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Original Article

In vitro analysis of PI3K pathway activation genes for exploring novel biomarkers and therapeutic targets in clear cell renal carcinoma

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Abstract: Objectives: The regulation of various cellular functions such as growth, proliferation, metabolism, and angiogenesis, is dependent on the PI3K pathway. Recent evidence has indicated that kidney renal clear cell carcinoma (KIRC) can be triggered by the deregulation of this pathway. The objective of this research was to investigate 25 genes associated with activation of the PI3K pathway in KIRC and control samples to identify four hub genes that might serve as novel molecular biomarkers and therapeutic targets for treating KIRC. Methods: Multi-omics in silico and in vitro analysis was employed to find hub genes related to the PI3K pathway that may be biomarkers and therapeutic targets for KIRC. Results: Using STRING software, a protein-protein interaction (PPI) network of 25 PI3K pathway-related genes was developed. Based on the degree scoring method, the top four hub genes were identified using Cytoscape's Cytohubba plug-in. TCGA datasets, KIRC (786-O and A-498), and normal (HK2) cells were used to validate the expression of hub genes. Additionally, further bioinformatic analyses were performed to investigate the mechanisms by which hub genes are involved in the development of KIRC. Out of a total of 25 PI3K pathway-related genes, we developed and validated a diagnostic and prognostic model based on the up-regulation of TP53 (tumor protein 53) and CCND1 (Cyclin D1) and the down-regulation of PTEN (Phosphatase and TENsin homolog deleted on chromosome 10), and GSK3B (Glycogen synthase kinase-3 beta) hub genes. The hub genes included in our model may be a novel therapeutic target for KIRC treatment. Additionally, associations between hub genes and infiltration of immune cells can enhance comprehension of immunotherapy for KIRC. Conclusion: We have created a new diagnostic and prognostic model for KIRC patients that uses PI3K pathway-related hub genes (TP53, PTEN, CCND1, and GSK3B). Nevertheless, further experimental studies are required to ascertain the efficacy of our model.

Keywords: KIRC, immunotherapy, biomarker, hub gene

Introduction

Kidney renal clear cell carcinoma (KIRC) originates in the proximal tubules of the kidney [1]. KIRC is the most common subtype of renal cell carcinoma and accounts for approximately 75% of all renal cell carcinomas [2]. KIRC is a highly aggressive and metastatic cancer that is often

resistant to conventional treatments such as chemotherapy and radiation [3]. Some of the major risk factors for KIRC include age, family history, smoking, obesity, high blood pressure, certain genetic syndromes, exposure to harmful chemicals like asbestos, and prolonged use of painkillers. Genetics also play a crucial role in increasing the risk of KIRC [4-7]. Besides these, individuals with end-stage renal disease are also more prone to developing KIRC [8].

Biomarkers are an essential component for early detection and diagnosis of KIRC [9, 10]. Several biomarkers have been identified for KIRC. One such biomarker is carbonic anhydrase IX (CAIX), which is a transmembrane glycoprotein that is expressed in KIRC cells, but not in normal renal tissue [11]. Studies have shown that CAIX expression is linked to the aggressiveness and metastasis of KIRC, and is, therefore valuable for diagnosis and treatment of KIRC [12]. Another biomarker for KIRC is vascular endothelial growth factor (VEGF), which is a protein that promotes the growth and proliferation of blood vessels [13]. KIRC is known to be a highly vascular cancer, and VEGF is expressed at high levels in KIRC tumor cells [14]. Therefore, VEGF can be valuable for early diagnosis of KIRC. Other candidate biomarkers for KIRC include soluble vascular cell adhesion molecule 1 (sVCAM-1), soluble intercellular adhesion molecule 1 (sICAM-1), and platelet-derived growth factor (PDGF) [15]. While biomarkers have shown promising results for detection of KIRC, still their accuracy is low. Therefore, the discovery of new molecular biomarkers of KIRC with better accuracy is required.

Previous reports have suggested that abnormal activation of the PI3K pathway can promote the development of cancer and tumor angiogenesis [16, 17]. In particular, activating missense mutations of PIK3CA have been detected in approximately 30% of breast cancer cases, providing cells with a growth advantage and contributing to tumorigenesis [18]. Dysregulated PI3K pathway signaling has also been linked to resistance to various conventional therapies in breast cancer, glioblastoma, and non-small cell lung cancer [19, 20]. This study aimed to assess 25 genes involved in PI3K pathway activation in KIRC and control samples. The results indicated that four hub genes exhibit significantly differential expression, suggesting their use as novel molecular biomarkers and therapeutic targets.

Methodology

PI3K pathway components selection

25 key genes that are known to be involved with PI3K pathway activation were selected for detailed analysis across KIRC. These proteins were AKTIP, ARP1, BAD, GSK3A, GSK3B, MERTK-1, PIK3CA, PRR5, PSTPIP2, PTEN, FOX1, RHEB, RPS6KB1, TSC1, TP53, BCL2, CCND1, WFIKKN2, CREBBP, caspase-9, PTK2, EGFR, FAS, CDKN1A and XIAP.

PPI and PI3K pathway-related hub gene identification

STRING is a bioinformatic database and web resource that provides information about protein-protein interactions (PPIs) [21]. It consolidates data from a diverse range of sources to create a comprehensive map of known and predicted interactions between proteins. STRING helps in interpreting experimental results, generating hypotheses for further investigation, and identifying therapeutic targets [21]. It also provides additional information such as functional annotations, protein domains, and interaction networks that can be used to better understand the biology of complex diseases. Herein, we used the STRING source for constructing the PPIs of PI3K pathway proteins.

Cytoscape software is a popular open-source tool used for network analysis and visualization [22]. This software caters to the needs of researchers and scientists involved in the study of molecular interactions, gene regulation, and protein networks. The software features an interactive platform that allows users to create, manipulate and analyze complex networks with ease [22]. Overall, Cytoscape software is a powerful tool that has revolutionized network analysis and visualization in biological sciences. The Cytohubba plugin application [23] of the Cytoscape platform was used to analyze the constructed PPI for identifying hub genes based on the degree method.

mRNA and protein expression profiling of hub genes

The UALCAN database [24] is a user-friendly online resource that provides comprehensive

information on cancer genomics and transcriptomics. This powerful tool enables researchers to access public cancer data in a quick and efficient manner, aiding in cancer research. The database allows users to explore gene expression profiles across various cancer types and their subgroups, while also providing the ability to examine the relationship between gene expression and clinical outcomes for specific cancer types [24]. The UALCAN database also offers valuable features such as the creation of basic graphs, data filters, and the ability to download data easily. This innovative platform can facilitate the discovery of novel therapeutic targets and biomarkers, leading to new cancer therapies and treatments. UALCAN was used in this work for mRNA and protein expression profiling of the PI3K pathway-related hub genes across KIRC samples relative to controls.

mRNA expression validation and survival analysis of hub genes using additional TCGA detests

GEPIA [25], OncoDB [26], and MuTarget [27] are web-based platforms that enable researchers to analyze and visualize large-scale cancer genomic data. These databases include genomic and transcriptomic data from various sources, such as The Cancer Genome Atlas (TCGA) project. With GEPIA, OncoDB, and MuTarget, researchers can perform various analyses, including expression analysis, survival analysis, and correlation analysis. The platform also offers interactive features such as clustering, principal component analysis, and gene ontology analysis. These tools provide easy access to comprehensive data, making it possible to perform detailed analyses and generate actionable insights for cancer research. In this work, GEPIA, OncoDB, and MuTarget databases were used for the expression validation analysis of the hub genes across KIRC samples relative to controls. Moreover, GEPIA databases was further utilized for survival analysis as well.

Subcellular localization and protein expression validation of hub genes

The HPA database is a valuable tool for researchers and scientists to study the human proteome [28]. The HPA database contains detailed information on the localization, abundance, and expression patterns of proteins, as well as their functions, interactions, and post-translational modifications [28]. The HPA database is freely accessible to all researchers, making it an essential resource for drug target identification, biomarker discovery, and disease diagnosis and treatment. In this work, the HPA database was used to identify the subcellular localization of the proteins encoded by the hub genes in KIRC cells as well as to validate hub gene expression at the protein level across KIRC samples relative to controls based on immunohistochemical images.

Development of hub gene-based prognostic model

The least absolute shrinkage and selection operator (Lasso) and multivariate Cox proportional hazard regression analysis were further developed to construct a prediction model with “survival” package in R language [29]. In this analysis, GSE167573 dataset was used a training dataset while TCGA_KIRC, ICGC_AU, GSE29609, and E_MTAB_1980 datasets were used as the validation datasets. The formula of the prognostic model of KIRC patients’ prognosis was as follows: risk score = the sum of the multivariate Cox regression coefficient variation of each mRNA.

Methylation analysis of hub genes

UALCAN [24] and OncoDB [26] databases were used in the present work for the methylation analysis of the hub genes across KIRC samples relative to controls.

Mutational analysis, mutation-based survival analysis, and co-expression gene analysis of hub genes

cBioPortal database is an open-access, web-based platform that provides integrated genomic data analysis for cancer research [30]. The database contains large-scale, multidimensional cancer genomic datasets that allow users to explore genetic alterations, gene expression, and clinical data across different cancer types. The cBioPortal database provides rich visualizations and interactive tools that facilitate data exploration and interpretation. Importantly, the database is designed to be intuitive and user-friendly, allowing researchers to quickly access and analyze complex genomic data [30]. Overall, cBioPortal repre-

sents a crucial resource for understanding cancer biology and developing effective treatments. In this work, this tool was used for the mutational analysis, mutation-based survival analysis, and co-expression gene analysis of hub genes in TCGA KIRC samples.

Functional enrichment analysis

The Gene Ontology (GO) analysis provides functional annotation of the gene(s) of interest [31]. KEGG (Kyoto Encyclopedia of Genes and Genomes) provides an interpretation of the user-defined genes in biologic pathways [32]. The GO and KEGG analysis of the hub was performed using the GSEA program [33].

Immune cell infiltration analysis

TIMER2.0 is an advanced computational tool designed to analyze tumor-infiltrating immune cells in a wide variety of cancers [34]. It is a powerful software that utilizes gene expression data to perform immune cell quantification and visualization. TIMER2.0 provides an accurate assessment of tumor-infiltrating lymphocytes (TILs) and other immune cells by combining multiple algorithms, including CIBERSORT and quanTIseq [34]. This analysis tool can enhance our understanding of the tumor microenvironment and its association with disease progression and treatment response. In this research, levels of immune cell infiltration in KIRC were plotted against hub gene expression.

miRNA network analysis

The ENCORI database is a valuable resource for researchers to access a comprehensive collection of RNA sequencing and microarray data [35]. It contains high-quality data from several model organisms, including humans and mice, making it an essential tool for studying changes in gene expression. The database offers a user-friendly interface that allows for easy exploration and analysis of the dataset [35]. Its comprehensive data, ease of use, and frequent updates make ENCORI a valuable resource for researchers in the field of genomics. In this investigation, the ENCORI database was used to create a miRNA network of the identified hub genes.

Hub genes' drug prediction analysis

DrugBank is a comprehensive, freely accessible, online database that contains detailed information on drugs, their targets and side effects [36]. It is aimed at healthcare professionals, researchers, and the general public, and provides detailed descriptions of over 11,000 drugs and their properties [36]. The easy-to-use search function allows users to quickly find the information they need, making it a valuable resource for pharmacology. We used the DrugBank database to uncover a variety of drugs associated with the identified hub genes that may be therapeutic targets.

In vitro validation of PI3K pathway-related hub genes

Cell lines: Human RCC cell lines (786-O and A-498), and normal renal tubular epithelial cell line (HK-2) were purchased from the American Type Culture Collection (ATCC, USA) and cultivated in accordance with the manufacturer's instructions.

Total RNA and RNA extraction: Total RNA extraction from both KIRC and normal cell lines was done by isopycnic centrifugation as described previously [37]. The extracted RNA was then processed for the DNA digestion step of incubation with RNase-free DNase I (Roche, Germany) at 37°C for 15 minutes. DNA extraction was done following the organic method [38]. The quality of the extracted RNA and DNA was checked by a 2100 Bioanalyzer (Agilent Technologies, Germany).

RNA sequencing (RNA-seq) and targeted bisulfite sequencing (targeted bisulfite-seq) analysis: RNA and DNA samples were sent to Beijing Genomics Institute (BGI) company for RNA-seq bisulfite-seq analysis. Following RNA-seq and targeted bisulfite-seq analyses, the gene expression values of the hub genes were normalized using reads per kilo base million reads (RPKM) and fragments per kilo base million reads (FPKM). Methylation values were normalized as beta values. The obtained FPKM, and beta values against hub genes in RCC and a normal control cell line were compared to identify differences in expression and methylation levels.

Statistical details

For enrichment analysis, we used Fisher's Exact test for computing statistical differences [39]. Correlational analyses were carried out using the Pearson method. For comparison, a

Student t-test was adopted. All the analyses were carried out in R version 3.6.3 software.

Results

Determination of PI3K pathway-related hub genes

The PPI network of 25 key proteins involved in PI3K pathway activation, including AKTIP, ARP1, BAD, GSK3A, GSK3B, MERTK-1, PIK3CA, PRR5, PSTPIP2, PTEN, FOX1, RHEB, RPS6KB1, TSC1, TP53, BCL2, CCND1, WFIKKN2, CREBBP, caspase-9, PTK2, EGFR, FAS, CDKN1A, and XIAP was constructed initially using the STRING database. After removing disconnected nodes, the resulting PPI network had 25 nodes and 87 edges (**Figure 1A**). To select hub genes, the constructed PPI was further analyzed using Cytohubba, resulting in the selection of TP53 (tumor protein 53), PTEN (Phosphatase and TENsin homolog deleted on chromosome 10), CCND1 (Cyclin D1), and GSK3B (Glycogen synthase kinase-3 beta) as the top four genes with the highest degree scores (**Figure 1B**).

Expression analysis of hub genes in TCGA

In order to analyze the expression of hub genes in TCGA, data for mRNA and protein expression of these genes was obtained from the ULACAN database for KIRC and control samples. Through evaluation of these data, it was determined that the expression of TP53 and CCND1 genes at both mRNA and protein levels were considerably up-regulated in terms of *p*-value. Conversely, PTEN and GSK3B genes were significantly down-regulated in *p*-value terms in KIRC samples relative to controls (**Figures 1C-E** and **2**).

By looking at the significant dysregulation of the hub genes, we further analyzed TP53, PTEN, CCND1, and GSK3B in KIRC patients with diverse clinical parameters to confirm the expression status of those genes. By this analysis, we further found that TP53 and CCND1 genes were significantly up-regulated, while the PTEN and GSK3B were significantly down-regulated in KIRC patients with different clinical variables relative to controls (**Figure 3**).

Validation and survival analysis results

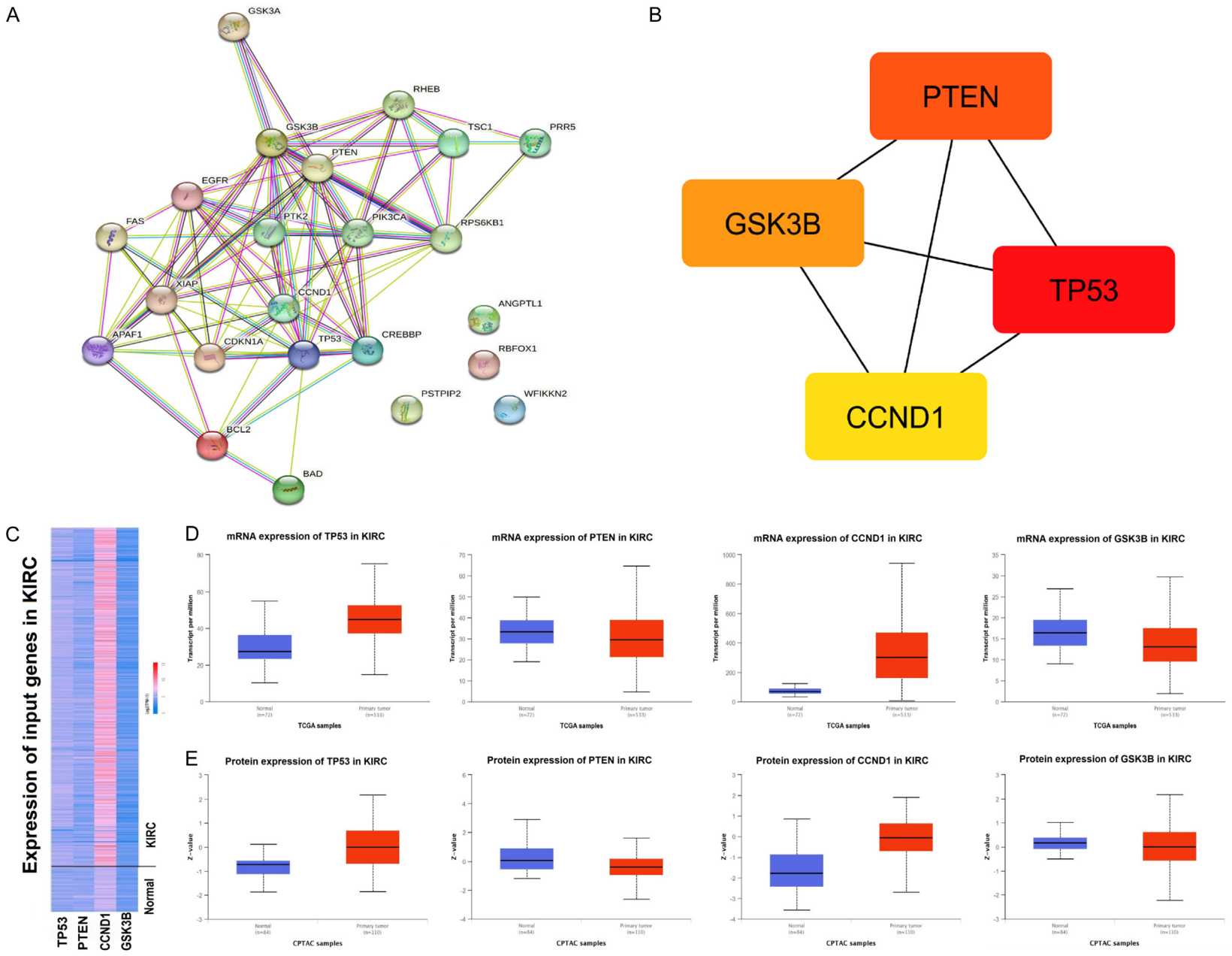
To ensure more accurate results, we conducted expression validation analysis of the hub genes (TP53, PTEN, CCND1, and GSK3B) on additional TCGA datasets using GEPIA, OncoDB, and MuTarget databases. Our findings revealed that TP53 and CCND1 gene expressions were significantly higher, whereas PTEN and GSK3B expressions were significantly lower in KIRC tissues compared to normal tissues (**Figure 4A-C**). Furthermore, we utilized the GEPIA database for survival analysis, which revealed that higher expressions of TP53 and CCND1, along with lower expressions of PTEN and GSK3B, were linked to negative prognosis in KIRC patients with significant *p*-values (**Figure 4D**).

Sub-cellular localization and protein expression validation analysis

First, sub-cellular localization analysis by HPA database analysis revealed that TP53 protein is mainly found in the nucleoplasm, vesicles, and cytosol (**Figure 5A**), PTEN protein was present in the cytosol and nucleoplasm (**Figure 5A**), while CCND1 was detected in the nucleoplasm (**Figure 5A**), and GSK3B was found in the nucleoplasm and cytosol (**Figure 5A**). Secondly, IHC-based expression analysis of four hub genes (TP53, PTEN, CCND1, and GSK3B) indicated that TP53 and CCND1 protein levels were higher in KIRC tissues compared to normal samples, as evidenced by IHC staining (**Figure 5B**). Conversely, the IHC staining of PTEN and GSK3B proteins in KIRC tissues were lower when compared to normal samples (**Figure 5B**). Taken together, these results demonstrate that TP53 and CCND1 are up-regulated while PTEN and GSK3B are down-regulated at the protein level in KIRC samples.

Methylation analysis, mutational analysis, and mutations-based survival analysis results of TP53, PTEN, CCND1, and GSK3B

The UALCAN and OncoDB databases were utilized to investigate the methylation levels of the promoter of hub genes. **Figure 6A** and **6B** illustrate that the KIRC samples exhibited noticeably decreased methylation levels of TP53 and CCND1 promoters, whereas the PTEN and GSK3B promoters displayed notably higher methylation levels relative to normal controls. Thus, it is proposed that there is a negative correlation between the expressions of TP53, PTEN, CCND1, and GSK3B hub genes and their promoter methylation levels in KIRC tissue samples.


A
GSK3A
RHEB
GSK3B
TSC1
PRR5
PTEN
EGFR
FAS
PTK2
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RPS6KB1
XIAP
CCND1
ANGPTL1
APAF1
CDKN1A
TP53
CREBBP
RBFOX1
PSTPIP2
WFIKKN2
BCL2
BAD
B
PTEN
GSK3B
TP53
CCND1
C
Expression of input genes in KIRC
TP53
PTEN
CCND1
GSK3B
KIRC
Normal
D
mRNA expression of TP53 in KIRC
mRNA expression of PTEN in KIRC
mRNA expression of CCND1 in KIRC
mRNA expression of GSK3B in KIRC
Transcript per million
TCGA samples
E
Protein expression of TP53 in KIRC
Protein expression of PTEN in KIRC
Protein expression of CCND1 in KIRC
Protein expression of GSK3B in KIRC
Z-value
CPTAC samples

Figure 1. PPI network of the PI3K pathway activation genes, hub genes, and results of their expression profiling using UALCAN. (A) PPI network of the PI3K pathway activation genes, (B) PPI network identified four hub genes, (C) A heatmap of hub genes in the KIRC sample group and normal control group, (D) Box plot presentation of hub gene mRNA expression in KIRC sample group and normal control group, and (E) Box plot presentation of hub gene protein expression in KIRC sample group and normal control group. PPI = Protein-protein interaction.

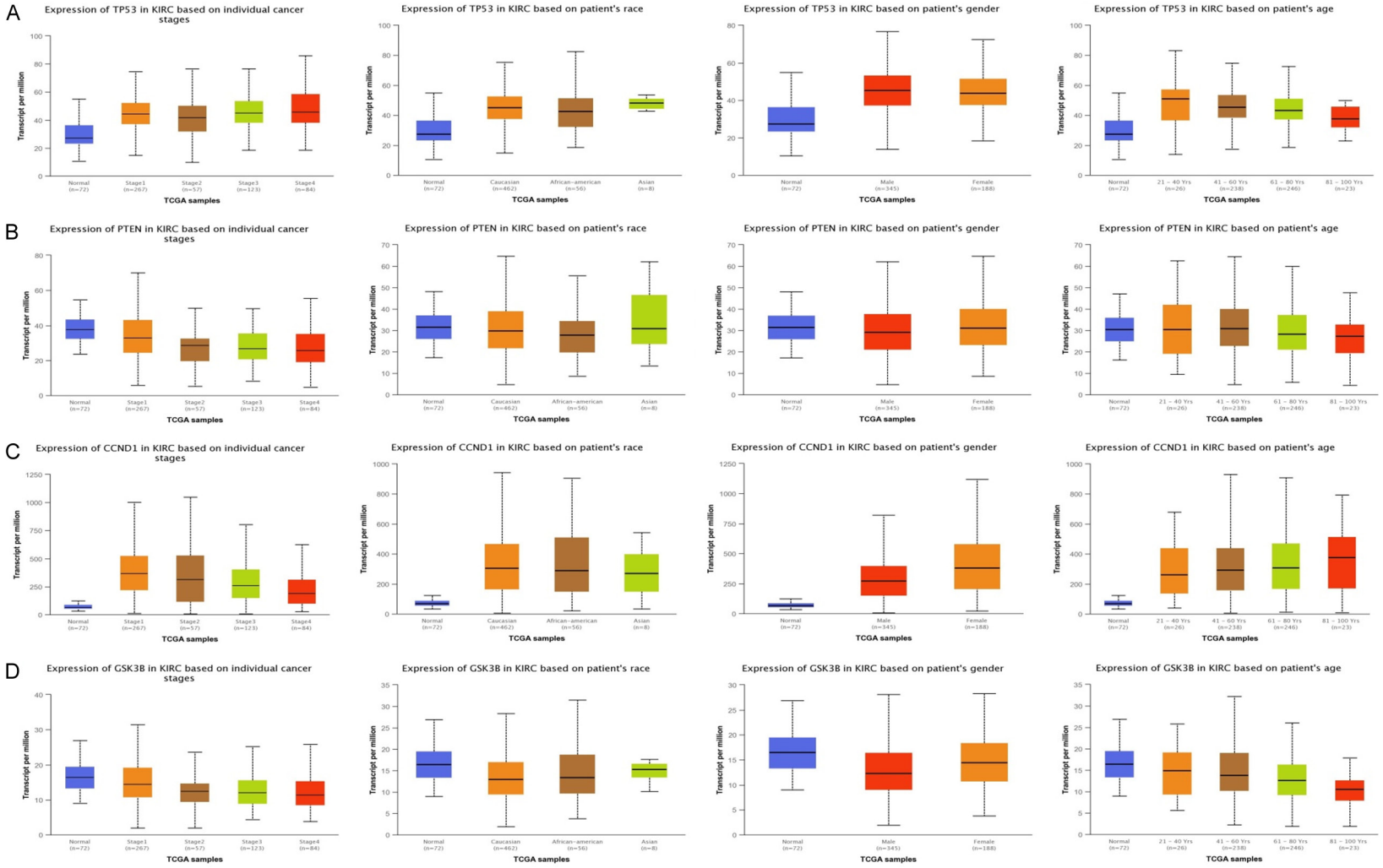

Figure 2. mRNA expression profiling of TP53, PTEN, CCND1, and GSK3B in KIRC samples of different clinical variables relative to controls by UALCAN. (A) Expression profiling of TP53 in KIRC samples of different clinical variables, (B) Expression profiling of PTEN in KIRC samples of different clinical variables, (C) Expression profiling of CCND1 in KIRC samples of different clinical variables, and (D) Expression profiling of GSK3B in KIRC samples of different clinical variables. TP53 = tumor protein 53, PTEN = Phosphatase and TENsin homolog deleted on chromosome 10, CCND1 = Cyclin D1, and GSK3B = Glycogen synthase kinase-3 beta, KIRC = Kidney renal cell carcinoma.

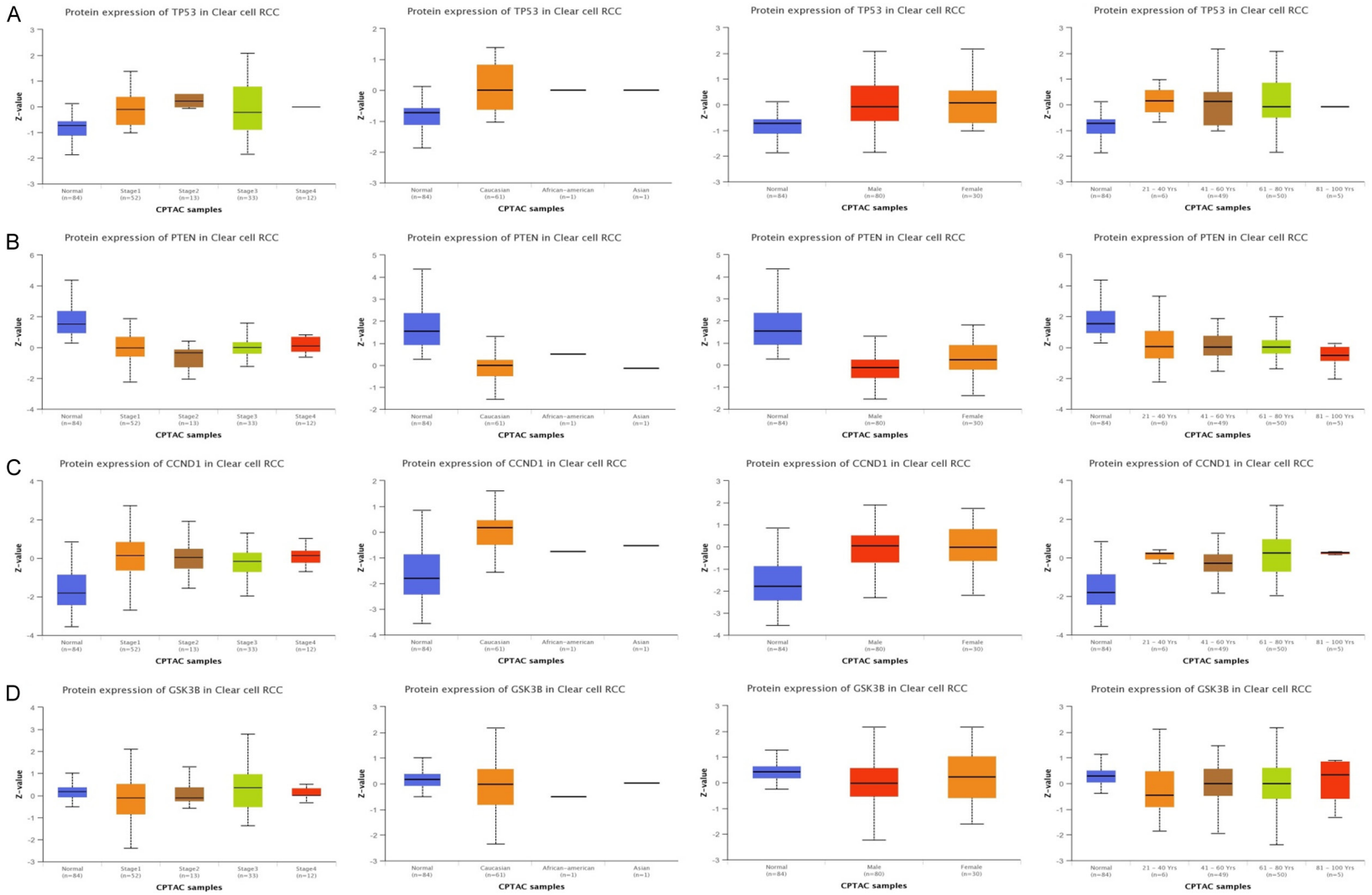

Figure 3. Protein expression profiling of TP53, PTEN, CCND1, and GSK3B in KIRC samples of different clinical variables relative to controls by UALCAN. (A) Expression profiling of TP53 in KIRC samples of different clinical variables, (B) Expression profiling of PTEN in KIRC samples of different clinical variables, (C) Expression profiling of CCND1 in KIRC samples of different clinical variables, and (D) Expression profiling of GSK3B in KIRC samples of different clinical variables.

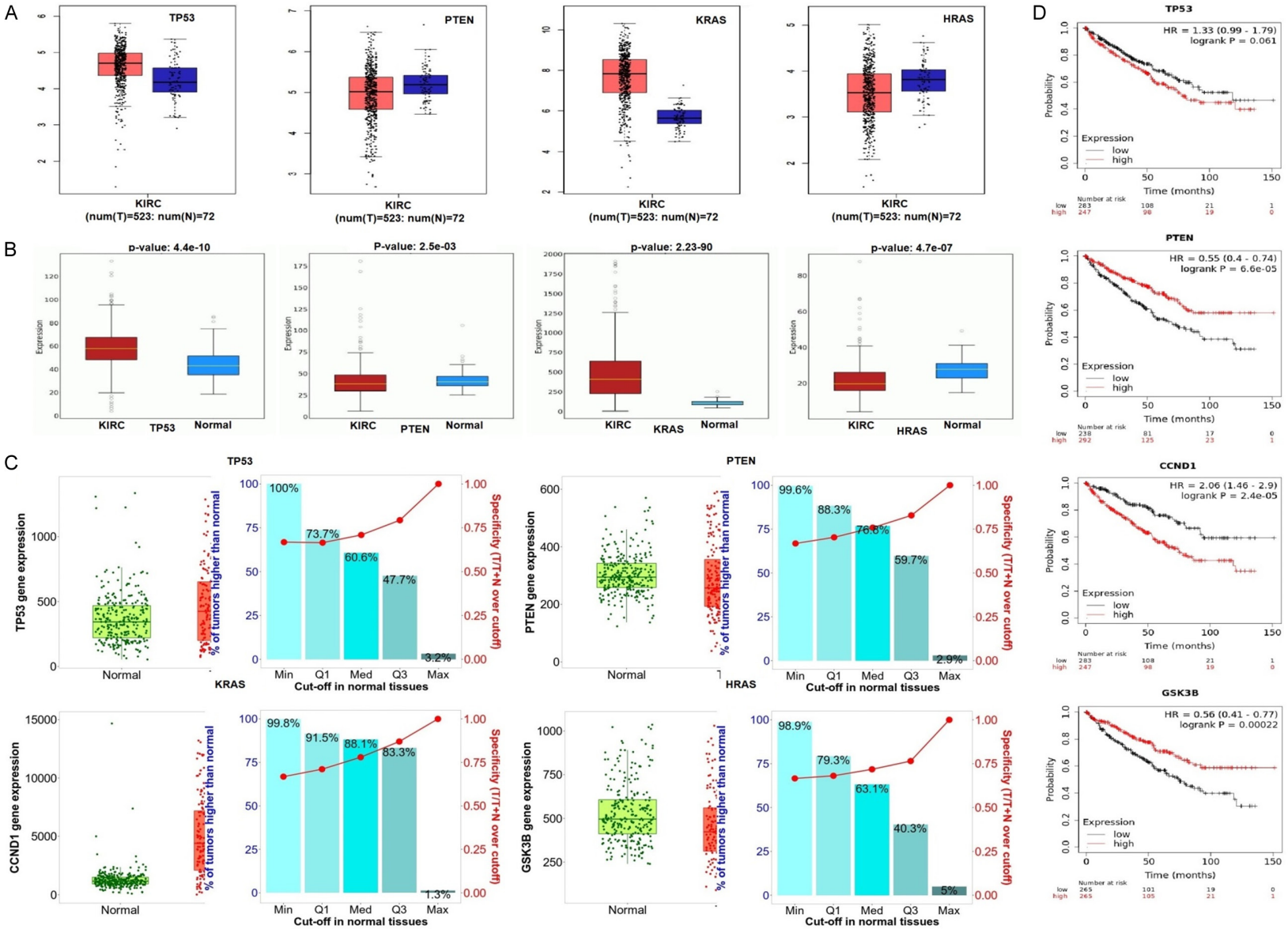

Figure 4. Expression validation and survival analysis of TP53, PTEN, CCND1, and GSK3B. (A) Expression validation TP53, PTEN, CCND1, and GSK3B in KIRC and normal samples through the GEPIA database, (B) Expression validation of TP53, PTEN, CCND1, and GSK3B in KIRC and normal samples through the OncoDB database, (C) Expression validation of TP53, PTEN, CCND1, and GSK3B in KIRC and normal samples through the Mutarget database, and (D) Survival analysis of TP53, PTEN, CCND1, and GSK3B in KIRC and normal samples through the GEPIA database. TP53 = tumor protein 53, PTEN = Phosphatase and TENsin homolog deleted on chromosome 10, CCND1 = Cyclin D1, and GSK3B = Glycogen synthase kinase-3 beta, KIRC = Kidney renal cell carcinoma.

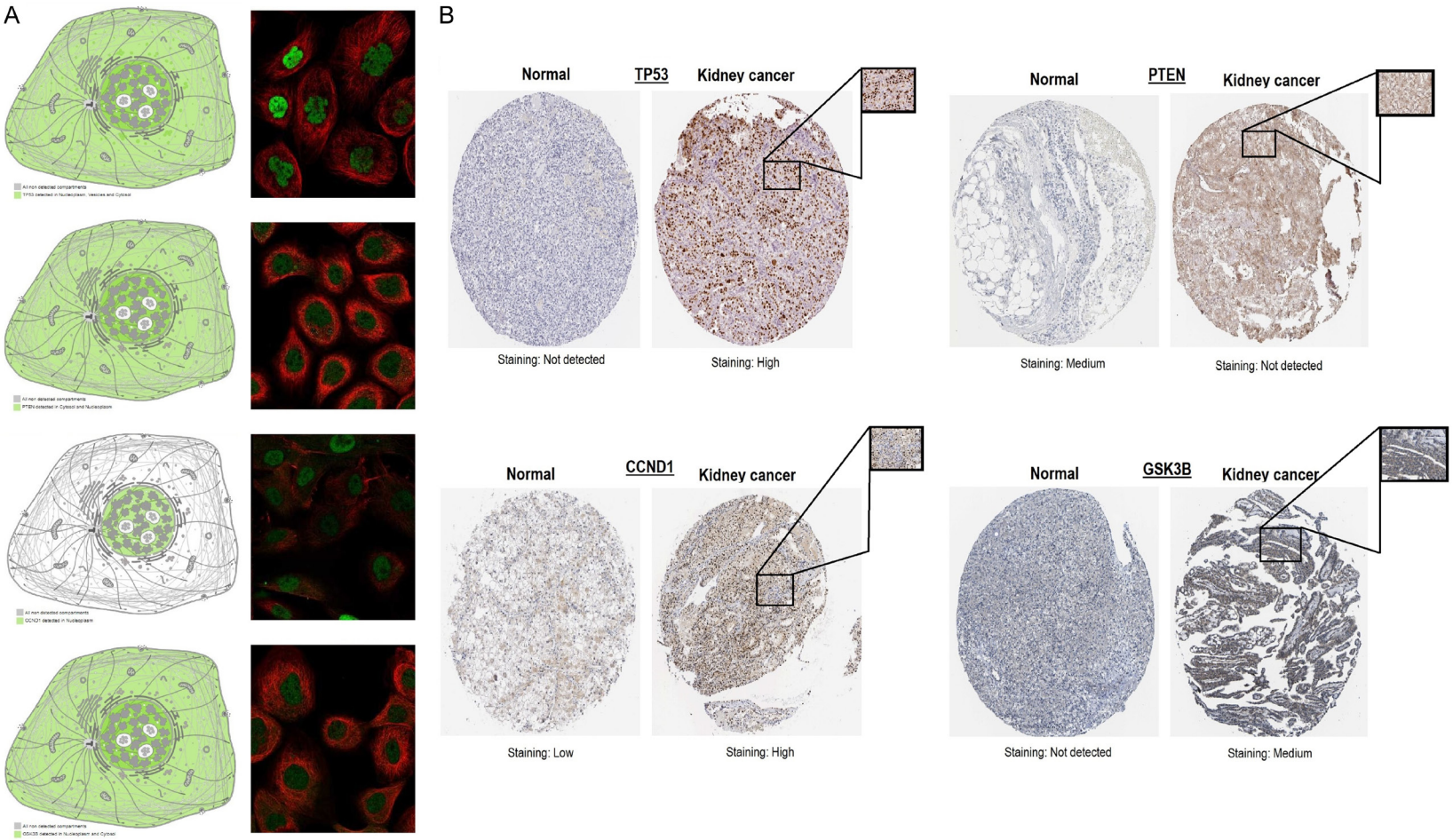

Figure 5. Subcellular localization and protein expression validation of TP53, PTEN, CCND1, and GSK3B through the HPA database. (A) Subcellular localization prediction of TP53, PTEN, CCND1, and GSK3B, and (B) Protein expression analysis of TP53, PTEN, CCND1, and GSK3B in KIRC and normal samples. TP53 = tumor protein 53, PTEN = Phosphatase and TENsin homolog deleted on chromosome 10, CCND1 = Cyclin D1, and GSK3B = Glycogen synthase kinase-3 beta, HPA = Human Protein Atlas.

Ferroptosis biomarker genes in renal cell carcinoma

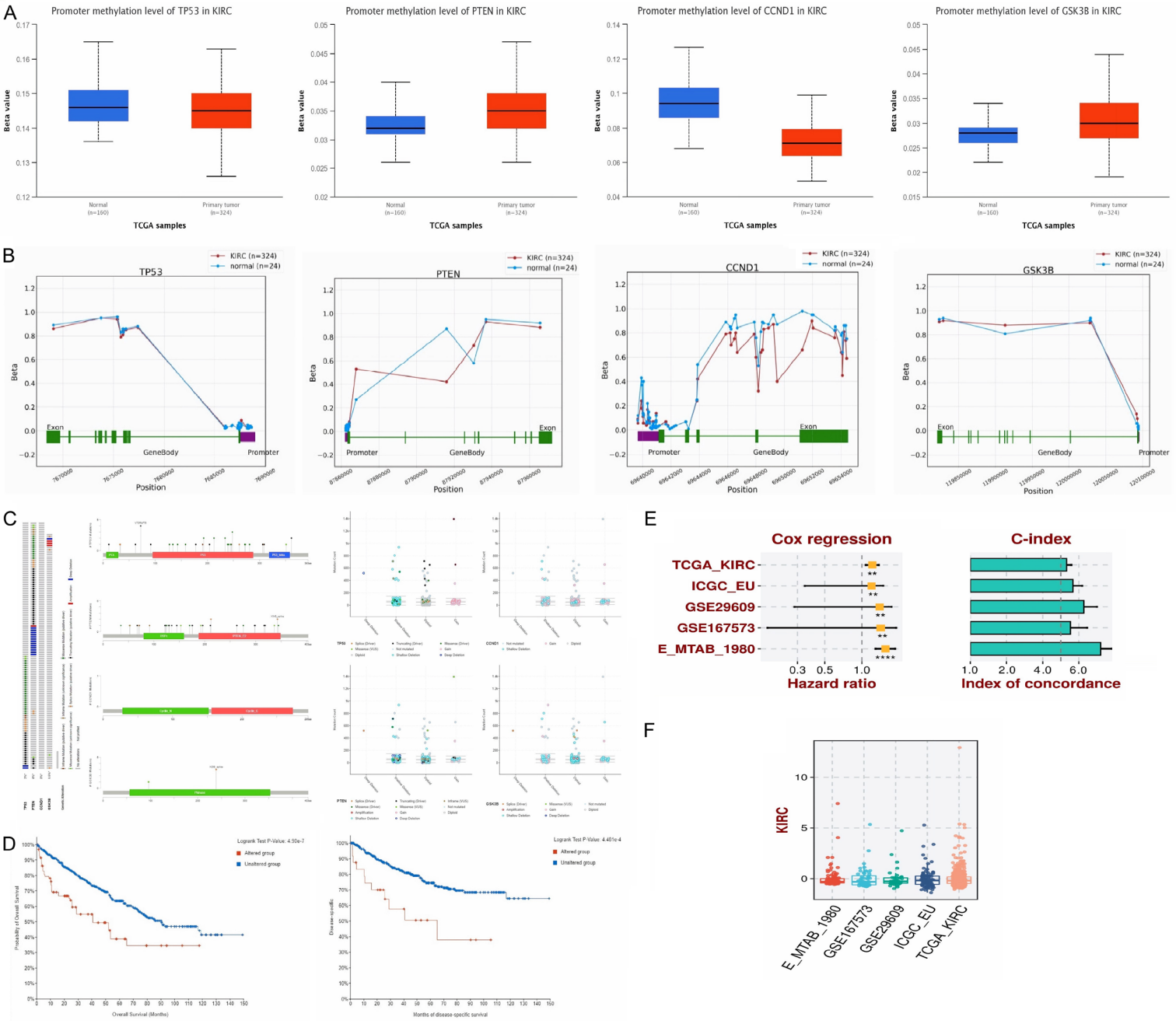

Figure 6. Methylation exploration, genetic alteration frequencies, mutational hotspots, OS, DSS analyses, and the construction of a hub genes (TP53, PTEN, CCND1, and GSK3B) based prognostic model. (A) Methylation status exploration of TP53, PTEN, CCND1, and GSK3B through UALCAN, and (B) Methylation status exploration of TP53, PTEN, CCND1, and GSK3B via OncoDB, (C) Types, frequencies, and location of the genetic alterations in TP53, PTEN, CCND1, and GSK3B, and (D) OS and DSS analysis of TP53, PTEN, CCND1, and GSK3B in genetically altered and unaltered KIRC groups, (E) Univariate Cox regression analysis, and c-index scores, and (F) Risk scores. TP53 = tumor protein 53, PTEN = Phosphatase and TENsin homolog deleted on chromosome 10, CCND1 = Cyclin D1, and GSK3B = Glycogen synthase kinase-3 beta, OS = Overall survival, DFS = Disease free survival.

In order to investigate the roles of genetic alterations in the dysregulation of TP53, PTEN, CCND1, and GSK3B, we utilized the cBioPortal database to further analyze their genetic alterations. Specifically, we selected all KIRC-associated datasets to explore the genetic alterations of each gene. Our analysis revealed that TP53, PTEN, CCND1, and GSK3B genetic alterations occurred at low frequencies in the KIRC samples of the TCGA dataset (3%, 4%, 0%, and 0.5%, respectively) (**Figure 6C**). The most common type of genetic alteration in TP53 was deep missense mutation, while truncated mutation was more frequently observed in PTEN. Additionally, missense mutation was the most common form of GSK3B gene alteration in KIRC patients. Interestingly, our analysis revealed that missense mutations were predominantly concentrated in the P53 domain of TP53, while truncated mutations were located in the DSPc and PTEN-c2 domains of PTEN (**Figure 6C**). Additionally, it was also revealed thorough survival analysis that a KIRC patient group with genetically altered hub genes had the worst overall and disease-specific survival as compared to the unaltered group (**Figure 6D**). This information showed mutations in the TP53, PTEN, CCND1, and GSK3B were associated with reduced survival time of KIRC patients.

Development of hub genes-based prognostic model

In the TP53, PTEN, CCND1, and GSK3B gene-based prognostic model analysis, the GSE-167573 dataset was used a training dataset while TCGA_KIRC, ICGC_AU, GSE29609, and E_MTAB_1980 datasets were used as the validation datasets. We constructed a stepwise Cox regression model including the parameters of the hazard ratio, c-index, and risk score. Evaluation of the prognostic model using a c-index revealed the efficacy and robustness of the model for predicting prognosis of KIRC patients (**Figure 6E**, **6F**).

Enrichment, miRNA, and immune cell infiltration analyses

GO analysis of TP53, PTEN, CCND1, and GSK3B hub genes indicated that those genes were involved in the “Beta-catenin destruction complex, Wnt signalosome, Cyclin-dependent protein kinase holoenzyme complex” etc., CC terms (**Figure 7A**), “Histone deacetylase regulator activity, TFIID-class transcription factor complex binding, MDM2/MDM4 family protein binding” etc., MF terms (**Figure 7B**), “ER overload response, Pos. reg. of protein export from nucleus” etc., BP terms (**Figure 7C**). KEGG analysis of TP53, PTEN, CCND1, and GSK3B showed that these genes were dominantly involved in the “Thyroid cancer, Endometrial cancer, Hedgehog signaling pathway, colorectal cancer, Basal cell carcinoma, prostate cancer, thyroid hormone signaling pathway” etc., pathways (**Figure 7D**).

Using ENCORI and Cytoscape, we constructed the miRNA-mRNA co-regulatory networks of the TP53, PTEN, CCND1, and GSK3B in KIRC. In the constructed networks, the total counts of miRNAs and mRNAs were 430, and 4, respectively (**Figure 7E**). Based on the constructed networks, we have identified one miRNA (hsa-mir-17-5p), that targets all hub genes simultaneously (**Figure 7F**). Therefore, we speculate that the identified has-mir-16-5p, and hub genes (TP53, PTEN, CCND1, and GSK3B) as an axis, might also be the potential inducers of the KIRC.

In this current study, we utilized the TIMER algorithm to further investigate the oncogenic roles of hub genes TP53, PTEN, CCND1, and GSK3B in KIRC. Specifically, we analyzed their correlation with immunocytotoxic infiltration. Surprisingly, our results revealed that the expressions of these hub genes were positively ($P < 0.05$) correlated with the infiltration of both CD4+ T and CD8+ T cells but negatively ($P < 0.05$) correlated with macrophage cell infiltration in KIRC samples (**Figure 8**). These findings

Ferroptosis biomarker genes in renal cell carcinoma

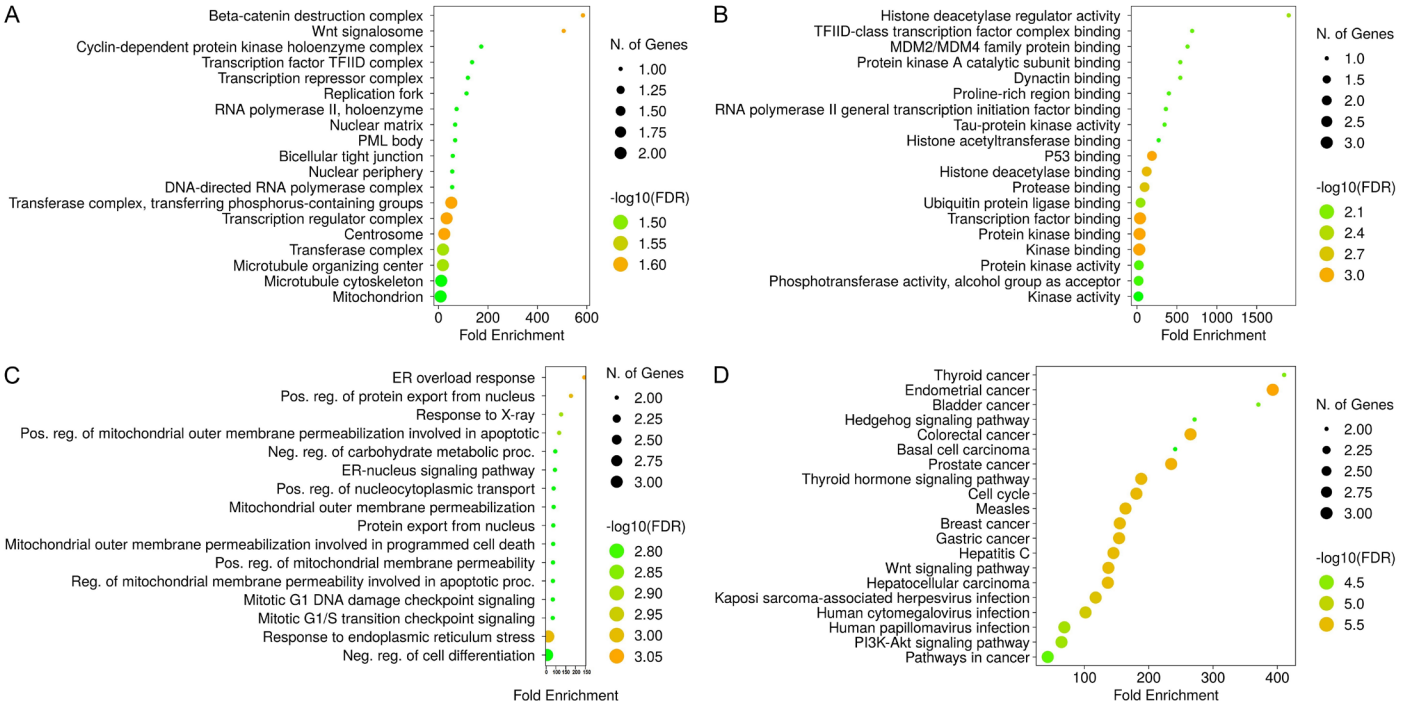

Figure 7. Gene enrichment and miRNA-mRNA co-regulatory network analyses of TP53, PTEN, CCND1, and GSK3B hub genes. (A) TP53, PTEN, CCND1, and GSK3B associated CC terms, (B) TP53, PTEN, CCND1, and GSK3B associated MF terms, (C) TP53, PTEN, CCND1, and GSK3B associated BP terms, and (D) TP53, PTEN, CCND1, and GSK3B associated KEGG terms, (E) A PPI of miRNAs targeting hub genes, and (F) A PPI highlighting most important miRNA (hsa-mir-17-5p) targeting all hub genes. TP53 = tumor protein 53, PTEN = Phosphatase and TENsin homolog deleted on chromosome 10, CCND1 = Cyclin D1, and GSK3B = Glycogen synthase kinase-3 beta, mRNA = Messenger RNA, miRNA = MicroRNA, CC = Cellular component, MF = Molecular function, BP = Biological process, KEGG = Kyoto Encyclopedia of Genes and Genomes.

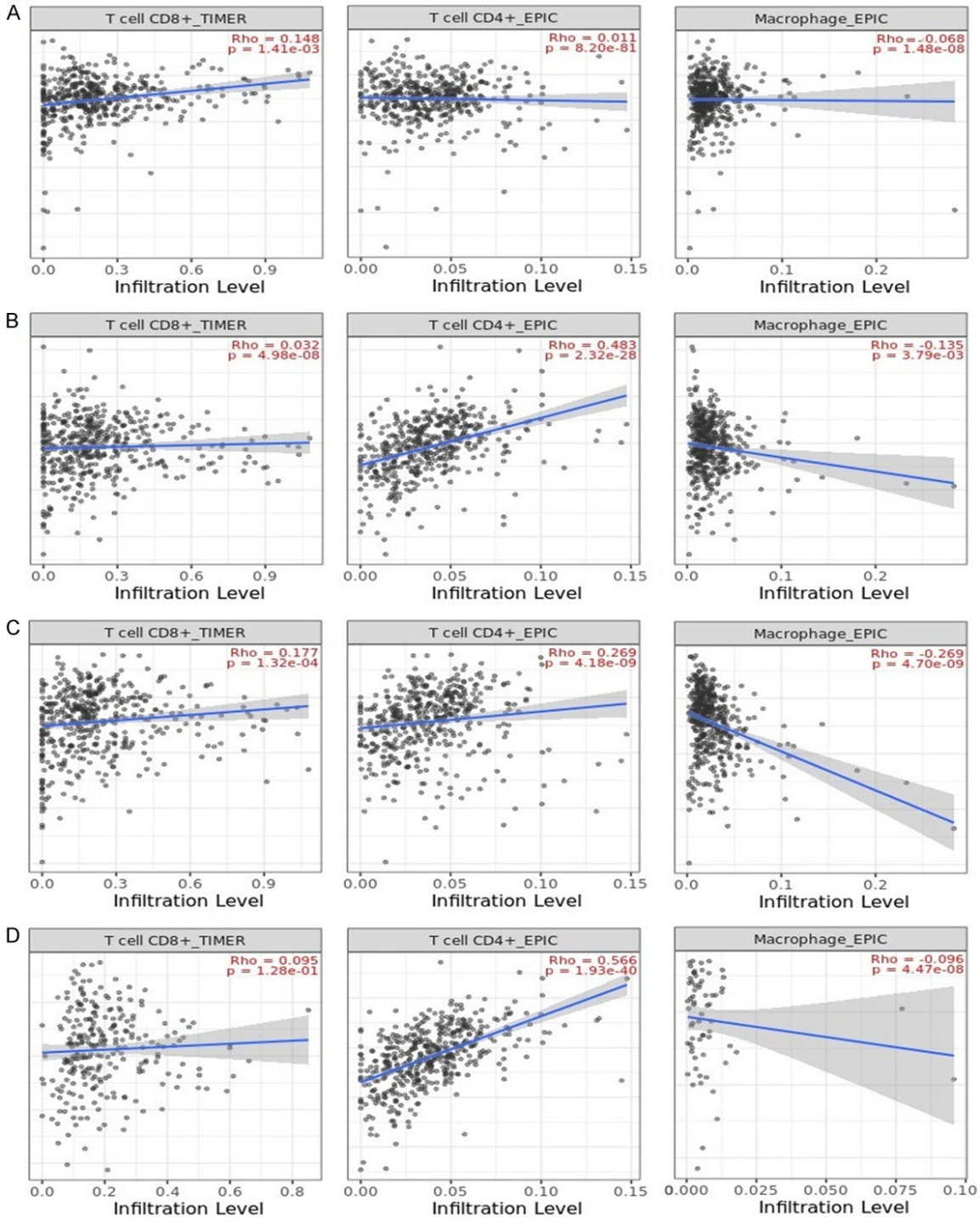

Figure 8. Correlation analysis of MCL1, TP53, PTEN, CCND1, and GSK3B hub genes expression with different immune cells (CD8+ T, CD4+ T, and macrophage) infiltration level. (A) TP53, (B) PTEN, (C) CCND1, and (D) GSK3B. TP53 = tumor protein 53, PTEN = Phosphatase and TENsin homolog deleted on chromosome 10, CCND1 = Cyclin D1, and GSK3B = Glycogen synthase kinase-3 beta.

Table 1. DrugBank-based TP53, PTEN, CCND1, and GSK3B-associated drugs

Sr. No	Hub gene	Drug name	Effect	Reference	Group
1	TP53	Acetaminophen	Decrease expression of TP53 mRNA	A20420	Approved
		Estradiol		A21152	
		Tretinoin		A20405	
2	PTEN	Estradiol	Increase expression of PTEN mRNA	A21179	Approved
		Azacitidine		A20985	
		Genistein		A22790	
		Resveratrol		A22790	
		Tretinoin		A24453	
3	CCND1	Acetaminophen	Decrease expression of CCND1 mRNA	A20420	Approved
		Acitretin		A20453	
		Cyclosporine		A20661	
4	GSK3B	Arsenic trioxide	Increase expression of GSK3B mRNA	A20706	Approved
		Dinitrochlorobenzene		A22388	

TP53 = tumor protein 53, PTEN = Phosphatase and TENsin homolog deleted on chromosome 10, CCND1 = Cyclin D1, and GSK3B = Glycogen synthase kinase-3 beta, mRNA = Messenger RNA.

provide further evidence of the oncogenic potential of TP53, PTEN, CCND1, and GSK3B in KIRC.

Drug prediction analysis of the hub genes

For patients suffering from KIRC, medical treatment is the first option for treatment. Therefore, a selection of appropriate candidate drugs is required. In the current study, using the DrugBank database, we explored some potential drugs, that can reverse the gene expressions of identified hub genes (TP53, PTEN, CCND1, and GSK3B) for the treatment of KIRC. We noted that many drugs are available in the DrugBank database including Acetaminophen, Estradiol, Acitretin, and Cyclosporine, that can reverse TP53, PTEN, CCND1, and GSK3B mRNA expressions and may be utilized as a new regimen for treating KIRC patients (**Table 1**).

In vitro validation of PI3K pathway activation hub genes

In the current study, by performing RNA-seq and targeted bisulfite-seq analyses of 2 RCC cell lines, including 786-O and A-498, and the normal renal tubular epithelial cell line HK-2, the expression and methylation levels of the TP53, PTEN, CCND1, and GSK3B were validated. The expression levels of these genes were validated using FPKM, while the methylation level was validated using beta values. Both FPKM and beta are quantitative values with widespread use in RNA-seq and bisulfite-seq analysis. As shown in **Figure 9A**, TP53, PTEN, CCND1, and GSK3B genes were expressed in both normal and RCC cell lines, and FPKM values of the TP53 and CCND1 were notably higher, while FPKM values of PTEN and GSK3B were notably lower in RCC cell lines (786-O and A-498) as compared to a normal cell line (HK-2) (**Figure 9A**). Moreover, the beta values of the TP53 and CCND1 were lower, while beta values of PTEN and GSK3B were higher in RCC cell lines (786-O and A-498) as compared to normal cell line (HK-2) (**Figure 9B**).

Discussion

This study investigated the role of 25 key genes involved in PI3K pathway activation for the identification of hub genes that could be novel molecular biomarkers or therapeutic targets for kidney clear cell renal cell (KIRC) treatment. Integrative analysis revealed four hub genes, including TP53, PTEN, CCND1, and GSK3B, between KIRC and normal samples. Next, using

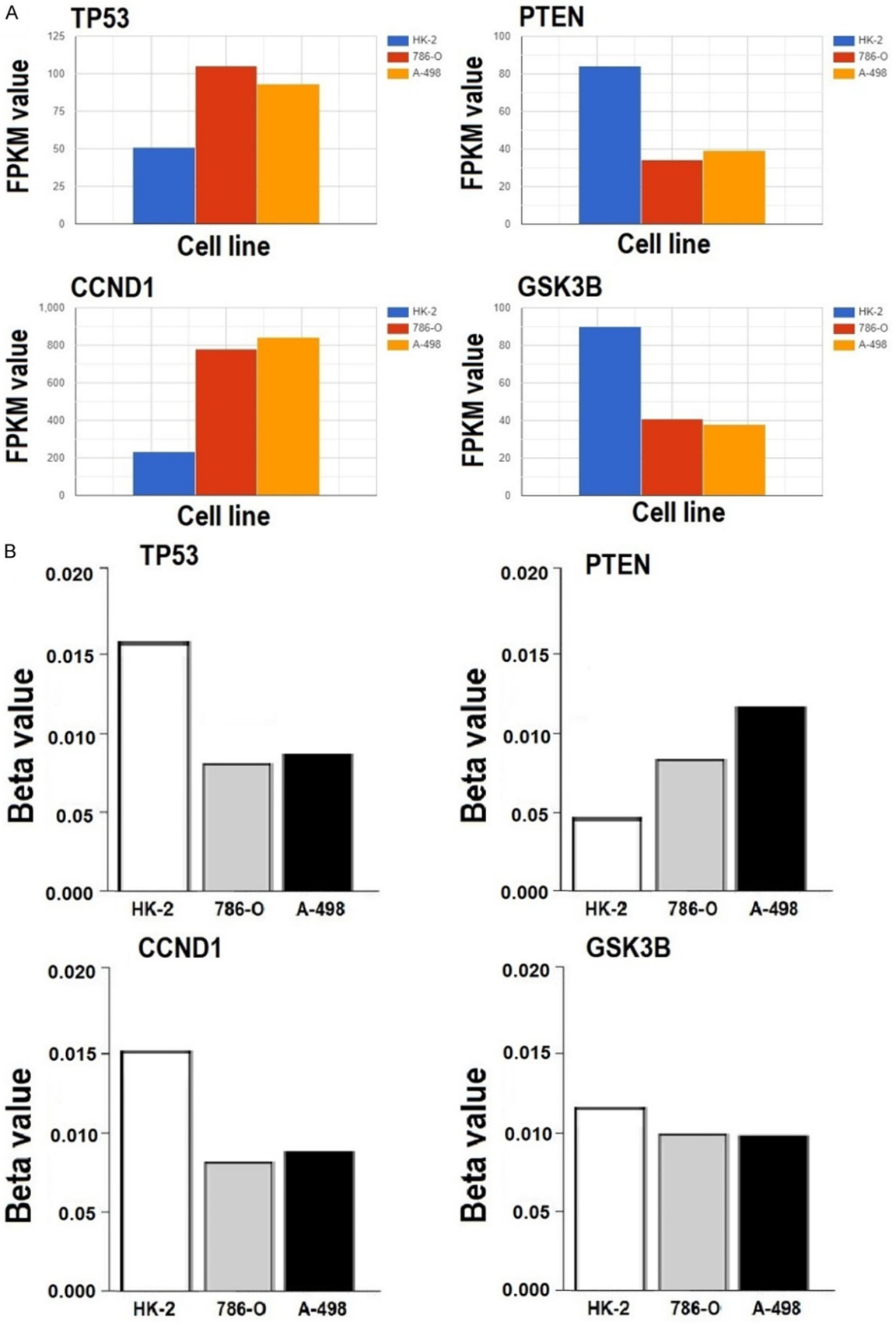
A
TP53
PTEN
CCND1
GSK3B
FPKM value
Cell line
HK-2
786-O
A-498
B
TP53
PTEN
CCND1
GSK3B
Beta value
HK-2
786-O
A-498

Figure 9. Validation of TP53, PTEN, CCND1, and GSK3B expression and promoter methylation levels in RCC cell lines (786-O and A-498), and the normal renal tubular epithelial cell line (HK-2) through RNA-seq and targeted bisulfite-seq analysis. (A) FPKM values-based expression validation of TP53, PTEN, CCND1, and GSK3B and (B) Beta values-based promoter methylation-based validation of TP53, PTEN, CCND1, and GSK3B. TP53 = tumor protein 53, PTEN = Phosphatase and TENsin homolog deleted on chromosome 10, CCND1 = Cyclin D1, and GSK3B = Glycogen synthase kinase-3 beta, RNA-seq = RNA sequencing, Bisulfite-seq = Bisulfite-sequencing, FPKM = Fragments Per Kilobase of transcript per Million mapped reads.

in silico and in vitro analyses, we confirmed the significant up-regulation of TP53 and CCND1, and significant down-regulation of PTEN and GSK3B in TCGA, in cell lines (786-O and A-498), and in samples of KIRC patients compared to controls. Dysregulated expressions of TP53, PTEN, CCND1, and GSK3B were significantly associated with poor survival of KIRC patients and abnormal promoter regions.

Tumor protein 53 (TP53), is a vital gene that plays a crucial role in preventing human cancer [40]. The TP53 gene helps to regulate cell division and prevent the development and growth of tumors by controlling the activity of other genes that are involved in cell growth and cell death [41]. When the TP53 gene is functioning properly, it works to repair damaged DNA and prevent formation of tumors [42]. However, when this gene is mutated or deleted, it can lead to uncontrolled cell division and cancer [42]. Studies have shown that TP53 mutations and dysregulation are common in many different types of cancer, including breast, colorectal, lung, and pancreatic cancer [43-45]. In addition to its role in preventing the development of cancer, TP53 is also important in the treatment of cancer. Many chemotherapy drugs work by damaging DNA, and the ability of TP53 to repair damaged DNA can affect the effectiveness of these treatments [46]. In some cases, cancer cells with TP53 mutations may be more resistant to chemotherapy, making it more difficult to treat the disease. In sum, the TP53 gene plays a critical role in preventing the development of cancer in humans by regulating cell growth and DNA repair. However, abnormal expression of this gene may be involved in ooncogenesis.

Phosphatase and Tensin homolog (PTEN), is a tumor suppressor gene that regulates many cellular processes [47]. PTEN plays a major role in human cancer by regulating cell growth, survival, and proliferation [48]. PTEN's tumor suppressor function is achieved through its phosphatase activity, which leads to the inhibition of the oncogenic PI3K/AKT/mTOR signaling pathway [47]. This pathway is frequently up-regulated in many types of human cancer and drives cancer cell growth and survival [49]. Loss of PTEN function has been linked to a wide variety of human cancers, including prostate, breast, lung, glioma, and endometrial cancer [50-52]. The loss of PTEN expression can occur through multiple mechanisms, including gene mutation, deletion or promoter methylation [52, 53]. Thus, PTEN is a critical tumor suppressor gene that plays a central role in human cancer by regulating multiple cellular processes, including cell growth, proliferation, and survival. Its loss leads to the activation of the PI3K/AKT/mTOR signaling pathway and is associated with a wide variety of human cancers.

The CCND1 gene plays a crucial role in the development of cancer. It encodes CCND1, which is a protein that plays a crucial role in the cell cycle [54]. CCND1 is responsible for driving the cell from the G1 phase to the S phase, where the cell replicates its DNA [55]. However, overexpression of CCND1 can lead to uncontrolled cell growth and division, resulting in development of cancer [56]. Numerous studies have shown that an amplification of the CCND1 gene is a common alteration in various types of cancer, including breast, lung, and prostate cancers [57-59]. Moreover, research indicates that CCND1 may play a role in regulating other cancer-causing genes, such as the c-Myc oncogene [60]. Furthermore, preclinical research has shown that CCND1 knockdown can lead to inhibition of cancer cell proliferation and suppression of tumor growth [61]. These findings suggest that targeting CCND1 may offer a promising strategy for the treatment of various cancers. In short, the amplification and overexpression of the CCND1 gene play an essential role in cancer development.

The GSK3B gene has a critical role in human cancer. GSK3B is a serine/threonine protein kinase that is involved in several cellular processes such as glycogen metabolism, cell divi-

sion, and gene expression [62]. Abnormal expression of this gene has been linked to the development and progression of several types of cancer, including breast, liver, and lung cancers [63-65]. The up-regulation of GSK3B in cancer cells leads to increased cell proliferation, angiogenesis, and tumor invasion [66]. In addition, high levels of GSK3B have been associated with drug resistance, making cancer treatment less effective [67]. On the other hand, the down-regulation of GSK3B can inhibit cancer cell growth and promote cell death [68]. Thus, GSK3B has emerged as a therapeutic target for cancer treatment. In a nutshell, the GSK3B gene plays a crucial role in human cancer and its dysregulation has been linked to tumor progression and drug resistance.

Prior investigations have demonstrated the crucial role of the immune system in regulating tumor growth and metastasis [69]. Immunotherapy has emerged as a promising treatment option for various cancers due to its noteworthy therapeutic potential [70]. In KIRC patients specifically, the prognosis improved with higher levels of CD8+ T immune cell infiltration [71]. Our study revealed a significant positive correlation between expression levels of TP53, PTEN, CCND1, and GSK3B hub genes and the infiltration levels of CD4+ T and CD8+ T immune cells, which were negatively correlated with macrophage cell infiltration in KIRC samples. These findings present novel avenues for designing immunotherapy for KIRC patients.

KEGG enrichment of TP53, PTEN, CCND1, and GSK3B genes highlighted that these genes have significant involvement in the "Thyroid cancer, endometrial cancer, hedgehog signaling, colorectal cancer, basal cell carcinoma, prostate cancer, thyroid hormone signaling" etc., pathways. The dysregulation of these pathways is already implicated in cancer development by previously published studies.

Through miRNA network analysis, we further noticed that TP53, PTEN, CCND1, and GSK3B genes expression were regulated simultaneously by hsa-mir-16-5p miRNA in KIRC patients. Hsa-mir-17-5p is a well-studied miRNA that is involved in various biologic processes, including human cancer [72]. It is one of the key players in the miR-17~92 cluster family that has been shown to act as oncogenes by promoting cell proliferation, survival, and migration in various human cancers [73]. Hsa-mir-17-5p has been found to be overexpressed in multiple cancers, including pancreatic cancer, breast cancer, ovarian cancer, and non-small cell lung cancer. Studies have shown that hsa-mir-17-5p directly targets tumor suppressor genes, such as TP53, PTEN, and TIMP2, leading to their down-regulation and promoting cancer cell proliferation and survival [74-76]. Additionally, it has been shown to play a crucial role in the metastatic process by regulating epithelial-mesenchymal transition (EMT) through the targeting of E-cadherin [77, 78]. However, any tumor suppressor or tumor-causing role of hsa-mir-16-5p in KIRC has not been reported. Therefore, we are the first to report hsa-mir-16-5p having a role in KIRC development in relation to the identified hub genes (TP53, PTEN, CCND1, and GSK3B).

Conclusion

In this research, a unique model was developed for diagnosing and predicting KIRC using biomarkers consisting of TP53, PTEN, CCND1, and GSK3B genes that activate the PI3K pathway. The TCGA data sets and cell lines indicated that the dysregulation of these genes was strongly linked to advancement of KIRC. Nevertheless, more studies are needed to validate the roles of these genes as biomarkers for KIRC.

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Disclosure of conflict of interest

None.

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