Original Article

Molecular signature of immune-related new survival predictions for subtype of renal cell carcinomas

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Background: Kidney renal papillary cell carcinoma (KIRP), kidney chromophobe (KICH), and kidney renal clear cell carcinoma (KIRC) are three most common subtypes of renal cell carcinomas (RCC), and its development is a multifaceted process that intricately involves the interplay of numerous genes. Despite recent advances in research on renal cell carcinoma, the prognosis of KIRC patients remains dismal. Therefore, there is an urgent need to explore new prognostic biomarkers and treatment strategies to help clinicians choose more effective treatment methods and accurately predict long-term efficacy. Our study aimed to systematically evaluate the gene expression profiles of three RCC subtypes, especially KIRC, and to identify survival-related biomarker.

Methods: In our present study, we systematically evaluate the genes expression profile difference among three subtypes of RCC, and identify the survival-related key genes signature based on GEPIA2. GeneMANIA was used to identify the functionality-related differentially expressed genes (DEGs). Furthermore, focusing on KIRC, we intersected functionality-related and survival-related DEGs based on two datasets.

Results: We ascertained five DEGs (*ANK3*, *FREM2*, *KIF13B*, *MPP7* and *SOX6*) as key survival-related genes in KIRC. High levels of these five DEGs expressions were strongly associated with favorable prognosis, but not correlated to metastasis. Downregulation of these five DEGs expressions was closely associated with immunomodulators, chemokines, and infiltrating levels of different immune cells, which indicated that these five DEGs were key immune-related novel prognostic biomarkers for KIRC.

Conclusions: The five identified DEGs serve as potential novel prognostic biomarkers for KIRC. However, the crucial factors that lead to the downregulation and functional inactivation of these five key genes need to be explored in future studies.

Keywords: Renal cell carcinomas (RCC); molecular signature; immune-related genes; immune-related marker

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Introduction

Kidney cancer stands among the leading ten malignancies worldwide, constituting 2% of the total global cancer incidence, with an increasing number observed each year. Renal cell carcinoma (RCC) is the predominant type of kidney cancer, comprising 2–3% of all non-cutaneous malignant neoplasms in adults (1). RCC are classified into three main subtypes, including kidney renal papillary cell carcinoma (KIRP), kidney chromophobe (KICH), and kidney renal clear cell carcinoma (KIRC). The most common subtype is KIRC, which accounts for ~70–75% of RCC (2). Despite significant advances in understanding the molecular mechanisms and therapy approaches for RCC in recent years, the prognosis for KIRC patients remains discouraging (3). Consequently, it is urgent to explore promising novel prognostic biomarkers and treatment strategies. These endeavors will help clinicians in selecting more effective treatment strategies, accurately predicting long-term prognosis, and ultimately benefiting individuals diagnosed with KIRC.

The molecular pathogenesis of cancer is complex, which is related to the inactivation and mutation of antioncogenes and the activation of oncogenes (4). RCC exhibits molecular diversity, so capturing relevant molecular features may improve outcome prediction (5). The application of gene sequencing technology has facilitated the identification of numerous mRNA molecules in RCC. Zhao *et al.* identified 259 genes correlated with survival in conventional renal cell carcinoma (cRCC) using DNA microarrays (5). Numerous studies have shown that multiple genes are associated with overall survival, such as genes related to cuproptosis, apoptosis, glycolysis, ferroptosis and phytanoyl-CoA 2-hydroxylase (PHYH) family genes, etc. (6-10). The molecular mechanisms affecting RCC development are still not well understood, due to the discontinuity of most studies. Recently, immunotherapy as a hot topic has been shown to be an appreciated treatment strategy for cancers including RCC (11,12). Programmed cell death protein 1/programmed cell death protein ligand 1 (PD-1/PD-L1) blockade showed modest anti-tumor efficacy in non-clear cell RCC, and exhibited a considerably higher response rate compared to patients with other RCC types (11). Nevertheless, the availability of viable immune-related biomarkers for predicting patient survival in these other RCC types remains scarce. Furthermore, the identification of potential novel immunotherapeutic targets is also lacking in this context.

Fortunately, with the advance of modern bioinformatics technologies, our understanding of the molecular basis of cancer has significantly improved. In our present study, we used multiple databases to explore the difference among three subtypes of RCC, and investigate key genes and mechanism related to survival in KIRC. In conclusion, five immune-related genes, including *ANK3*, *FREM2*, *KIF13B*, *MPP7* and *SOX6*, were identified to be as immune-related biomarkers for KIRC, and these genes may be feasible for predicting prognosis and immunotherapy efficacy. Our findings improve our understanding of the molecular basis of RCC, and provide the indication of targeting immune-related genes in RCC therapy. We present this article in accordance with the TRIPOD reporting checklist (available at https://tau.amegroups.com/article/view/10.21037/tau-24-225/rc).

Highlight box

Key findings

- This study identified five key survival-related genes (*ANK3*, *FREM2*, *KIF13B*, *MPP7* and *SOX6*) in renal clear cell carcinoma (KIRC) that are associated with favorable prognosis.
- Higher expression levels of these genes are associated with better survival outcomes, whereas downregulation is associated with immune regulation and infiltration of various immune cells.
- This study highlighted these genes as potential novel prognostic biomarkers for KIRC.

What is known and what is new?

- Renal cell carcinoma (RCC) encompasses various subtypes, including KIRC, kidney renal papillary cell carcinoma, and kidney chromophobe, and is influenced by multiple genetic factors. Previous studies have identified various genes associated with RCC prognosis and immune response.
- Despite recent advances in renal cell carcinoma, patients with KIRC have a poor prognosis, and new prognostic biomarkers and treatment strategies are urgently needed.
- This paper systematically evaluated the gene expression profiles of RCC subtypes and identified specific differentially expressed genes associated with survival in KIRC.
- It provides new insights into the relationship between the expression of the identified DEGs and the immune response, suggesting their role as prognostic biomarkers.

What is the implication, and what should change now?

- *ANK3*, *FREM2*, *KIF13B*, *MPP7* and *SOX6* can be used as biomarkers for the prognosis of KIRC, which may affect treatment decisions and patient management.
- Future research should focus on understanding the mechanisms behind the downregulation and functional inactivation of these genes to explore their therapeutic potential.

Methods

Identification of differentially expressed genes (DEGs)

GEPIA2 website was used to analyze tumor and normal gene expression based on The Cancer Genome Atlas (TCGA) and Genotype-Tissue Expression data (13). The cut-off value of $\log_2$FC was set as 1, and q-value cutoff was set to 0.01. Next, a volcano plot and Venn diagram of DEGs were created by the Genescloud (https://www.genescloud.cn). The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

Functional enrichment analysis

FLAME provides a combined approach through merging and visualizing results from extensively used functional enrichment analysis following a variety of input options (14). In our present study, FLAME was used to perform Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis, and the heatmap of KEGG pathways enriched in DEGs was also analyzed using FLAME database. GeneMANIA is an available, user-friendly website, which can provide a large amount of functional association data (15). GeneMANIA was used to establish gene networks and predict the gene enriched in KICH, KIRC and KIRP. In our study, those identified key genes were submitted to the GeneMANIA to illustrate the functional association network. The weighing method used was automatically selected.

Survival analysis

Survival analysis and the differential survival genes were obtained from GEPIA2 (http://gepia2.cancer-pku.cn/#survival). These genes were downloaded with the top 500 genes most relevant to overall survival in KIRP, KICH and KIRC, separately. The cutoff was defined based on the median. The survival analysis of five key genes screened by GEPIA2 and PrognoScan databases was further confirmed using UALCAN database. A value of P<0.05 was considered significant.

PrognoScan (http://dna00.bio.kyutech.ac.jp/PrognoScan/index.html) was used to further identify the key genes associated with KIRC prognosis, and evaluate the relationships between these key genes and patient outcomes in KIRC (16). The threshold of Cox P value was set as <0.05.

UALCAN is a comprehensive and interactive website for analyzing tumor data according to the TCGA project (17). UALCAN was applied to analyze the protein expression of key genes and validate the survival value of these genes.

Analysis of metastasis potential

The Cancer Dependency Map offers an available online database based on large-scale multi-omics screening projects, including Cancer Cell Line Encyclopedia (CCLE) (18). We used DepMap to analyze the dependencies between the key genes associated with survival and cancer cell lines. In the Correlation analysis of relative metastatic potential, we use Depmap (https://depmap.org/portal/data_explorer_2), in the X Axis module of plot Configuration, Expression was selected as Data Type, and MPP7, SOX6, ANK3, FREM2 and KIF13B were selected as Feature.

Immune cell infiltration analysis

To investigate the association between the levels of ANK3, FREM2, KIF13B, MPP7, SOX6 and immune infiltration in KIRC patients, the immune cell infiltration profile was identified via the Tumor Immune Estimation Resource (TIMER) and Translational Medicine Integrated Database (TISIDB) web portal resources. TISIDB incorporates numerous heterogeneous data types (19), and TIMER offers comprehensive analysis and visualization of cancer-immune system interactions (20).

Statistical analysis

All data were presented as the mean and standard deviation (SD). The Student's *t*-test was used for comparing the difference between two groups. The log-rank test was used to analyze the survival curve. GraphPad Prism 8 (GraphPad Software) was used to create chart and common data analysis. The P value <0.05 was regarded as the level of statistically significant.

Results

Screening of DEGs

The GEPIA2 database was used to characterize DEGs profile in RCC and normal tissues. In total, 4,204, 2,408 and 2,955 DEGs were obtained for KICH, KIRP and KIRC, respectively (*Figure 1A-1C*). Result showed that KICH may be the most major subtype of RCC with the largest number of DEGs. Based on the DEGs for different

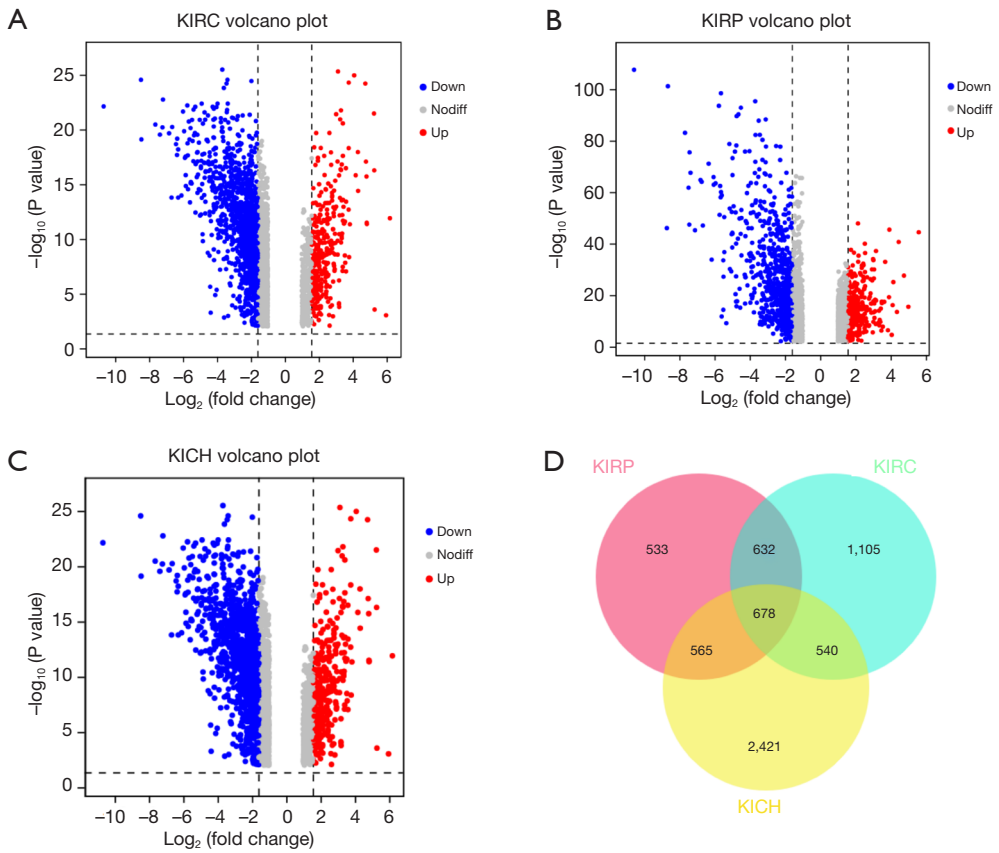

Figure 1 The volcano plot and Veen of DEGs. Volcano plots quantifying differential expression for identified DEGs in KIRC (A), KIRP (B) and KICH (C) compared to control. The red dots indicate up regulation. The blue dots indicate downregulation. The gray dots indicate no significant difference. (D) Venn diagram showing the shared and unique DEGs among different groups. DEGs, differentially expressed genes; KIRC, kidney renal clear cell carcinoma; KIRP, kidney renal papillary cell carcinoma; KICH, kidney chromophobe.

RCC subtypes in the GEPIA2 dataset separately, we found that the intersection of the results provided 678 overlapping DEGs using Venn tool (*Figure 1D*). Among these DEGs, Venn diagram revealed that 533 DEGs were sole for KIRP, 1,105 DEGs were sole for KIRC, while 2,421 DEGs were solely for KICH (*Figure 1D*).

Visualization of KEGG enrichment analysis

KEGG pathway analysis was applied to display functions of DEGs. The DEGs in three RCC subtypes were involved in different pathways. Results showed that 4,204 DEGs for KICH were predominantly involved in tumor necrosis factor (TNF) signaling pathway, Rap1 signaling pathway and metabolic pathway (*Figure 2A*), and 2,408 DEGs for KIRP were mainly involved in Rap1 signaling pathway, calcium signaling pathway, metabolic pathway and phosphatidylinositol-3-kinase-protein kinase B (PI3K-Akt) pathway (*Figure 2B*), and 2,955 DEGs for KIRC were mainly involved in Rap1 and HIF-1 signaling pathway and PI3K-Akt pathway (*Figure 2C*). While 678 overlapping DEGs, among three different RCC subtypes, were mainly involved in metabolic pathway and PI3K-Akt pathway (*Figure 2D*). In addition, we generated a KEGG pathway heatmap based on the 678 overlapping DEGs. This analysis revealed that these DEGs significantly intersected with ten main pathways, including retinol metabolism, mineral absorption, metabolism of xenobiotics by cytochrome P450, PI3K-Akt signaling pathway, metabolic pathways, drug metabolism by cytochrome P450, complement and coagulation cascades, collecting duct acid secretion, cell adhesion molecules (CAMs), and arachidonic acid metabolism (*Figure 2E*).

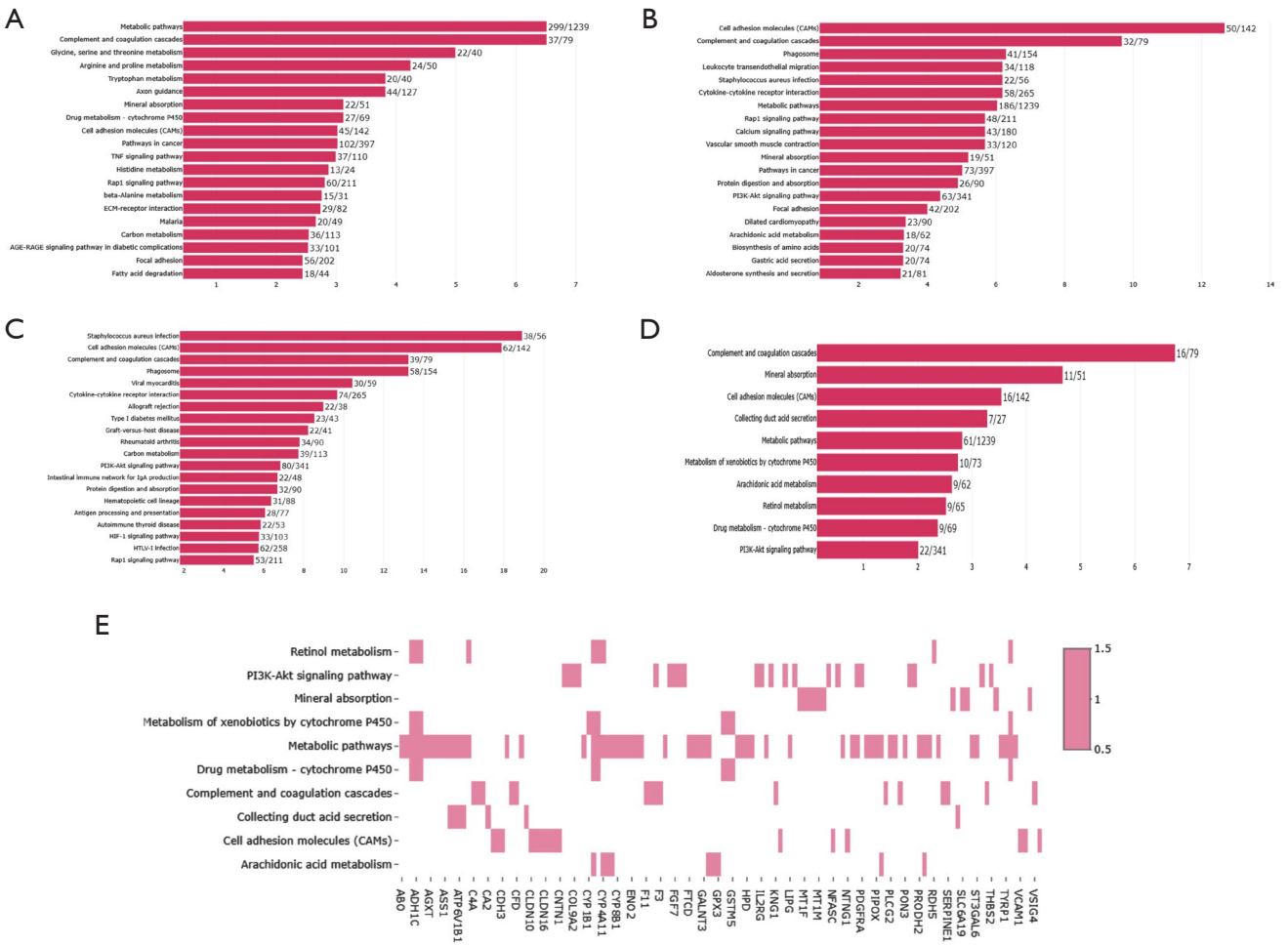

Figure 2 KEGG pathway enrichment of DEGs obtained from KICH (A), KIRP (B) and KIRC (C), separately. (D) KEGG pathway enrichment of the shared 678 DEGs in three groups. For (A)-(D), x-axis indicates –lg(P value). (E) Heatmap of the shared 678 DEGs and function interaction. KEGG, Kyoto Encyclopedia of Genes and Genomes; DEGs, differentially expressed genes; KIRC, kidney renal clear cell carcinoma; KIRP, kidney renal papillary cell carcinoma; KICH, kidney chromophobe; HIF-1, hypoxia inducible factor-1; IgA, immunoglobulin A; ECM, extracellular matrix.

These results showed that tumors raised in the same organ had poor gene consistency due to transcription difference. These results indicated that transcriptomic profiling may contribute to identifying different RCC subtypes.

Correlation of DEGs with survival

Differential survival genes were obtained from the "Expression Analysis-Survival Analysis" module of GEPIA2. Furthermore, we intersected DEGs of transcriptome and overall survival (OS)-related differential survival genes. A total of 81 overlapping differential genes were identified between KIRC DEGs and KIRC OS (*Figure 3A*, and Table S1), while 68 overlapping differential genes were identified between DEGs and OS both in KICH and KIRP (*Figure 3B*,*3C*, and Tables S2,S3). Moreover, the correlation was analyzed based on the survival P value and $|\log_2$ fold change$|$ of these overlapping differential genes. As shown in *Figure 3*, there was no direct relationship between the fold change of DEGs and the survival associations (*Figure 3D-3F*).

Protein-protein interactions (PPI) and prediction of gene function

A query gene list including all those overlapping genes

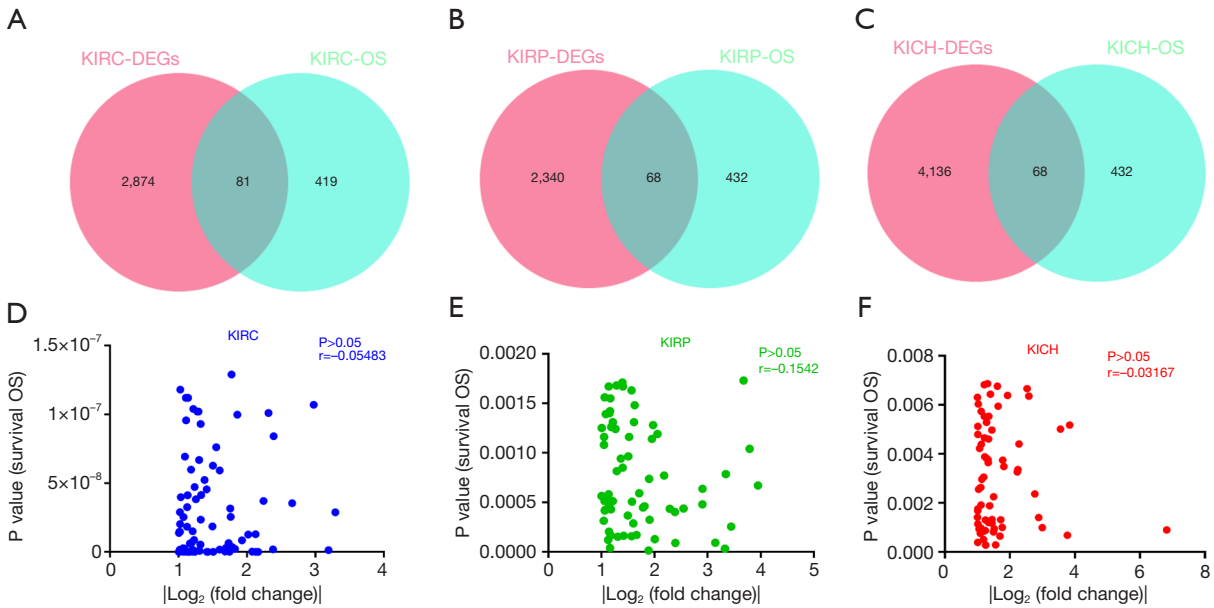

Figure 3 The Venn diagram showing the shared and unique genes between DEGs and genes associated with survival in KIRC (A), KIRP (B) and KICH (C), separately. The correlation of DEGs and genes associated with survival in KIRC (D), KIRP (E) and KICH (F), respectively. OS, overall survival-related differential survival genes; DEGs, differentially expressed genes; KIRC, kidney renal clear cell carcinoma; KIRP, kidney renal papillary cell carcinoma; KICH, kidney chromophobe.

identified above is shown in *Figure 3*. GeneMANIA was used to analyze the interactions between protein and predict gene function. The network showed co-expression 77.06%, co-localization 19.45%, shared protein domains 2.53%, and genetic interactions 0.96% based on the 81 overlapping genes in KIRC group (*Figure 4A*). The networks represented co-expression 81.92%, physical interactions 15.66%, co-localization 2.06% and pathways 0.07% based on the 68 overlapping genes in KIRP group (*Figure 4B*). While the networks represented only two tasks including co-expression 91.64% and co-localization 8.36% based on the 68 overlapping genes in KICH group (*Figure 4C*). KIRC is the most typical subtype of RCC, and accounts for 75% of all renal cancers (21). Thus, we next performed a more in-depth analysis focusing on KIRC. According to GEPIA2 database, 81 overlapping genes were obtained and further filtrated according to p value of overall survival, and the top 20 overlapping genes were further crossed with PrognoScan database. Finally, five key genes (ANK3, FREM2, KIF13B, MPP7 and SOX6) were confirmed to be strongly associated with prognosis in KIRC, according to the results of two interactive databases (Table S4). The survival value of these key genes was also validated using the UALCAN database (*Figure 5A-5E*), and we also utilized the Kaplan-Meier plotter dataset to evaluate the prognostic relevance of 5 key genes in KIRC based on their expression levels, revealing that their increase was all significantly linked with a greater overall survival in KIRC (*Figure 5F-5J*). Our analysis indicated that the low-expression group exhibited a shorter survival time.

Validation and analysis of key genes expression

To obtain a comprehensive understanding of the expression levels of these key genes, we used UALCAN database to analyze the protein levels in KIRC. The results obtained from UALCAN showed that ANK3, FREM2, KIF13B, MPP7 and SOX6 were all significantly downregulated in KIRC (n=110), compared with the normal control (n=84) (*Figure 6A-6E*).

Key genes expression in different RCC and correlation with metastasis potential

We then analyzed the 5 key genes level in different renal cancer cell lines, and investigated the correlation between gene expression and renal cancer metastasis potential using Depmap database. The data for all renal cancer cell lines available in Depmap showed that the expression of 5 key genes were different in various renal cancer cell lines

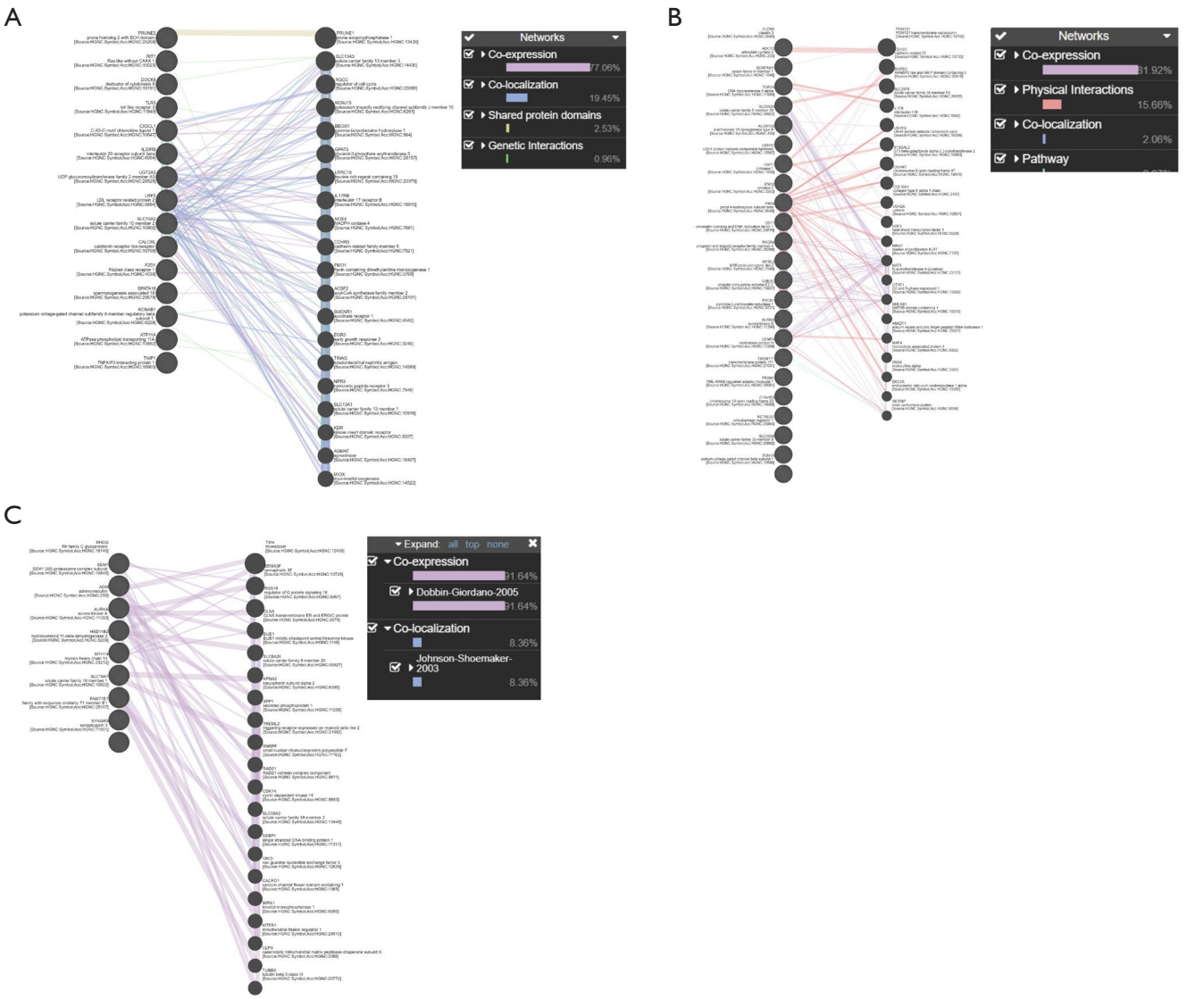

Figure 4 The interactions between gene and gene and prediction of gene function in KIRC (A), KIRP (B) and KICH (C) according to GeneMANIA database. KIRC, kidney renal clear cell carcinoma; KIRP, kidney renal papillary cell carcinoma; KICH, kidney chromophobe.

(*Figure 7*), although with lower expression in tumor tissue in KIRC (*Figure 6*). Metastasis potential results showed that there was no significant correlation with *ANK3*, *FREM2*, *KIF13B*, *MPP7* and *SOX6* expression in renal cancer cell lines (*Figure 7*).

Correlation between downregulated key genes expression and chemokines receptors in KIRC patients

The correlation of the expression levels of *ANK3*, *FREM2*, *KIF13B*, *MPP7* and *SOX6* with chemokines receptors in KIRC was demonstrated in this study. Results implicated that the 5 key genes were interrelated with different chemokines receptors in KIRC (P<5.12E–12). *ANK3* was mainly correlated with CCR10 (rho =–0.359) (*Figure 8A*), *KIF13B* (rho =–0.353), *MPP7* (rho =–0.346) and *SOX6* (rho =–0.369) were all significantly correlated with CXCR4, while FREM2 was significantly connected with CXCR5 (rho =–0.294) (*Figure 8*). These findings further revealed that all these 5 key genes may serve as immunoregulatory elements in KIRC.

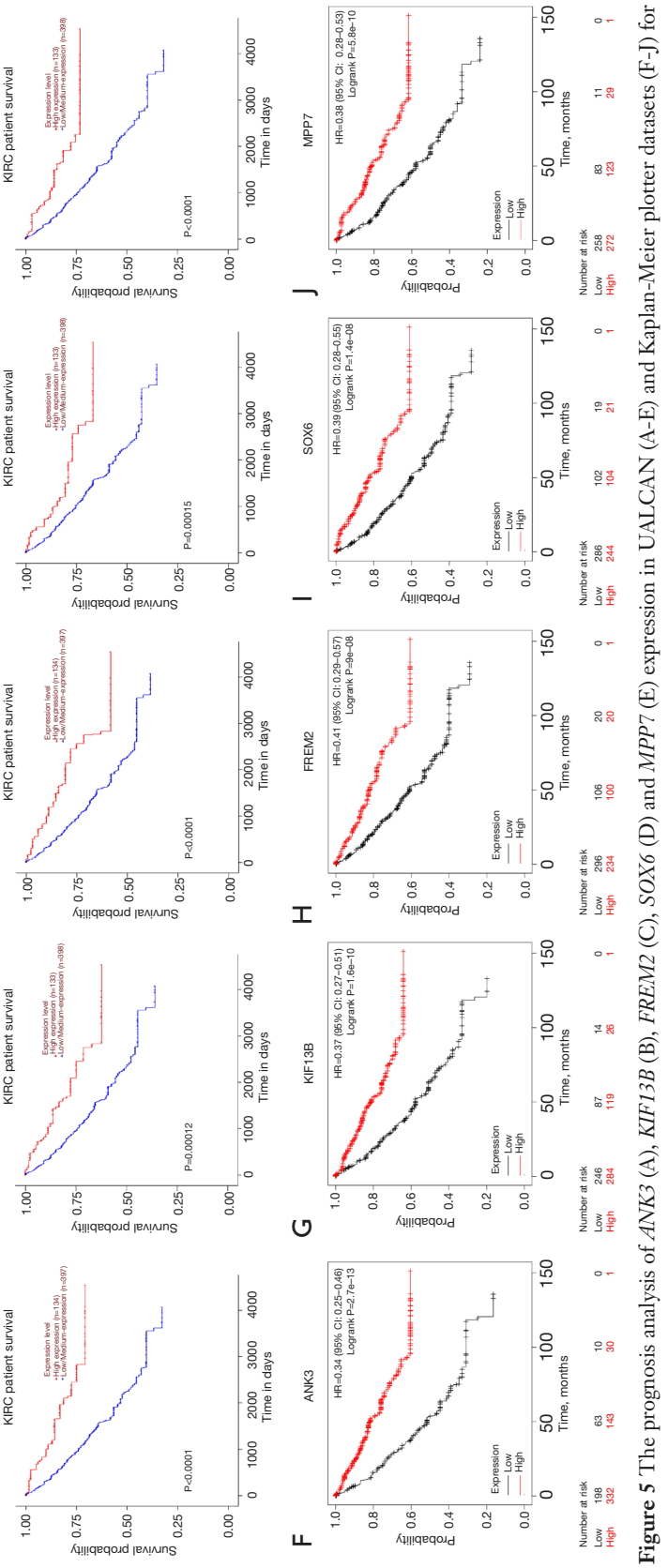

Figure 5 The prognosis analysis of *ANK3* (A), *KIF13B* (B), *FREM2* (C), *SOX6* (D) and *MPP7* (E) expression in UALCAN (A-E) and Kaplan-Meier plotter datasets (F-J) for KIRC. P value <0.05 was defined as statistically significant. KIRC, kidney renal clear cell carcinoma; HR, hazard ratio; CI, confidence interval.

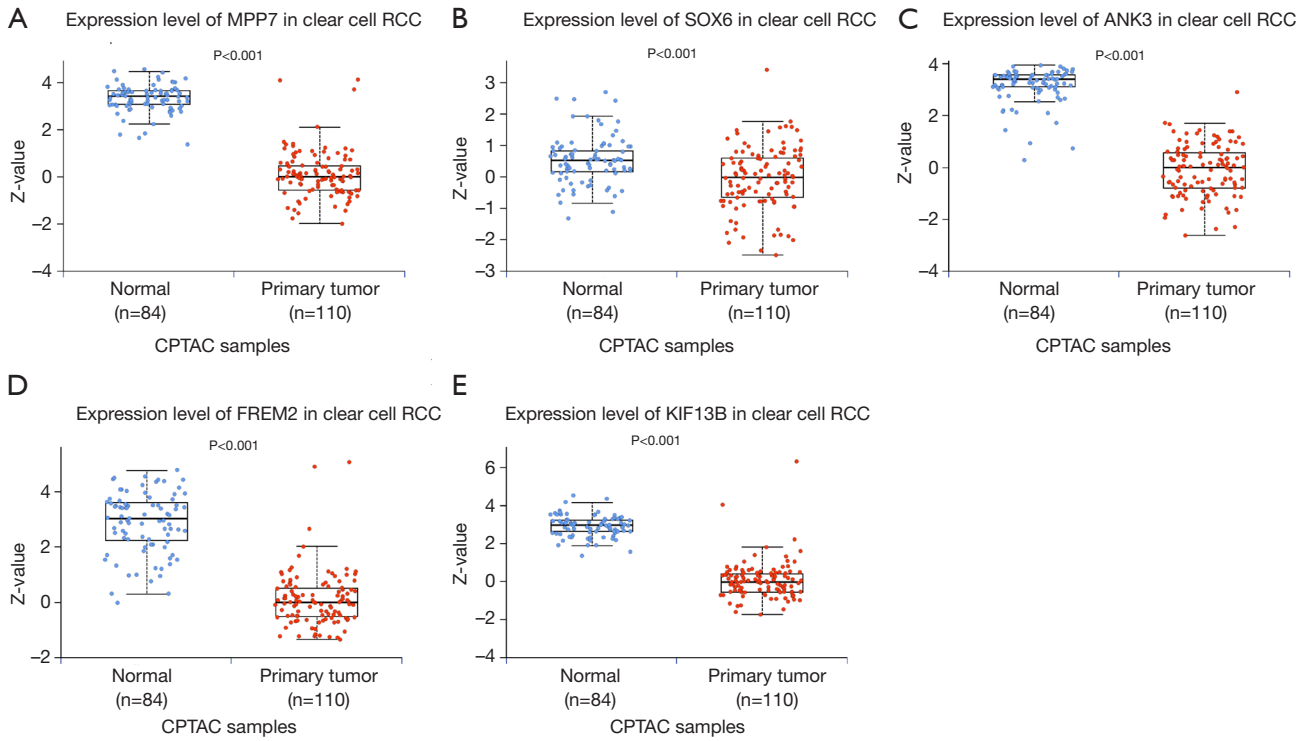

Figure 6 Jitter plots showing the protein expressions of MPP7 (A), SOX6 (B), ANK3 (C), FREM2 (D) and KIF13B (E) in normal and KIRC samples. Z-values represent standard deviations from the median across samples for the given cancer type. Log_2 spectral count ratio values from CPTAC were first normalized within each sample profile, then normalized across samples. Students' *t*-test provided P values. P value <0.05 was defined as statistically significant. RCC, renal cell carcinoma; KIRC, kidney renal clear cell carcinoma; CPTAC, Clinical Proteomic Tumor Analysis Consortium.

Correlation between downregulated key genes expression and immune infiltration in KIRC

Immune infiltration around tumors is a crucial factor correlated with tumor progression. Therefore, 5 key genes were submitted to the TIMER database to explore their associations with 6 tumor-infiltrating immune cells ($CD8^+$ T cells, B cells, $CD4^+$ T cells, neutrophil, macrophage and dendritic cells), and tumor purity. Our analysis results found that ANK3 was positively associated with B cells (*Figure 9A*), while KIF13B was positively correlated with $CD4^+$ T cells (*Figure 9B*). FREM2 was positively correlated with macrophages, as was MPP7 and SOX6 (*Figure 9C-9E*). These results showed that 5 key genes expression were all correlated with immune infiltration in KIRC, which might be a potential mechanism to exert the effects on prognosis.

Discussion

Bioinformatics, as an interdisciplinary field of science, provides more significant insights and data related to molecular mechanisms of cancer progression. The identification of potential key genes and understanding their interactions contribute significantly to the discovery of promising prognostic, predictive biomarkers and novel clues for therapies in RCC. In our study, to explore the unique and shared genes signature in different subtype of RCC, we first performed RNA-seq analysis among KIRC, KIRP and KICH samples from gepia2 database. In our present study, 4,204, 2,408 and 2,955 DEGs were identified in KICH, KIRP and KIRC, respectively. Guo *et al.* identified 5,699, 4,896 and 5,759 DEGs in KIRC, KIRP and KICH from TCGA cohort, respectively, and found that cell migration and immune response process were mainly enriched in KIRC and KIRP, but more synaptic plasticity and kidney morphogenesis were involved in KICH (22). These results indicated that RCC patients of different subtypes with different originating cell types had different transcript response, although tumors occurred in the same organ.

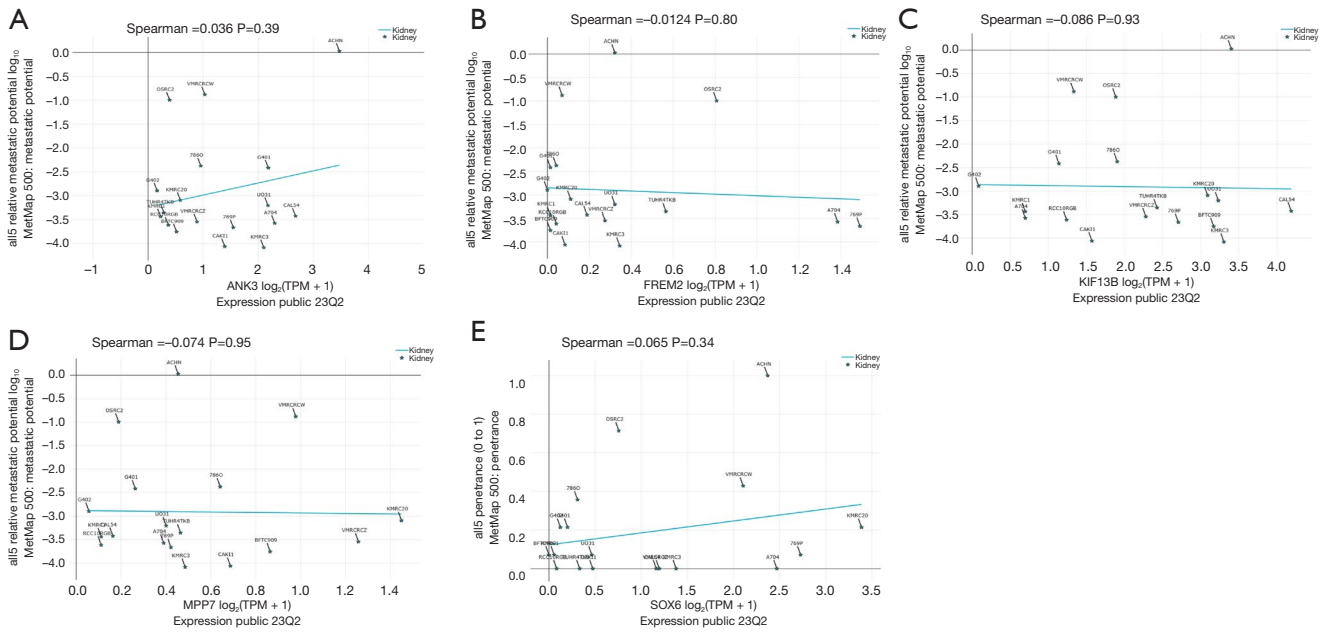

Figure 7 Correlation analysis of relative metastatic potential (MetMap 500: metastatic potential) with ANK3 (A), FREM2 (B), KIF13B (C), MPP7 (D) and SOX6 (E) expression (Depmap: expression 21Q3 public) in several kidney cell lines (n=18). TPM, transcript per million.

The most typical subtype of RCC is KIRC, which accounts for about 80–90% of RCC with a poor prognosis (23). In the meantime, the pathogenesis of KIRC remains unclear, and no sensitive biomarkers have been discovered. Therefore, we paid more attention to KIRC in our research. We further screened 5 key genes associated with KIRC survival through cross-database analysis. Our results showed that 5 key genes had a similar trend in KIRC, compared to normal control. FREM2, MPP7 and SOX6 were suggested to be potential candidates for KIRC prognosis prediction (24-27), which is consistent with our present results. Although ANK3 was reported to be only associated with RCC in previous publication (28), our study further proved that ANK3 was a novel survival-associated gene in KIRC. Previous studies have reported that low FREM2 expression in KIRC patients showed a high propensity for metastasis and poor prognosis (29). MPP7 and SOX6 were also identified to be associated with breast cancer and liver metastasis (30,31). These results showed that five key genes associated with metastasis are potential immune-related biomarkers in multiple cancers. Furthermore, our present study systematically explored the relationships between these key genes expression and KIRC in bioinformatics manner, and our results showed that all these 5 key genes expressions were related with different chemokines (receptors) (*Figure 8*) and infiltration of different immune cells (*Figure 9*).

Numerous studies have proved that chemokines and their receptors modulate tumor microenvironment (TME) and affect the development of cancers and the therapeutic outcomes (32-34). In our present study, we found that all 5 key genes were correlated with different chemokines and chemokines receptors (*Figure 8*). Enhanced immune infiltration in tumors has been proved to be typically associated with good prognosis in tumor patients, including KIRC (26,35). Furthermore, we found that these 5 key genes were closely correlated with different immune cells (*Figure 9*). In addition, we found no correlations between these genes and metastasis potential (*Figure 7*). These results revealed that *ANK3*, *FREM2*, *KIF13B*, *SOX6* and *MPP7* may serve as crucial immunoregulatory elements in KIRC patients, and we speculated that these novel survival-related genes may be involved in the development of KIRC through immunomodulation.

Undeniably, our present study has certain limitations. Although we conducted integrated bioinformatics analysis on multiple datasets to explore the differences among three subtypes of RCC, and mainly focused on analysis of key

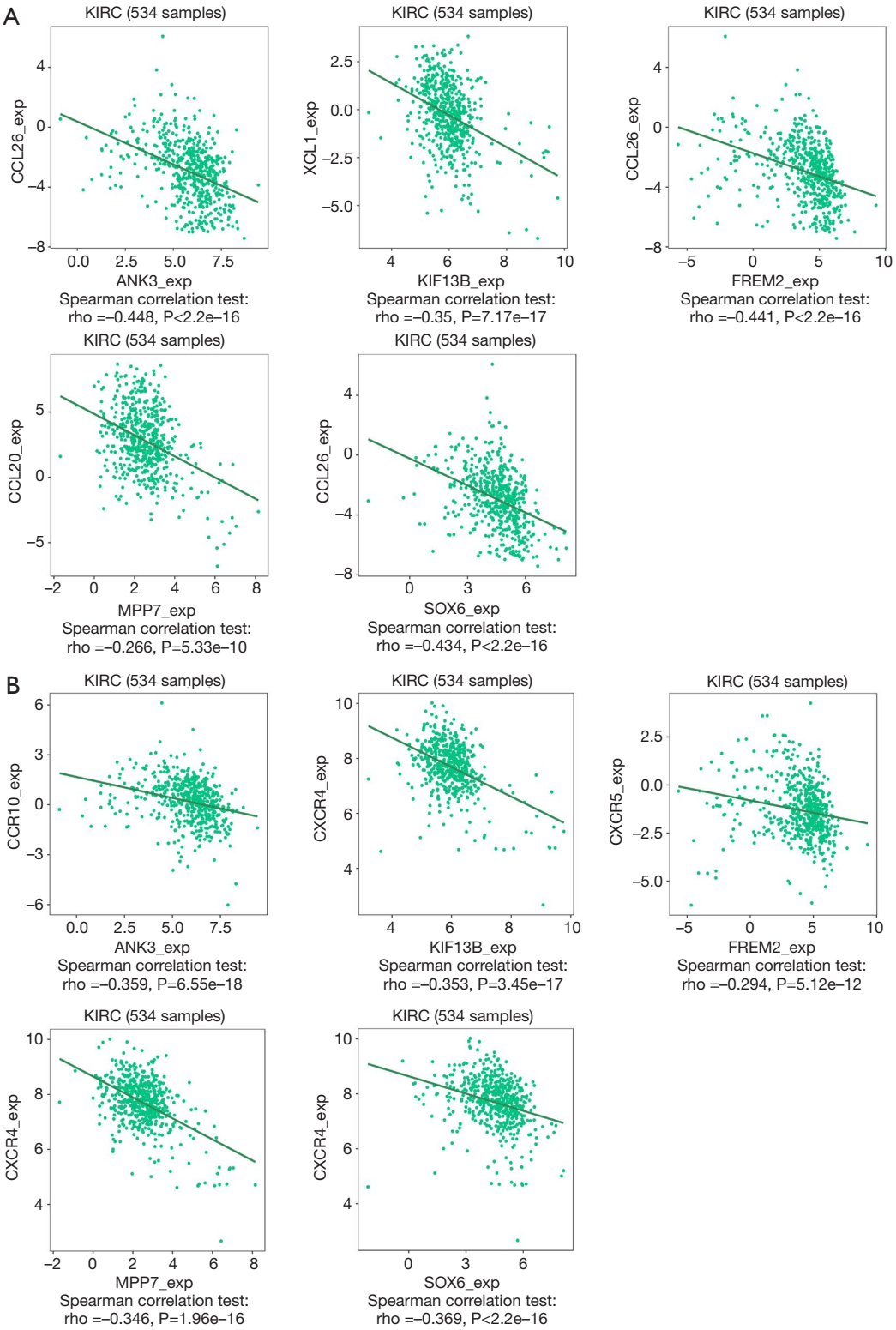

Figure 8 Correlation between the expression of 5 key genes and chemokines in KIRC. Correlation between ANK3, KIF13B, FREM2, MPP7 and SOX6 expression and chemokines (A) and chemokine receptors (B) in KIRC available at TISIDB database. Color images are available online. KIRC, kidney renal clear cell carcinoma; TISIDB, Translational Medicine Integrated Database.

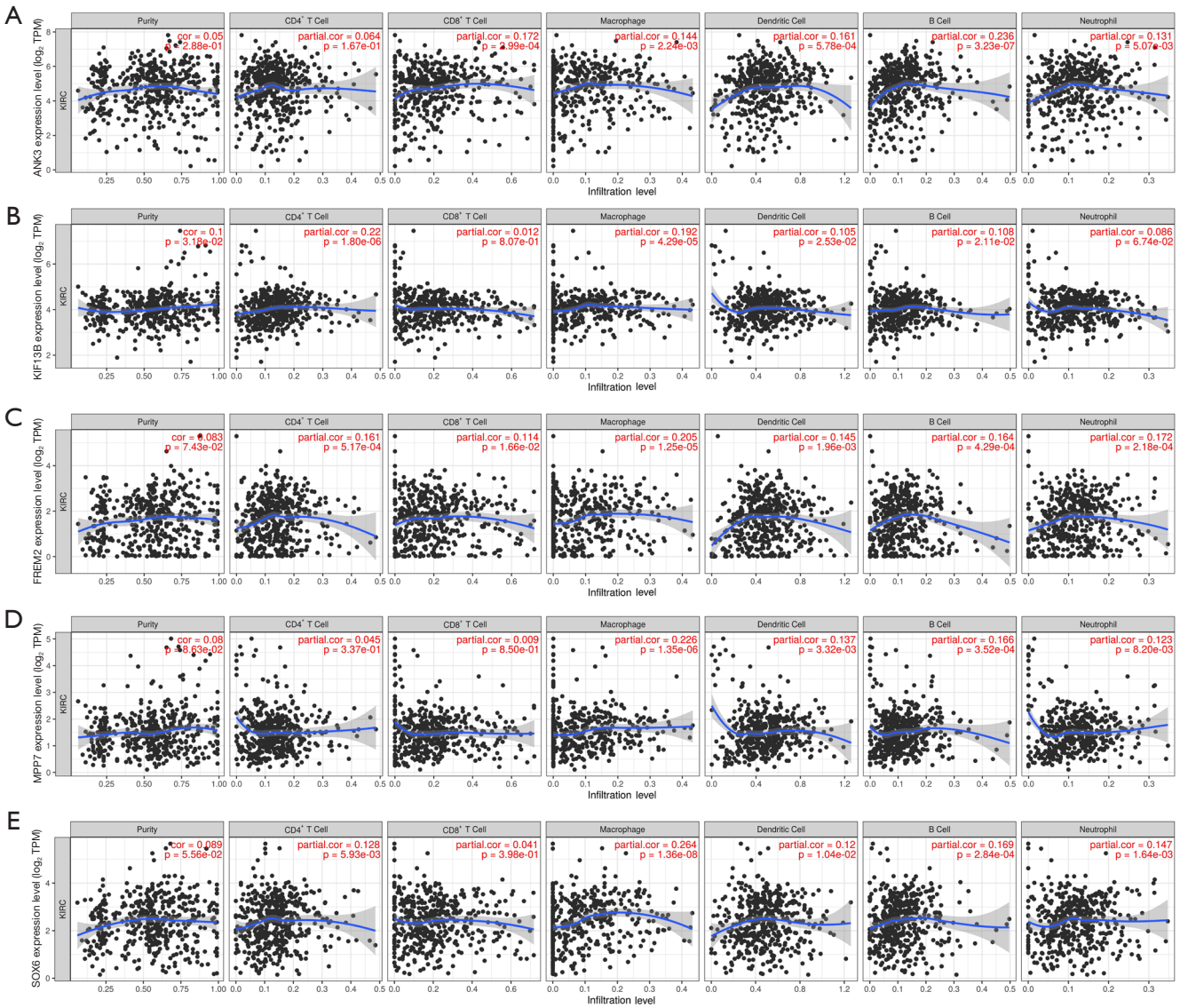

Figure 9 The correlation between different expressed genes, including ANK3 (A), KIF13B (B), FRREM2 (C), MPP7 (D), SOX6 (E), and immune cell infiltration in KIRC. The correlation was adjusted by purity. KIRC, kidney renal clear cell carcinoma; TPM, transcript per million.

genes and mechanisms related to survival in KIRC, we did not carry out corresponding experimental verification *in vitro* or *in vivo*, which will be improved in future research. In conclusion, our present findings provide valuable clues for future studies, and foundation for our further validation of the present findings, and a deeper understanding of the mechanisms.

Conclusions

In summary, we identified five key genes that were correlated with prognosis and immunotherapy for KIRC. Our findings improve our understanding of the function of these immune-related novel prognostic biomarkers in KIRC, while the crucial factors that lead to the downregulation and functional inactivation of these five

key new immune-related prognostic biomarkers need to be explored in future studies.

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Footnote

Reporting Checklist: The authors have completed the TRIPOD reporting checklist. Available at https://tau.amegroups.com/article/view/10.21037/tau-24-225/rc

Peer Review File: Available at https://tau.amegroups.com/article/view/10.21037/tau-24-225/prf

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tau.amegroups.com/article/view/10.21037/tau-24-225/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

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