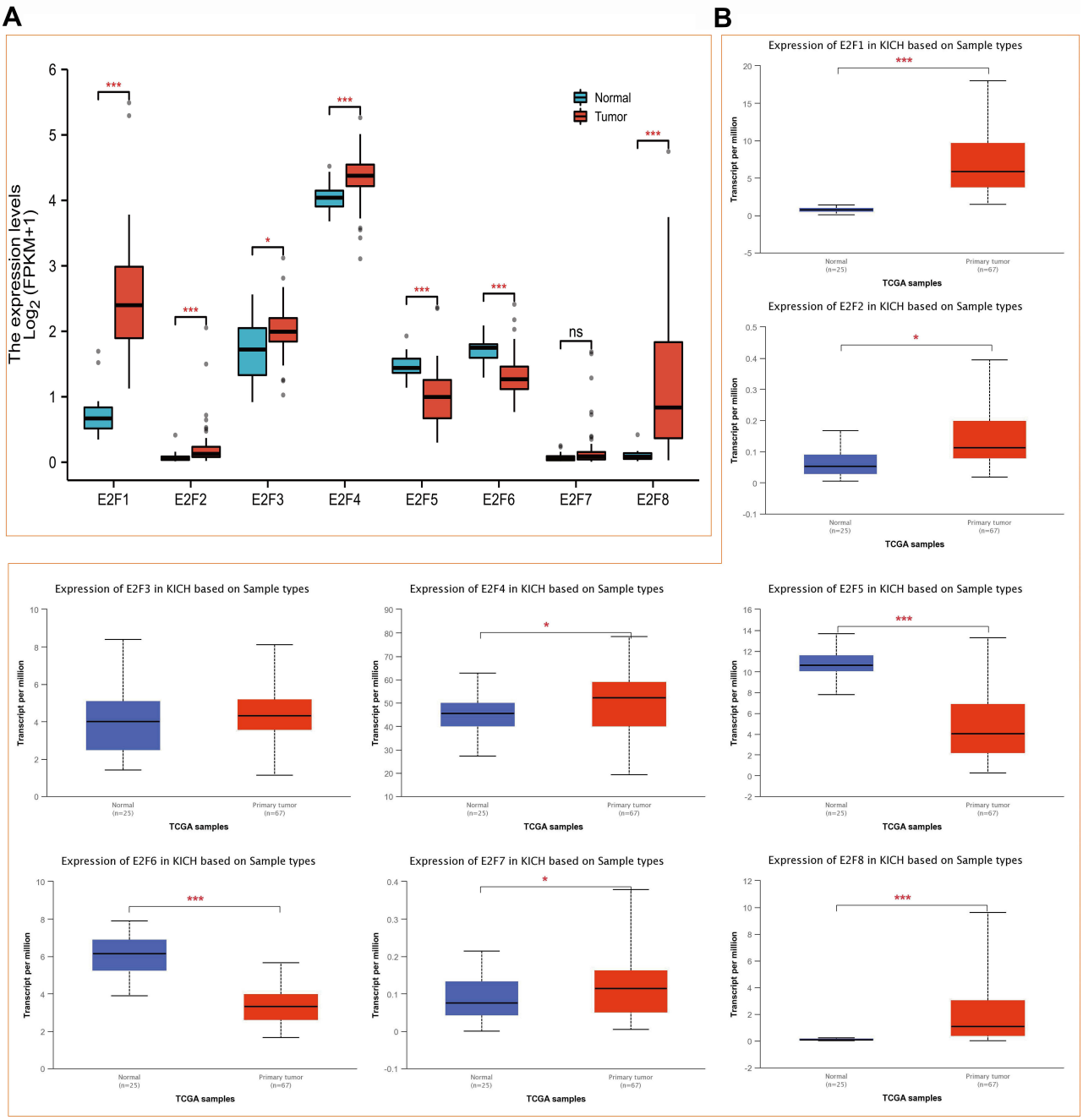


**Figure 1** Expression of distinct E2Fs family members in chRCC tissues and adjacent normal kidney tissues. (**A**) Differential expression analysis of RNAseq data of E2Fs expression in chRCC and normal samples from TCGA. (**B**) The transcriptional levels of E2Fs in chRCC and normal samples (UALCAN). ns, p≥0.05; *p< 0.05; ***p<0.001.

proved that high mRNA expressions of E2F1 (HR=7.311, 95% CI: 1.401–38.16, p=0.018) and E2F2 (HR=12.885, 95% CI: 1.831–90.671, p=0.01) were significantly correlated to worse OS of chRCC patients (Table S3).

Besides, based on the raw counts of RNA-sequencing data and the corresponding clinical data of 65 chRCC patients retrieved from the TCGA dataset. The KM survival and Time-dependent ROC analysis with the Log rank test were applied to compare the survival difference between differential expression of E2F1/2/7/8 and the predictive accuracy and risk score of E2F1/2/7/8 in chRCC patients. As presented in Figure 4A, D, and G, we found that after sorting the E2F1/2/7 expression from low to high, the corresponding middle scatter plots show the trend of more and more patients dying and shorter survival time from left to right. As shown in Figure 4B, E, and H, we found that higher expression of E2F1/2/7 (p=3e-04, p=0.00013, and p=0.013, respectively) are risk factors of chRCC, the higher the gene expression, the

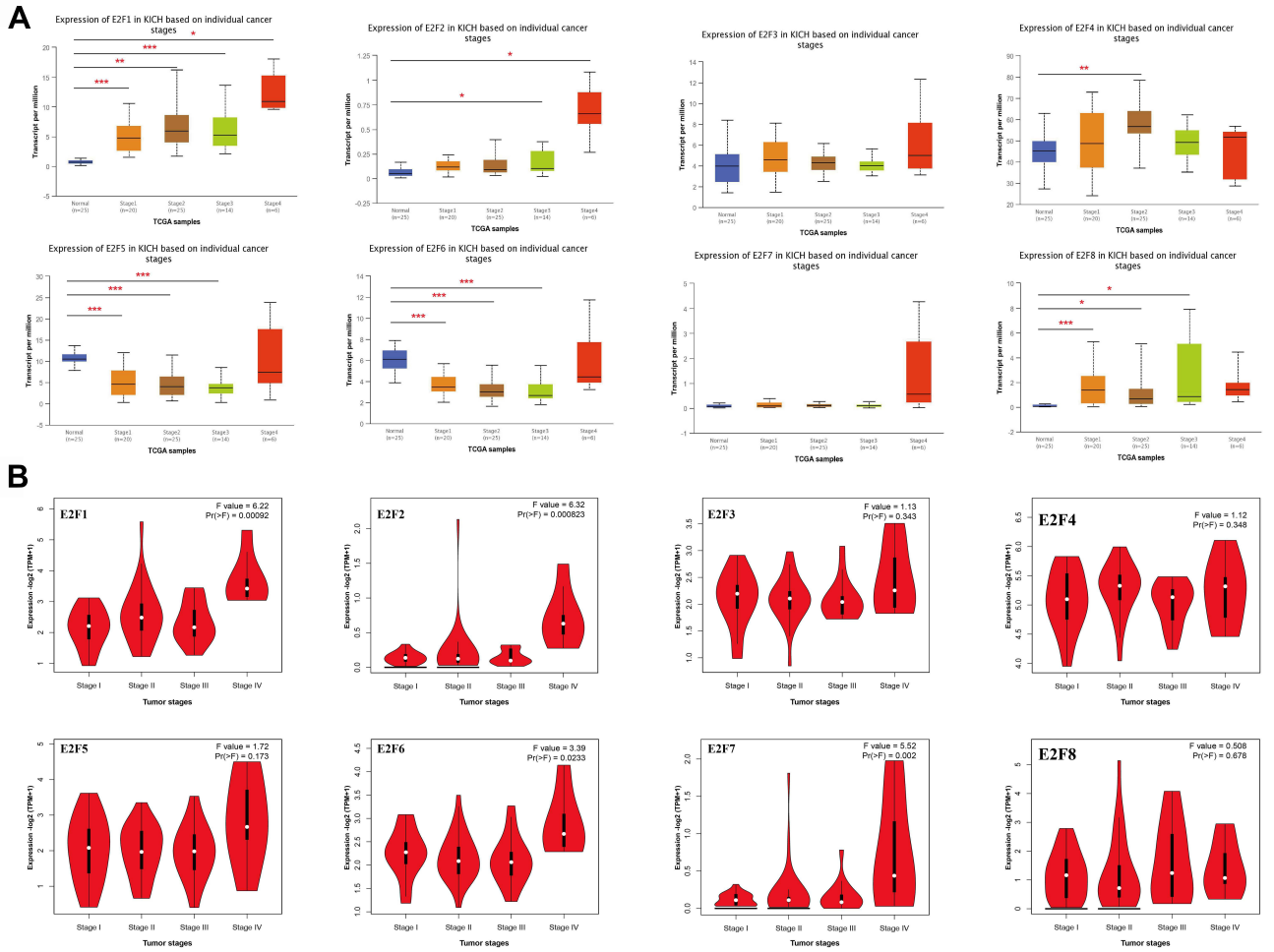


**Figure 2** Correlation between E2Fs expression and pathologic stage in chRCC patients. (**A**) Relationship between mRNA expression of distinct E2Fs family members in normal and individual cancer stages of chRCC (UALCAN). (**B**) Correlation between different expressed E2Fs and the pathological stage of chRCC patients (GEPIA). *p< 0.05; **p<0.01; ***p<0.001.

worse the overall survival. Time-dependent ROC analysis suggests that E2F1, E2F2, and E2F7 have a great potential to predict the survival of chRCC patients. The AUC of 1-year, 3-years and 5- years were 0.983, 0.839, and 0.881 for E2F1; 0.983, 0.822, and 0.869 for E2F2, and 0.983, 0.830, and 0.903 for E2F7, respectively (Figure 4C, F, and I). Of interest, no statistical significance was noticed for E2F8 in the KM survival and Time-dependent ROC analysis (Figure 4J, K, and L, p=0.19). Taken together, we found that E2F1/2 could act as the potential prognostic biomarkers in chRCC.

## Genetic Alteration in E2Fs and Their Associations with OS, DFS, DSS, and FP of chRCC Patients

We analyzed the genetic alterations of differentially expressed E2Fs family members using the cBioPortal online tool for chRCC. As shown in Figure 5A, E2F1, E2F2, E2F3, E2F4, E2F5, E2F6, E2F7, and E2F8 were altered in 5%, 3%, 11%, 8%, 11%, 9%, 11%, and 8% in 22 samples of the 65 sequenced of patients (34%). The mRNA expression z-scores of E2Fs relative to normal samples were presented in Figure 5B. In addition, the cBioPortal online tool also provides a Kaplan-Meier plot and Log rank test to analyze the association between genetic mutations and the prognosis of patients. We found that the genetic alteration in E2Fs was significantly correlated to the shorter OS (Figure 5C, p=8.893e-5; Figure 5G, p<0.001), DSS (Figure 5E, p=1.514e-3; Figure 5I, p<0.001), and FP (Figure 5F, p=0.0169; Figure 5H, p<0.001) of chRCC patients. While no association was noticed between the genetic alterations of E2Fs and DFS in chRCC (Figure 5D, p=0.388). Therefore, the prognosis of chRCC patients could also be statistically affected by the genetic alteration of E2Fs.

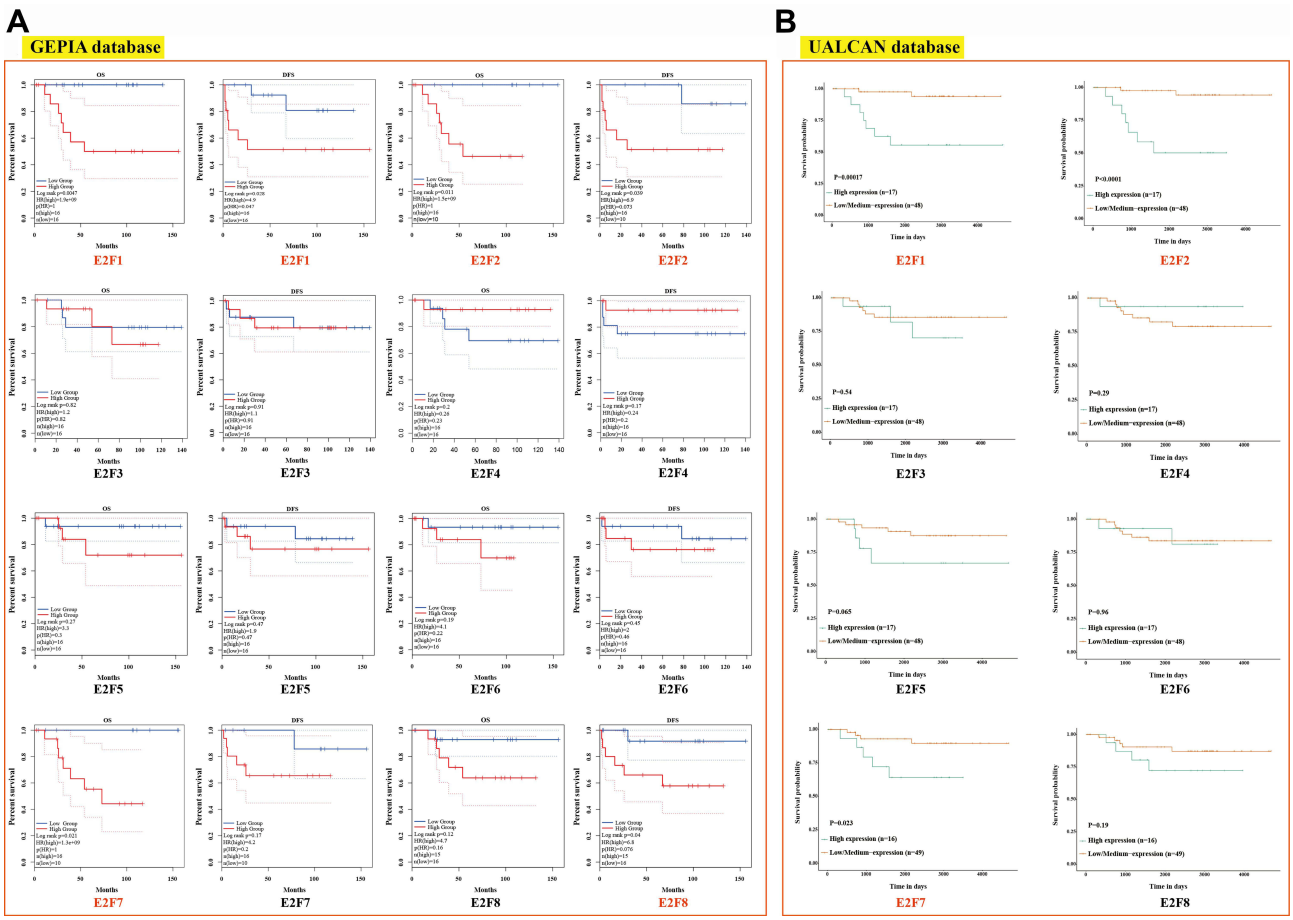


**Figure 3** Prognostic role of E2Fs expression in chRCC patients (**A:** GEPIA, **B**) UALCAN).

## Co-Expression, Neighbor Gene Network, and Interaction Analyses of E2Fs in chRCC Patients

After exploring the genetic alteration patterns in E2Fs and their prognostic value in chRCC patients, we next analyzed the potential co-expression of the differentially expressed E2Fs in chRCC. There was a low to moderate positive correlation between the expression of E2F3 and E2F6, E2F1 and E2F8, E2F2 and E2F5, and between E2F7 and E2F8. While moderate to high positive correlations were found among E2F1, E2F2, and E2F7 (Figure 6A, $p<0.05$ for all). Interestingly, we noticed negative correlations between E2F3 and E2F8, E2F4 and E2F5, E2F6 and E2F7, E2F6 and E2F8 (Figure 6A, $p<0.05$ for all). The correlations between E2F1-8 and their most correlated genes were presented in Figure 6B–I. We then constructed a PPI network analysis to explore the potential interactions among E2Fs and their most correlated genes (Figure 7A). Using the plug-in MCODE of Cytoscape, we detected the hub genes with a higher degree of connectivity. As shown in Figure 7B and Table S4, the protein-coding genes, including PLK1, CCNA2, CDC20, BIRC5, CDCA8, RRM2, BUB1B, TOP2A, TPX2, and CDT1 were mainly associated with the regulation and function of the differentially expressed E2Fs in chRCC.

## Functional Enrichment Analysis of E2Fs in Patients with chRCC

Using the online tool of Metascape, we performed the functional and pathway enrichment analysis to explore the biological classification of E2Fs and their most correlated genes. GO analysis results performed in Figure 7B show that changes in biological processes of E2Fs and their correlated genes were significantly enriched in GO: 0140014 (Mitotic nuclear division), GO: 0000280 (Nuclear division), GO: 0048285 (organelle fission), GO: 0000070 (Mitotic sister chromatid segregation), and GO: 0000819 (Sister chromatid segregation) (Figure 7C, Table

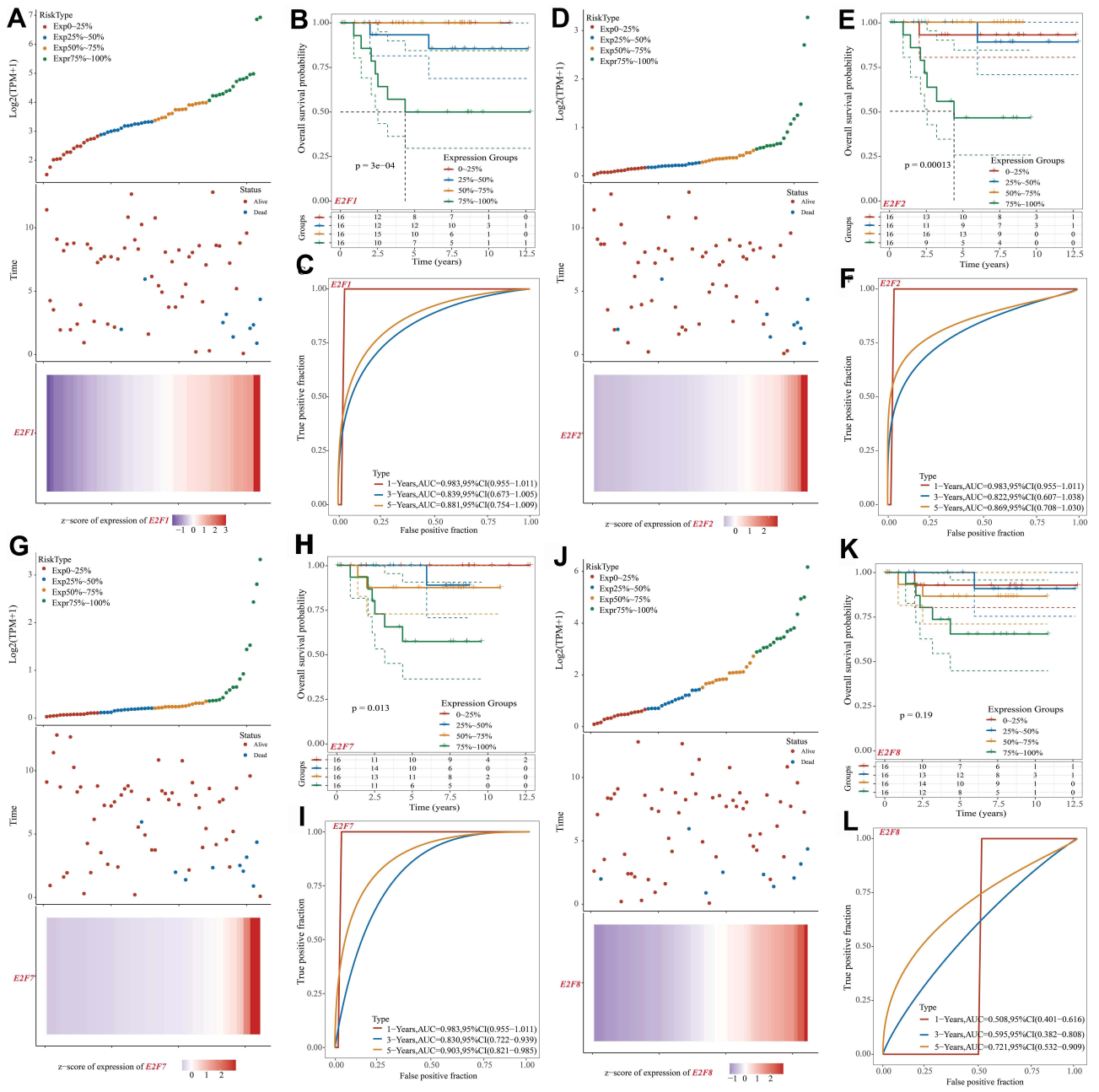


**Figure 4** Prognostic analysis of gene signature in the TCGA set. The dotted line represented the risk score and divided the patients into Exp 0–25% group, 25–50% group, 50–75% group, and 75–100% group. (**A, D, G** and **J**) The curve of risk score. Survival status of the patients. More dead patients corresponding to the higher risk score. Heatmap of the expression profiles of the E2F1/2/7/8 in Exp 0–25% group, 25–50% group, 50–75% group, and 75–100% group. (**B, E, H** and **K**) Kaplan-Meier survival analysis of E2F1/2/7/8. (**C, F, I** and **L**) Time-dependent ROC analysis of E2F1/2/7/8. ROC: receiver operating characteristic.

S5). Changes in cellular components were primarily enriched in GO: 0005819 (Spindle), GO: 0072686 (Mitotic spindle), GO: 0005876 (Spindle microtubule), GO: 0000779 (Condensed chromosome, centromeric region), and GO: 0000775 (Chromosome, centromeric region) (Figure 7D, Table S5). While the changes in molecular function were mainly enriched in GO: 0008017 (Microtubule binding), GO: 0035173 (Histone kinase activity), GO: 0015631 (Tubulin binding), GO: 0008009 (Chemokine activity), and GO: 0003774 (Motor activity) (Figure 7E, Table S5). KEGG pathway analysis can be used to define the functions of E2Fs and their most correlated genes. Results shown in Figure 7F and Table S5 revealed that E2Fs and their correlated genes were mostly enriched in has: 04110 (Cell cycle), has: 04218 (Cellular senescence), has: 04114 (Oocyte meiosis), has: 04061

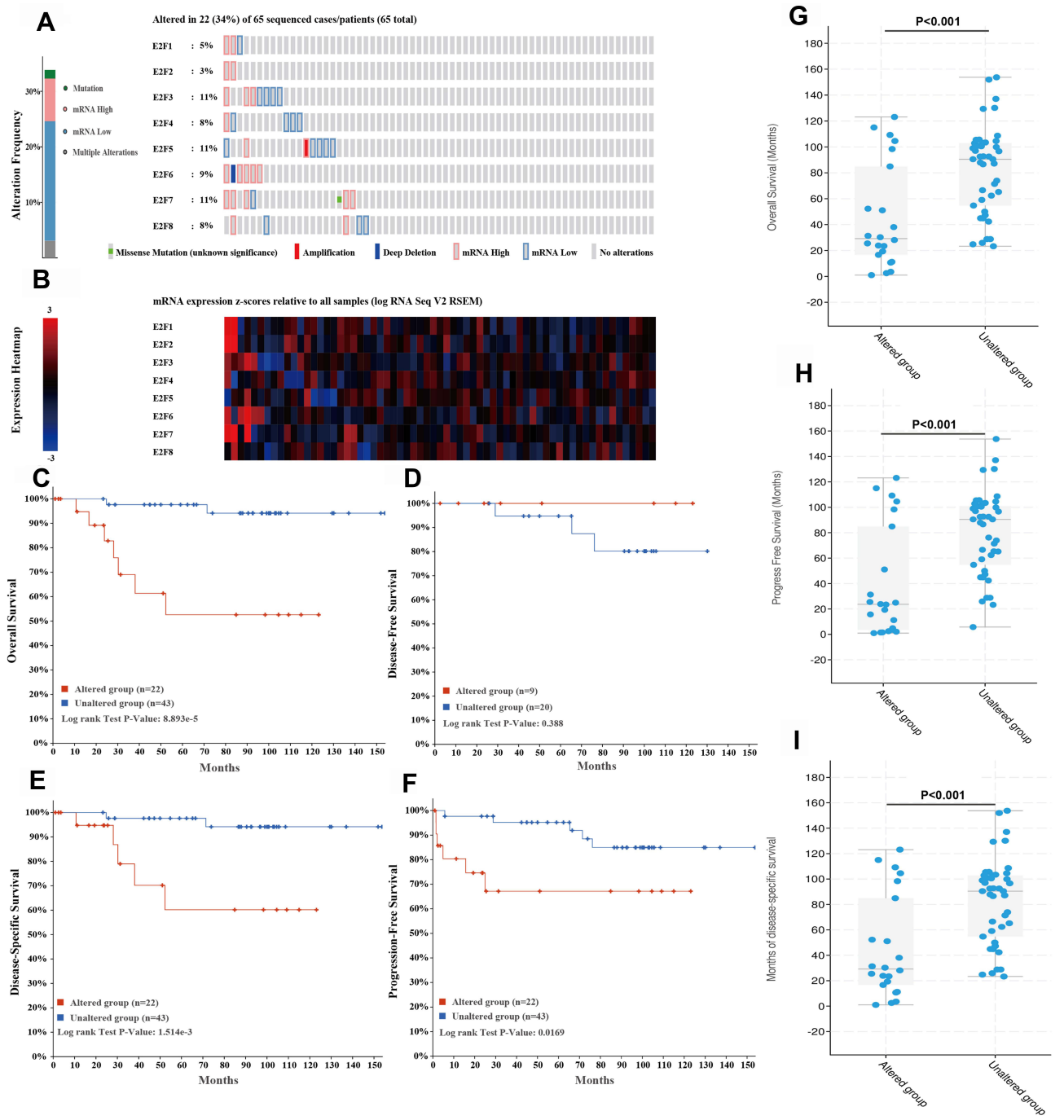


**Figure 5** Genetic mutations in E2Fs and their association with OS, DFS, DSS, and FP of chRCC patients (cBioPortal). A high mutation rate (34%) of E2Fs was observed in chRCC patients. E2F3/5/8 ranked the highest three genes of genetic alterations, and their mutation rates were 11%, 11%, and 11%, respectively (**A**). mRNA expression z-scores of E2Fs relative to normal samples (**B**). Genetic alterations in E2Fs were associated with shorter overall survival (OS) 1.514 (**C** and **G**), Disease-Specific Survival (DFS) (**E** and **I**), and Progression-Free Survival (FP) (**F** and **H**) of chRCC patients. No association was noticed between the genetic alterations of E2Fs and Disease-Free Survival (DFS) in chRCC (**D**).

(Viral protein interaction with cytokine and cytokine receptor), and has: 03320 (PPAR signaling pathway), which were significantly correlated to the tumorigenesis and progression of chRCC. The KEGG pathways with a higher gene ratio (has: 04110, has: 04218) were presented in Figure S2A and Figure S2B. Moreover, evidence from GeneMANIA also confirmed that the E2F family genes were crucially involved in the activity of transcription regulator complex, cell cycle G1/S phase transition, DNA damage response, signal transduction by

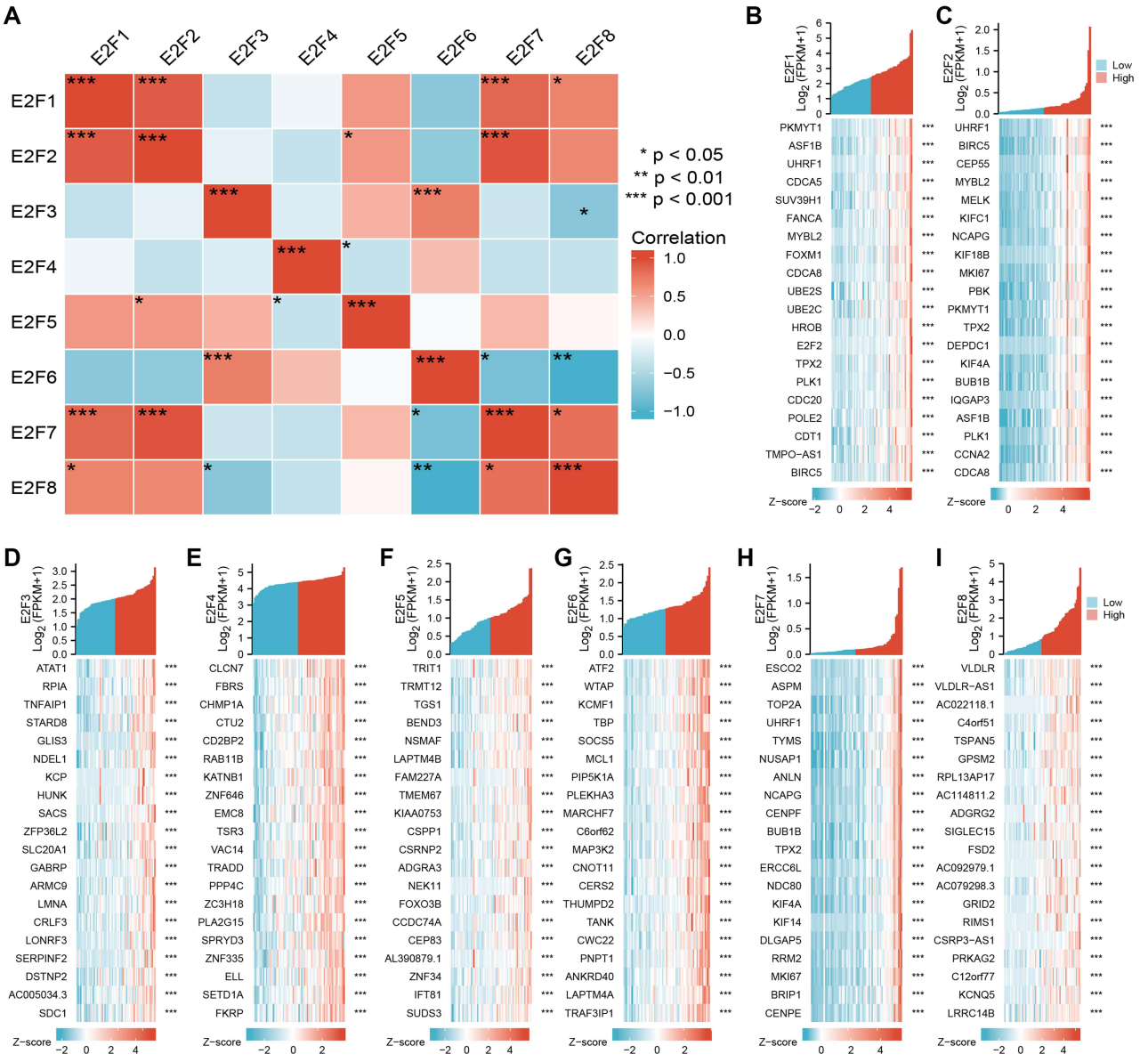


**Figure 6** Correlation heatmaps of E2Fs and their most correlated genes in chRCC. (**A**) Correlation heatmap of different expressed E2Fs in chRCC. Red and blue cells indicate co-occurrence and mutual exclusivity, respectively. (**B–I**) Correlation heatmap of E2F1-8 expression and their top 20 correlated genes. *p< 0.05; **p<0.01; ***p<0.001.

p53 class mediator, and mitotic G1 DNA damage checkpoint (Figure S2C).

## Correlation Between E2Fs Expression and Immune Cell Infiltration in chRCC

In the TIMER database, we explored the correlation of E2Fs expression and the immune cell infiltration level in chRCC. As shown in Figure 8A and Figure S3B, positive correlations were noticed between E2F2 and the infiltration of CD4+ T cells. While the expression of E2F3 and E2F6 were statistically associated with the infiltration of B cells, CD8+ T cells, Macrophage, and Dendritic Cells (Figure S3C, S3F). The expression of E2F4 and E2F7 were positively correlated to the infiltration of CD8+ T cells and Macrophage (Figure S3D, S3G). E2F5 expression was positively correlated to the infiltration of B cells, CD8+ T cells, and Dendritic Cells (Figure S3E). Besides, there was no relationship between E2F1, E2F8, and the six typical infiltrating immune cells (Figure S3A, S3H, p>0.05 for all). The correlation between E2Fs expression and immune cell infiltration in chRCC was also explored

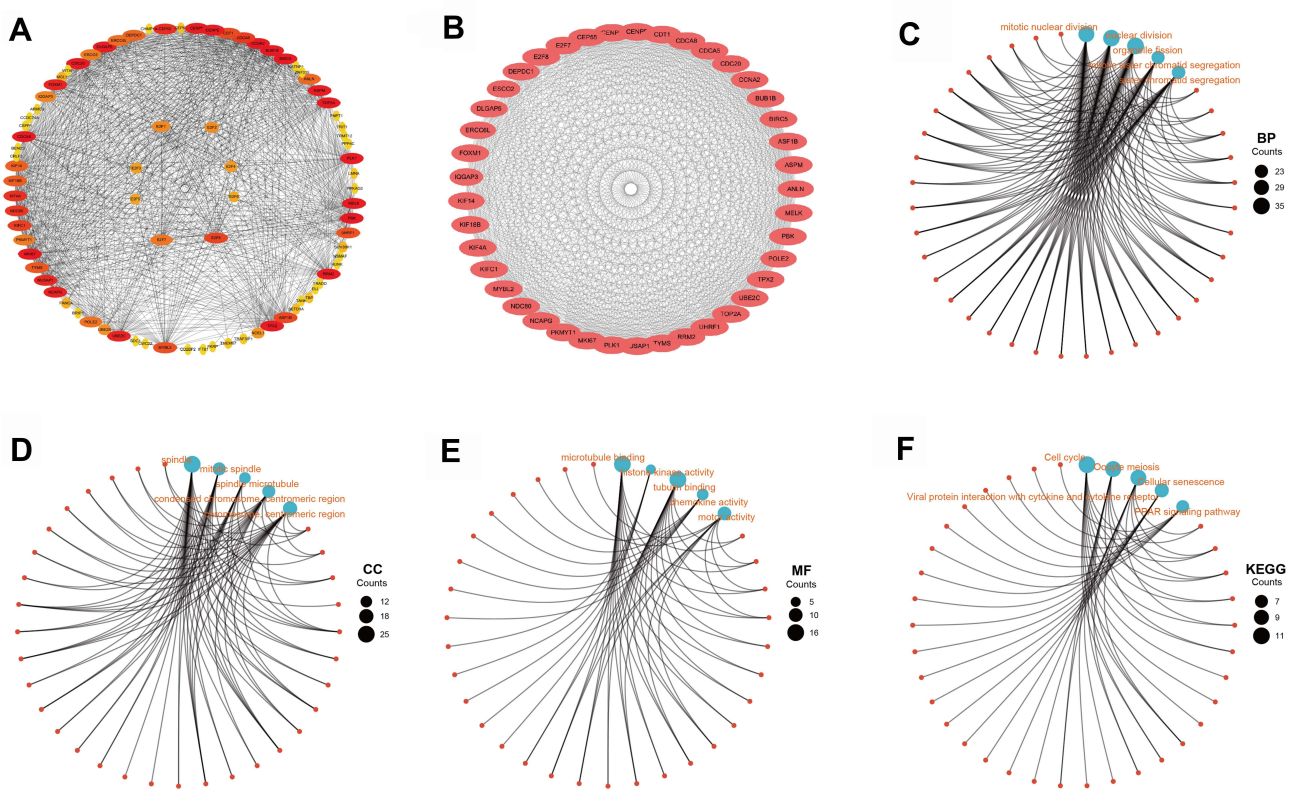


**Figure 7** Enrichment analysis of E2Fs and their most correlated genes in chRCC. (**A**) Gene-gene interaction network for E2Fs and most correlated genes (Cytoscape). (**B**) Interaction network of hub genes with a higher degree of connectivity. (**C–F**) The functions of E2Fs and their most correlated genes were predicted by the analysis of gene ontology (GO, and Kyoto Encyclopedia of Gene and Genomes (KEGG) by Metascape tolls). Go and KEGG enrichment analysis predicted the functional roles of target host genes based on four aspects, including (**C**) biological process, (**D**) cellular components, (**E**) molecular functions, and (**F**) KEGG pathway analysis.

using the TIMER database and generated as Kaplan-Meier plots. As presented in Figure S4, a significant correlation was observed for E2F7 expression and chRCC prognosis (p=0.002). While no association was noticed between immune cell infiltration and chRCC prognosis (p>0.05 for all). The above results suggest that although the immune cell infiltration was associated with E2Fs expression, particularly E2F3/4/5/6, they do not primarily participate in the prognosis of patients with chRCC.

## Correlation Between E2Fs Expression and Representative Immune Markers in chRCC

Although the above studies suggested that E2Fs were not crucially involved in the prognosis of chRCC by interacting with immune cell infiltration. We further explored the correlation between E2Fs expression and typical immune markers of specific immune cells. As shown in Figure 8B and Table S6, significant correlations were observed mainly for E2F3, E2F4, and Neutrophil markers, for E2F5 and Macrophage markers, for E2F6 and T helper 17 cells (Th17) markers. For E2F1/2/7/8, significant correlations were only observed between 2 of the 45 gene markers and E2F1 expression, between 4 of the 45 gene markers and E2F2 expression, between 8 of the 45 gene markers and E2F7 expression, and between 2 of the 45 gene markers and E2F8 expression in chRCC.

## Correlation Between E2Fs Expression, Tumor-Infiltrating Lymphocytes (TILs), and Immunomodulators in chRCC

Previously studies have reported that TILs could serve as independent prognostic predictors in several cancers,[35,36] and the above analysis revealed that E2F1/2/7/8 may participate in the prognosis of chRCC, we further explored the association between the immune-related signatures of 28 TIL types, three kinds of immunomodulators (immunoinhibitors, immunostimulators, and MHC molecules) and E2F1/2/7/8 expression utilizing TISIDB database. As

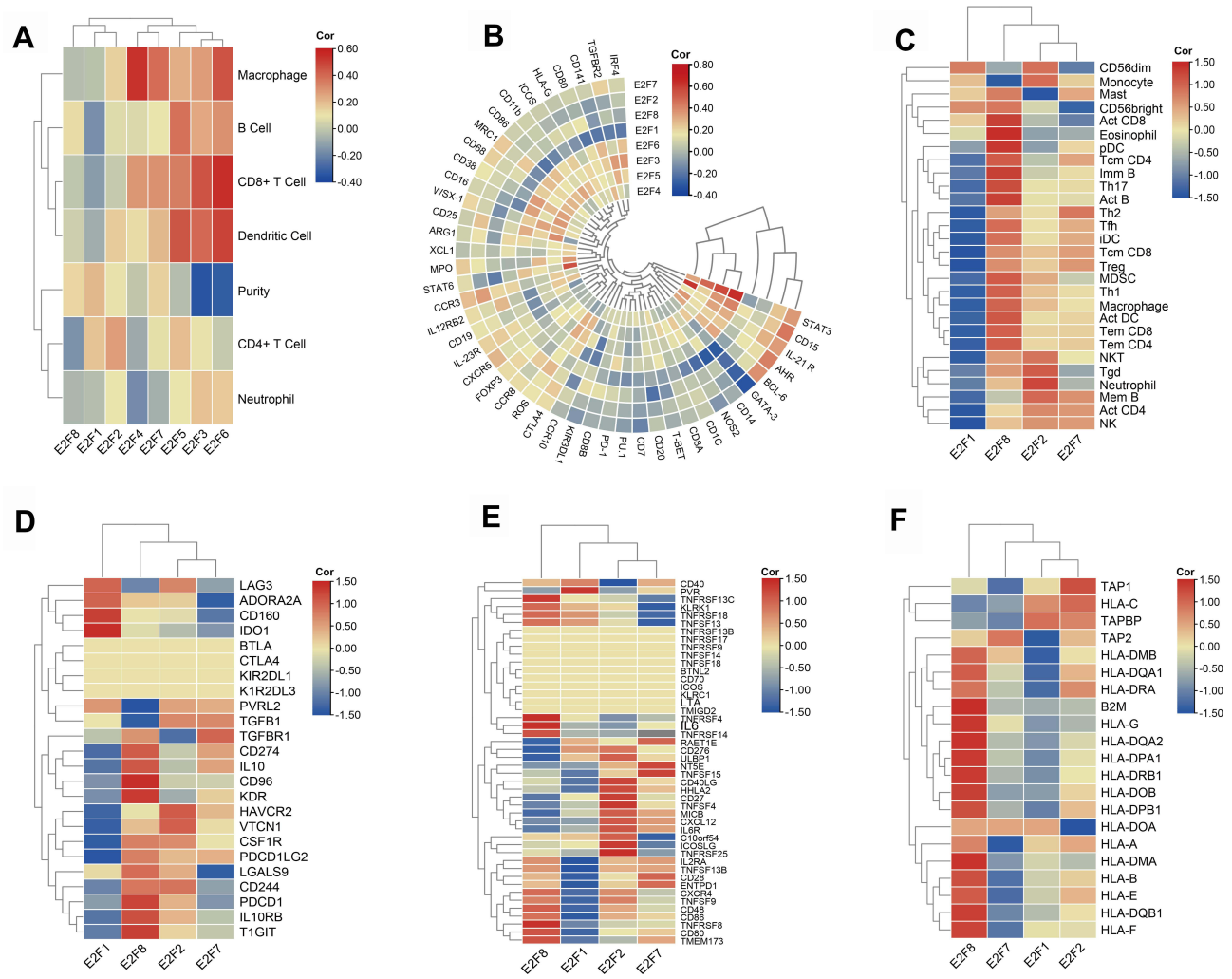


**Figure 8** Interactions of E2Fs expression and immune system. (**A**) Correlation heatmap of E2Fs expression and immune cell infiltration in chRCC. (**B**) Correlation heatmap of E2Fs expression and immune cells related gene markers. (**C**) Correlation heatmap of E2F1/2/7/8 expression and 28 tumor-infiltrating lymphocytes (TILs) types. (**D**) Correlation heatmap of E2F1/2/7/8 expression and 24 immunoinhibitor types. (**E**) Correlation heatmap of E2F1/2/7/8 expression and 46 immunostimulator types. (**F**) Correlation heatmap of E2F1/2/7/8 expression and 36 MHC molecule types. The correlation values were normalized by row scale (**C**–**F**).

performed in Figure 8C–F and Table S7, E2F1 was negatively correlated to 14 of the 28 TIL types, 8 of the 24 immunoinhibitor types, 10 of the 46 immunostimulator types, and 13 of the 36 MHC molecule types. While only 2 TIL types, 2 immunoinhibitor types, 5 immunostimulator types, and 5 MHC molecule types were correlated to E2F2 expression. For E2F7, we found that only 3 TIL types, 4 immunoinhibitor types, 6 immunostimulator types were correlated to gene expression; however, 16 MHC molecule types we noticed negatively correlated to E2F7 expression. At last, only 1 TILs type, 2 immunoinhibitor types, 3 immunostimulator types, and 1 MHC molecule type were correlated to E2F8 expression. According to the above findings, we hypothesis that E2F1 may participate in the tumor-specific immune response by regulating the TILs and immunomodulators, whereas E2F7 may participate in the immune response mainly through the interaction with the MHC molecules.

## Correlation Between E2Fs Expression and Methylation in chRCC

Recently, epigenetic factors like DNA methylation were reported crucially involved in the carcinogenesis and development of cancer by regulating gene expression.[37] We then explored the correlation between the levels of E2Fs expression and methylation using GSCALite and MEXPRESS databases. As shown in Figure 9A and B, negative correlations between E2F1/3/4/5/6/8 expression and methylation were observed, while no relationship between the expression of E2F2/7 and methylation was found.

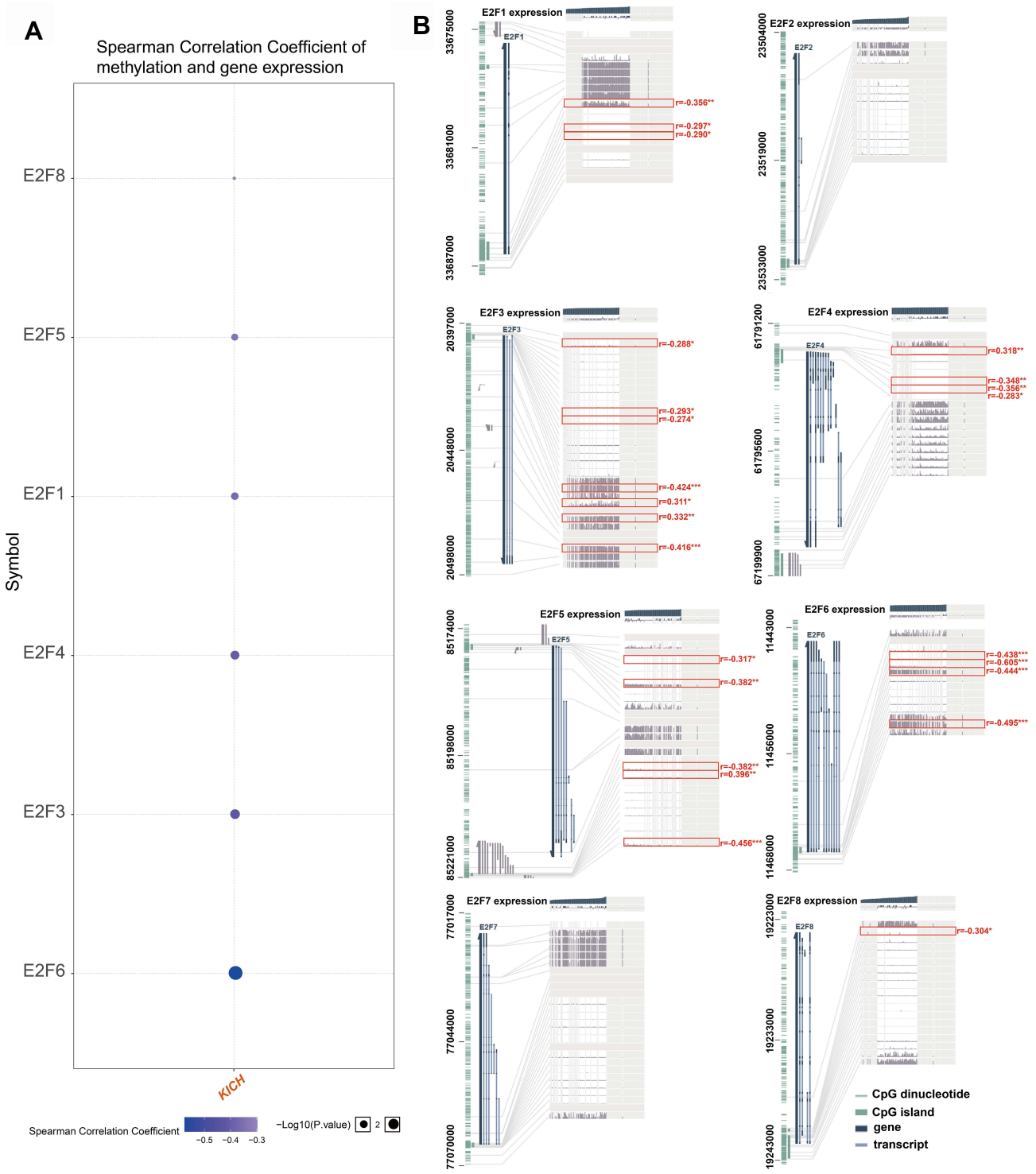


**Figure 9** Correlation between E2Fs expression and methylation levels. (**A**) Spearman correlation between methylation and expression of E2Fs (GSCALite, only positive genes were presented). (**B**) Association between E2Fs expression and DNA methylation, the Benjamini-Hochberg-adjusted p-value, and the Pearson correlation coefficients (r) are displayed (MEXPRESS). *p< 0.05; **p<0.01; ***p<0.001.

## Discussion

Accumulating evidence has implied that E2Fs were involved not only in tumorigenesis and tumor cell proliferation but also in tumor metastasis.[38,39] Previously studies have reported the expression of E2Fs was of clinical significance in various cancers; however, the prognostic value and biological function of E2Fs in chRCC remained to be elucidated. In the current study, we used

multiple online databases to explore the mRNA expression, genetic alteration, methylation, and biological function of E2Fs in chRCC patients, and its association with the occurrence, progress, prognosis, and immune cell infiltration in patients with chRCC.

E2F1 is the most thoroughly studied member of the E2Fs family. Accumulating evidence implied that E2F1 could induce several cell-cycle related proteins, such as CDC2, and cyclin E, which play an essential role in regulating the process of G1/S transition, and finally result in diverse aberrant transcription processes which could dominate carcinomas.[40] It was reported that the overexpression of E2F1 could enhance the expression of Nanog and contribute to the tumorigenesis and progression of breast cancer.[41] Besides, the overexpression of E2F1 was associated with the development and metastasis of various carcinomas, including ccRCC.[42] In the current study, we found a higher expression of E2F1 in chRCC patients in different stages compared with that in adjacent normal samples. Evidence from GEPIA and UALCAN databases suggested that high E2F1 expression was associated with worse OS and DFS in chRCC patients, indicating E2F1 took an important part in the occurrence and prognosis of chRCC. Interestingly, in different cancer types, E2F2 could serve as either a tumor suppressor (eg, in colon cancer) or an activator (eg, in breast cancer and lung cancer).[43–45] In our study, significantly higher expression of E2F2 was found in chRCC tissues, and mRNA expression of E2F2 was associated with the patient's pathological stages. Besides, higher expression of E2F2 was correlated to worse OS and DFS of chRCC patients, suggesting an oncogenic role of E2F2 in chRCC.

As one of the transcription activators (E2F1-3), the overexpression of E2F3 has been detected in many types of cancers, including bladder cancer,[46] lung cancer,[43] and breast cancer.[47] One of the possible mechanisms for the association between the tumorigenesis of diverse cancers and E2F3 could be that E2F3 plays an important role in controlling cycle progression and proliferation in both neoplastic and non-neoplastic cells.[48] Besides, in ccRCC, the expression of E2F3 was an independent prognostic factor. However, in the current report, there was no correlation between E2F3 expression and the tumor stage of chRCC. Moreover, the expression of E2F3 was not associated with the clinical outcomes of chRCC patients. Higher expression of E2F4 was reported in prostate and breast cancers than that in normal tissues, suggesting that E2F4 may act as oncogenes in the progression of carcinomas.[47,49] E2F5 and E2F6 also function as transcriptional repressors.[16] Higher expression of E2F5 was detected in prostate cancer, and glioblastoma compared with that in normal tissue.[50] Nevertheless, Xu et al[51] discovered that expression of E2F5 in MCF7 cells was downregulated, and such aberrant regulation could inhibit cell proliferation, invasion, and migration in vitro. In our report, no relationship between the expression of E2F4/5/6 and the survival of chRCC patients was detected. However, lower mRNA expression of E2F5 and E2F6 was noticed in chRCC samples than that in adjacent normal tissues, and their expressions were significantly associated with individual stages of chRCC (during the early stages of tumor evolution), suggesting a suppressor role of E2F5 and E2F6 in chRCC. These data suggested that E2F5 and E2F6 could serve as early screening indicators for tumor progression of chRCC patients.

E2F7 and E2F8 were also recognized as transcriptional repressors in the E2Fs family, previous studies reveal that E2F7 and E2F8 could inhibit DNA replication in keratinocytes when DNA was damaged, indicating a suppressor role of E2F7/8 in the tumorigenesis of carcinoma by inducing a cell-cycle arrest.[52,53] Interestingly, E2F7 and E2F8 were also recognized as transcriptional activators. Overexpression of E2F7 and E2F8 were observed in breast cancer, and by regulating the G1/S phase transition, E2F8 could promote the tumorigenicity and cell proliferation of breast cancer.[47,53] In the current work, higher mRNA expression of E2F7 and E2F8 was found in chRCC tissues compared to adjacent normal tissues. Moreover, E2F8 expression was significantly correlated to patients' individual cancer stages. Besides, E2F7 was also significantly associated with poorer OS of chRCC patients, while E2F8 was significantly correlated to worse DFS of chRCC patients. However, based on the evidence from Multivariate Cox regression analysis, only E2F1 and E2F2 were independent prognostic factors in chRCC. Taken together, we found that E2F1 and E2F2 have a great potential to be the prognostic biomarkers in chRCC.

It has been reported that during the process of cancer initiation, progression, and treatment, the interaction between the tumor and the immune system plays an important role.[54,55] Previous studies have found that the expression of E2Fs was significantly associated with the immune infiltration in Pancreatic adenocarcinoma

(PAAD), suggested a regulation role of E2Fs in the tumor immunity of PAAD.[56] In the current study, a statistical correlation was observed between immune cell infiltration and E2F3/4/5/6 expression. For E2F1/2/7/8, such correlation was not strong. Besides, we found low to moderate negative correlations between E2F1 expression, TILs, and immunomodulators, as well as between E2F7 expression and MHC molecules. For E2F2 and E2F8, such correlation was not evident. Moreover, we observed that the expression of E2F1 and E2F8 were negatively correlated to the DNA methylation; however, for E2F2 and E2F7, no correlation exists between the levels of methylation and gene expression. This suggests that besides the immunological and methylation factors, there might be other factors (such as inactivating with the Rb family) that may essentially contribute to the role of E2F1 and E2F2 in the prognosis of chRCC. However, more in-depth experiments are required to illuminate the underlying mechanisms of E2Fs in the occurrence and prognosis of chRCC.

## Limitations

Some limitations need to be recognized in the current study. Firstly, all the data explored in our analysis were retrieved from publicly available databases, even the results indicate that the high expression of E2F1/2 could act as independent prognostic factors in chRCC patients, further studies are required to validate our findings and illuminate the clinical application of E2Fs in the treatment of chRCC. Secondly, we did not explore the potential diagnostic and therapeutic values of E2Fs in chRCC patients, whether the expression of E2Fs could serve as diagnostic markers and therapeutic targets in chRCC patients or not remains unclear.

## Conclusion

In conclusion, the systematic analysis suggests that the expression of E2F1/2/4/7/8, were significantly up-regulated in chRCC patients, and the expression was associated with individual cancer stages. Additionally, higher expression of E2F1/2/7 was found to be significantly related to shorter OS of chRCC patients, and higher expression of E2F1/2/8 was remarkably associated with shorter DFS. Cox regression and Time-dependent ROC analysis also facilitate that mRNA expression of E2F1/2 could serve as independent prognostic factors for chRCC patients. Moreover, significant correlations were observed between E2F1 expression, TILs, and immunomodulators, between E2F7 expression and MHC molecules, and between E2F1/8 expression and methylation levels. Other factors that play a more important role for E2F1 and E2F2 expression in the prognosis of chRCC required to be identified. Our results provided a novel understanding of the complexity and heterogeneity of the molecular biological criteria of chRCC and implied that E2F1/2 could act as prognostic biomarkers for survivals of chRCC patients.

## Data Sharing Statement

The data sets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Ethics Approval

Ethical approval for our study was granted by The Committee on Medical Ethics of The First Affiliated Hospital of Anhui Medical University (Reference number: Quick-PJ2021-08-29). Since all the data used in the current study was available online, and no individual patient was involved, it could be confirmed we have obtained all the written informed consent.

## Consent for Participate

There are no patients and public were involved in this research; no participants consent was required.

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## 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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Youth from the First Affiliated Hospital of Anhui Medical University.

## Disclosure

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