

# Identification of biomarkers for prognosis and immunotherapy in clear cell renal cell carcinoma

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## Research Article

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# Abstract

**Background:** Clear cell renal cell carcinoma (ccRCC) is the dominating subtype of renal cancer with high malignancy and poor prognosis.

**Objective:** To identify novel potential biomarkers for the prognosis and immunotherapy of ccRCC patients.

**Methods:** We screened out differentially expressed genes (DEGs) between ccRCC tissues and adjacent normal tissues with 4 microarray series from GEO database and took function analysis of DEGs with GO and KEGG pathway. We constructed PPI network and identified target genes with prognostic value in ccRCC, performed immunological correlation analysis of the candidate genes to seek potential biomarkers of immunotherapy.

**Results:** In total, 382 DEGs were screened out and 11 genes were found to be associated with prognoses of ccRCC patients. Down-regulation of PCK1, HMGCS2, and up-regulation of RRM2 were significantly correlated with poorer prognoses both in overall survival and disease-free survival in ccRCC. Further immunological correlation analysis showed RRM2 expression was correlated with immune infiltration and immunological checkpoint in ccRCC.

**Conclusions:** We identified PCK1, HMGCS2 and RRM2 as potential markers for the prognosis of ccRCC patients with bioinformatic analyses. RRM2 may play an important role in the oncogenicity of ccRCC and maybe a potential target for immunotherapy of ccRCC patients.

## Background

The most aggressive histological subtype of renal cancer, accounting for 75-80% of all renal cancer patients, is clear cell renal cell carcinoma (ccRCC), which presents a higher rate of metastasis and a poorer prognosis among common renal malignancies[1]. As the mechanism of occurrence, progression and metastasis of tumors refer to a mass of genes, multifarious signal pathways, and the entire genes regulatory network, etiology and tumorigenesis of ccRCC remain indistinct. Surgical resection remains the best curative option for patients with localized disease, but to the advanced tumors, systemic therapy still does not significantly improve the prognosis[2]. However, recent remarkable efficacy of immunotherapy in the treatment of malignant tumor has heightened the need for seeking new biomarkers for the management of advanced renal cancer [3]. Thus, it is essential to probe into rooted molecular mechanisms of occurrence and development of ccRCC and identify potential biomarkers for early diagnosis, prognosis and precise therapy.

Bioinformatics, the integration of biology, computer informatics and statistics, make it possible to analyze and excavate the vast biological data obtained. With the assistance of bioinformatics analysis, researchers can analyze abundant gene expression data obtained by high-throughput microarray expression profile to discern key molecules in the process of oncogenesis and tumor progression, and seek for potential therapeutic targets of tumors[4].

In this study, we screened out 4 microarray series from the National Center for Biotechnology Information's (NCBI) Gene Expression Omnibus (GEO), and acquired differentially expressed genes (DEGs) between ccRCC tissues and adjacent normal tissues. We further identified the hub genes with prognostic value in the gene regulatory network of ccRCC and performed the immunological correlation analysis of the candidate biomarkers to seek potential molecular targets of immunotherapy in ccRCC.

## Methods

### Data collection in the GEO database

We screened the mRNA microarray series containing ccRCC tissues and adjacent normal tissues in the GEO database (<https://www.ncbi.nlm.nih.gov/geo/>) and selected four gene expression datasets (GSE6344, GSE47032, GSE53757, GSE66270) for further analysis[5-8]. GSE6344 and GSE47032 each has 10 paired of ccRCC tissues and adjacent normal tissues, and the corresponding platforms are GPL96 (Affymetrix Human Genome U133A Array) and GPL5188 [Affymetrix Human Exon1.0ST Array, probe set (exon version)], respectively. Platforms of GSE53757 and GSE66270 are both GPL570 (Affymetrix Human Genome U133 Plus2.0 Array), with 72 pairs and 14 pairs of paired specimens, respectively.

### Screening and identification of DEGs

To acquire genes differentially expressed, we use GEO2R to compare matched ccRCC samples in the four series. Default statistical parameters were used and probe sets with vacant gene symbols or genes with more than one probe set were removed or averaged

accordingly. The adjusted P-values (adj.P) <0.01 and the |logFC| (fold changes) >2 were considered statistically significant. Venn diagram was used to present the results of the filtering.

### Function enrichment analysis of DEGs

GO enrichment analysis provides the investigators structured and computable annotations regarding the functions of genes and gene products from three orientations: cellular component (CC), molecular function (MF) and biological process (BP). KEGG is an accessible database for systematic analysis of gene functions and advanced functions of biological systems from genomic and molecular level [9]. We exploited the DAVID (Database for Annotation, Visualization and Integrated Discovery, <https://david.ncifcrf.gov/>, version 6.8) to analyze the functions of DEGs acquired. FDR (false discovery rate) <0.05 was considered statistically significant.

### PPI network and module analysis

The STRING website (<https://www.string-db.org/>, version 11.0) was utilized to explore the interactions of DEGs with the minimum required interaction score of 0.4. Whereafter, Cytoscape (version 3.8.0) was applied for visualizing molecular interaction networks and the plug-in Molecular Complex Detection (MCODE) (version 2.0.0) in Cytoscape was ran for identifying the most significant module in the PPI network. Parameters was set as degree cutoff =2, node score cutoff=0.2, K-score=2 and Max depth=100. GO and KEGG analysis of the genes in the significant module were analyzed using DAVID. Statistical significance was set as FDR<0.05.

### Hub genes screening and survival analysis of candidate genes

The hub genes were identified by means of the CytoHubba in Cytoscape, a plug-in which predicts and explores important nodes and sub-networks in a given network by several topological algorithms. The hub genes were filtrated with degrees >10. Subsequently, the obtained hub genes were further screened for their prognostic values through three websites: UALCAN (<http://ualcan.path.uab.edu/>), GEPIA (Gene Expression Profiling Interactive Analysis, <http://gepia.cancer-pku.cn/>) and HPA (the Human Protein Atlas, <https://www.proteinatlas.org/>). In all screening procedures, P<0.05 was considered statistically significant. Results in which the expression level in the tumor was inconsistent with the prognosis were discarded.

### Verification of expression levels of candidate genes

The mRNA level of candidate genes in ccRCC tissues and normal kidney tissues were verified by GEPIA by matching TCGA normal and GTEx data. Meanwhile, CCLE (Cancer Cell Line Encyclopedia, <https://portals.broadinstitute.org/ccle/>) was utilized to inspect the variations of candidate genes in renal cancer cell lines at mRNA level. Outcomes of inconsistent changes of mRNA levels in ccRCC tissues and renal cancer cell lines were rejected. The protein expressions of candidate genes in ccRCC and paracancerous tissues were tested using CPTAC (Clinical Proteomic Tumor Analysis Consortium) confirmatory or discovery datasets via UALCAN, and reconfirmed in the HPA website.

### Potential target for immunotherapy

We applied two databases simultaneously to evaluate the potential of candidate genes as immunotherapeutic targets. TIMER (Tumor Immune Estimation Resource, <https://cistrome.shinyapps.io/timer/>) was adopted to investigate the correlations of candidate genes with immune infiltration level in ccRCC and the relevance between target genes and PD-1 (Programmed Cell Death 1), PDL-1 (Programmed Cell Death 1 Ligand 1) and CTLA-4. When |correlation coefficient (cor)| ≥ 0.2 and P < 0.05, it would be considered statistically significant. Synchronously, we utilized TISIDB (<http://cis.hku.hk/TISIDB/index.php>) to inspect the relation between abundance of TILs (tumor-infiltrating lymphocytes) and expression of target gene in 28 types of human cancers. The correlations between target gene and immunoinhibitors were tested moreover. Drugs targeting the target gene collected from DrugBank database were also presented. Spearman's test was used in all tests and P < 0.05 was considered significant.

## Results

### Identification of DEGs in the ccRCC

After calculation with GEO2R, the volcano plots of four selected gene expression datasets were plotted and shown in Figure 1A. The numbers of DEGs in GSE6344, GSE47032, GSE53757 and GSE66270 after further screening were 701, 1830, 1281 and 2042, respectively. There were 382 overlapped genes in the four datasets, as shown in Figure 1B with Venn diagram, containing 159 up-regulated genes and 223 down-regulated genes in ccRCC tissues compared to adjacent normal tissues.

## Functional annotations of DEGs

We performed GO and KEGG pathway analysis of the screened up-regulated genes and down-regulated genes with DAVID. The results were shown in Table1 and Table2. Results of Go analysis revealed that DEGs are mostly enriched in extracellular space, integral component of plasma membrane, cell surface. The biological processes of DEGs are enriched in response to hypoxia, regulation of immune response, angiogenesis, inflammatory response, cell adhesion, excretion, gluconeogenesis. Regarding to the molecular function of DEGs, the changes are principally involved in protein homodimerization activity, protein binding, receptor binding, protease binding, receptor activity. The KEGG analysis indicated that changes of DEGs are engaged in focal adhesion, phagosome, HIF-1 signaling pathway, Toll-like receptor signaling pathway, metabolic pathways, glycolysis/gluconeogenesis.

## PPI network construction and the most significant module analysis

With the construction using STRING online database and Cytoscape software, the PPI network of the 382 DEGs was shown in Figure1C. After calculation with MCODE, the most significant module in the network arose, as shown in Figure2A. **Function annotations** of the genes in the most significant module was also performed, shown in Figure2B-E. These genes are prevalingly enriched in endoplasmic reticulum lumen, post-translational protein modification, cellular protein metabolic process, serine-type endopeptidase activity, endopeptidase activity, and complement and coagulation cascades.

## Hub genes analysis and the prognostic value assessment

We **figured out** the hub genes of the DEGs with degree>10 using CytoHubba plug-in in Cytoscape and further screened the hub genes utilizing UALCAN, GEPIA and HPA simultaneously for their prognostic values in ccRCC. The candidate hub genes associated with prognoses in ccRCC and the P values of **statistical analysis** were listed in Table3. A total of 11 genes including TIMP1, PCK1, HMGCS2, G6PC, FBP1, ACAA1, HADH, HAO2, TGFBI, RRM2 and SUCLG1 were found to be associated with prognoses of ccRCC patients verified by three databases. Five genes including CASR, SLC34A1, ALDOB, FABP1 and MYCN were found to be associated with ccRCC patients' prognoses when evaluated using UALCAN and GEPIA, but not when using HPA website. Three genes including ACAA1, HADH and SUCLG1 were abandoned as their expression changes in cancer cell lines are inconsistent with that in cancer tissues. Particular researches regarding TIMP1, G6PC, FBP1, HAO2 and TGFBI in Pubmed have emerged. Based on the above analysis and previous researches on related genes in Pubmed literature, we choose PCK1, HMGCS2 and RRM2 for further explorations. Survival curves of PCK1, HMGCS2 and RRM2 in ccRCC patients were shown in Figure3. The survival analysis had shown that ccRCC patients with low expression of PCK1 or HMGCS2 have significantly poorer prognoses both in overall survival and disease-free survival while high RRM2 expression was correlated with worse survival.

## Confirmation of the mRNA and protein expression of 3 candidate genes

We analyzed the mRNA expression levels of candidate genes in ccRCC tissues and renal cancer cell lines using GEPIA and CCLE, respectively. The mRNA levels of PCK1, HMGCS2 and RRM2 in ccRCC tissues and renal cancer cell lines were shown in Figure4. PCK1 and HMGCS2 are down-regulated both in ccRCC tissues and kidney cancer cell lines while RRM2 is up-regulated, as shown in Figure4A. These genes were also found to be associated with pathological stages of ccRCC patients, as shown in Figure4B. Consistent results were obtained in kidney cancer cell lines, as shown in Figure4C. The protein expression of target genes was verified with CPTAC datasets and HPA immunohistochemical staining. As shown in Figure5A, protein expression of RRM2 in ccRCC tissues is higher than normal tissues, while PCK1 and HMGCS2 are down-regulated in cancer tissues with CPTAC datasets analysis. Interestingly, as shown in Figure 5B, staining degrees of PCK1 and HMGCS2 in renal cancer tissues are distinctly lower than normal tissues, while RRM2 staining in renal cancer cells and paracancer tissues are both not obvious in HPA immunohistochemical staining, which may due to the concentration or specificity of the antibody.

## RRM2 expression was correlated with immune infiltration and immunological checkpoint in ccRCC

We further excavated the relationships between the expressions of candidate genes and tumor immune infiltration to seek potential targets for immunotherapy of ccRCC patients. With TIMER analysis, RRM2 expression is positively correlated to infiltration levels of immune cells, including B cells (cor=0.377, P<0.001), CD8+ T cells (cor=0.209, P<0.001), macrophage (cor=0.272, P<0.001), neutrophil (cor=0.37, P<0.001) and dendritic cells (cor=0.418, P<0.001), while no significant correlations were observed as to PCK1 and HMGCS2 (Figure6A). Furthermore, the relevance between candidate genes and PD-1(CD274), PDL-1(PDCD1) and CTLA4 expression were explored and the results displayed low expression of HMGCS2 is associated with CTLA4 (cor=0.203, P<0.001) while RRM2 expression is positively linked to PDL-1 (cor=0.363, P<0.001) and CTLA4 (cor=0.343, P<0.001) (Figure6B). Therewith, we applied TISIDB website to verify the

correlation between RRM2 and abundance of tumor-infiltrating lymphocytes (TILs) across 28 types of human heterogeneous cancers. The results, as shown in Figure7A-B, indicated that RRM2 expression level significantly correlate with abundance of activated CD8 T cells (Act\_CD8,  $\rho=0.427$ ,  $P<0.001$ ), central memory CD8 T cells (Tcm\_CD8,  $\rho=0.462$ ,  $P<0.001$ ), activated CD4 T cells (Act\_CD4,  $\rho=0.762$ ,  $P<0.001$ ), Type 2 T helper cells (Th2,  $\rho=0.45$ ,  $P<0.001$ ), memory B cells (Mem\_B,  $\rho=0.474$ ,  $P<0.001$ ), activated dendritic cells (Act\_DC,  $\rho=0.451$ ,  $P<0.001$ ) in ccRCC. Relations between immunoinhibitors and RRM2 expression in ccRCC displayed that RRM2 correlated with expressions of CD96 ( $\rho=0.433$ ,  $P<0.001$ ), LAG3 ( $\rho=0.48$ ,  $P<0.001$ ), LGALS9 ( $\rho=0.413$ ,  $P<0.001$ ), PDCD1 ( $\rho=0.4$ ,  $P<0.001$ ) and TIGIT ( $\rho=0.462$ ,  $P<0.001$ ) (Figure7C-D). Drugs targeting RRM2 collected from DrugBank database were Cladribine, Imexon, Gallium nitrate, motexafin gadolinium and GTI 2040, as shown in Figure6C.

## Discussion

As the most common pathological subtype of renal cell carcinoma, ccRCC displayed high malignancy, easy metastasis and poor prognosis, accounting for the majority of kidney cancer deaths. Until now, mutations of certain genes such as VHL, PBRM1, SETD2 and BAP1, have been found to be related to the oncogenic driving events of ccRCC. Nonetheless the contributions of their mutations in the tumorigenesis and progression of ccRCC and their values as prognostic biomarkers are still not entirely clear.

In the beginning of this research, we combined four mRNA microarray series containing 106 pairs of ccRCC tissues and adjacent normal tissues from GEO database to obtain the significant DEGs in ccRCC. Subsequently, GO and KEGG analysis of the DEGs indicated that they are principally involved in response to hypoxia, regulation of immune response, angiogenesis, inflammatory response, cell adhesion, metabolic pathways and glycolysis/gluconeogenesis. The results of functional annotation of DEGs, on the other hand, proved that many classical metabolic pathways are reprogrammed in order to empower frantic proliferation of cancer cells in ccRCC, as previous studies have revealed [10]. Then, we used CytoHubba to identify the hub genes, obtain candidate genes related to the prognoses of ccRCC. The results displayed that TIMP1, PCK1, HMGCS2, G6PC, FBP1, ACAA1, HADH, HAO2, TGFBI, RRM2 and SUCLG1 are of prognostic significance in ccRCC patients, which were consistent with partial results of previous researches [11]. Whereafter, we verified the mRNA and protein expression of these genes in different ways, thus obtained three target genes, namely PCK1, HMGCS2 and RRM2. Survival analysis with three websites indicated that low expression of PCK1 or HMGCS2 and high expression of RRM2 have significantly correlated to poorer prognoses in ccRCC patients regardless of overall survival or disease-free survival. The results were consistent with some previous studies, hinting that PCK1, HMGCS2 and RRM2 could be potential prognostic biomarkers for ccRCC patients [12-14]. Furthermore, we investigated the relationships between target genes and immune infiltration and immunological checkpoints in ccRCC emphatically. Analysis of TIMER revealed that RRM2 expression is related to infiltration levels of B cells, CD8+ T Cells, macrophage, neutrophil and dendritic cells, as well as the expression of PDL-1 and CTLA4. Consistent results were observed when calculating in TISIDB website, suggesting that RRM2 may play a crucial part in the occurrence and progression of ccRCC and it maybe a potential target for immunotherapy of ccRCC patients.

PCK1 (Phosphoenolpyruvate Carboxykinase 1) is the first rate-limiting enzyme of gluconeogenesis in cytoplasm, catalyzing the transformation from oxaloacetate to phosphoenolpyruvate. Previous studies have established that PCK1 increase in gastric cancer, colon carcinoma, and tumor-repopulating cells from liver cancer, melanoma and lymphoma [15]. However, PCK1 was found down-regulated in hepatocellular carcinoma and renal cancer, and low expression of this gene is related to worse survival in hepatocellular carcinoma patients [16]. HMGCS2 (3-Hydroxy-3-Methylglutaryl-CoA Synthase 2) encodes a rate-limiting enzyme that catalyzes the first reaction of ketogenesis in mitochondrial. Researchers have discovered that HMGCS2 is reduced in colorectal cancer, prostate cancer, hepatocellular carcinoma, esophageal squamous cell carcinoma and correlated with the prognoses of patients in these cancers [17]. Herein, we discovered both PCK1 and HMGCS2 are reduced in ccRCC and correlated to the prognoses of ccRCC patients. All these discoveries indicated that cancer cells have different metabolic pathways compared to normal cells and the metabolic rewiring appears to be advantageous for cancer proliferation and progression [18]. Notably, ccRCC is one of the most studied malignancies characterized by metabolic reprogramming. Such alterations of metabolism in ccRCC have provided us new thoughts for the exploitation of new targets for oncotherapy [19].

RRM2 (Ribonucleotide Reductase Regulatory Subunit M2) is one of the two subunits of Ribonucleotide reductase (RR), which is the rate-limiting enzyme catalyzing the formation of deoxyribonucleotides from ribonucleotides. Other than the constant expression of RRM1 throughout the whole cell life, RRM2 expression changes dynamically with incentives [20]. Considerable researches have confirmed that RRM2 expression is dysregulated in multiple cancer types, including liver cancer, breast cancer, lung adenocarcinoma and glioblastoma [21]. As a tumor promotor, RRM2 enhances the proliferation, invasion of cancer cells and the resistance to chemotherapeutic drugs, could become the predictor for chemosensitivity and prognosis [22]. In this study, we discovered the expression of RRM2 in ccRCC is

significantly increased. Patients with high expression of RRM2 had relatively poor prognosis, which was consistent with previous results in other cancer types, suggesting that it could be a promising biomarker for the prognostic assessment for ccRCC patients. Additionally, we also discovered a significant correlation between RRM2 expression and immune cell infiltration and immunological checkpoints, extrapolating that it may play an important role in immune response of ccRCC. Numerous studies have proven that ccRCC is an immune tumor with a synergistic effect of angiogenesis and immuno-suppression [23]. Combination of antiangiogenics and targeted immunotherapy to overcome resistance has been proposed as an option in first line treatment of advanced ccRCC currently.

The present study also has certain limitations. Firstly, samples enrolled in the datasets from GEO is relatively few, and lack more validations from databases such as ICGC and TCGA. Secondly, although the expression of DEGs were validated with several online database, there is no experimental verification on samples. Additionally, the functions and regulatory pathways of the RRM2 in ccRCC need verifications in vitro or vivo experiments.

## Conclusion

We revealed that RRM2 is probably a new oncogene involved in the immune regulation mechanism and a promising biomarker for ccRCC immunotherapy. Certainly, further research should be undertaken to investigate its specific role in the occurrence and progression of ccRCC.

## Declarations

### Author contributions

Zuhu Yu designed the study and wrote the manuscript. Bin Lu and Hong Gao completed the data analysis. All authors approved the final version submitted for publication.

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### Conflicts of interest statement

The authors declare that they have no competing interests.

### Data sharing statement

Data are available from GEO database (<https://www.ncbi.nlm.nih.gov/geo/>).

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## Tables

**Table1** The Gene ontology analysis of differentially expressed genes in the ccRCC.

Gene expression	Category	Term	Count	%	P Value	FDR
<b>Up-regulated genes</b>	GOTERM_CC_DIRECT	GO:0005615~extracellular space	42	26.41509434	6.61E-13	1.33E-10
	GOTERM_CC_DIRECT	GO:0005576~extracellular region	42	26.41509434	1.84E-10	1.85E-08
	GOTERM_CC_DIRECT	GO:0005887~integral component of plasma membrane	35	22.01257862	4.02E-08	2.53E-06
	GOTERM_CC_DIRECT	GO:0009986~cell surface	21	13.20754717	5.04E-08	2.53E-06
	GOTERM_CC_DIRECT	GO:0016020~membrane	44	27.67295597	1.73E-07	6.94E-06
	GOTERM_BP_DIRECT	GO:0001666~response to hypoxia	18	11.32075472	2.51E-13	3.52E-10
	GOTERM_BP_DIRECT	GO:0050776~regulation of immune response	14	8.805031447	7.71E-09	5.40E-06
	GOTERM_BP_DIRECT	GO:0001525~angiogenesis	15	9.433962264	1.40E-08	6.53E-06
	GOTERM_BP_DIRECT	GO:0006954~inflammatory response	17	10.6918239	3.17E-07	8.88E-05
	GOTERM_BP_DIRECT	GO:0007155~cell adhesion	18	11.32075472	8.31E-07	1.66E-04
	GOTERM_MF_DIRECT	GO:0042803~protein homodimerization activity	24	15.09433962	7.35E-08	2.70E-05
	GOTERM_MF_DIRECT	GO:0005515~protein binding	107	67.29559748	2.75E-07	5.06E-05
	GOTERM_MF_DIRECT	GO:0005102~receptor binding	15	9.433962264	2.36E-06	2.89E-04
	GOTERM_MF_DIRECT	GO:0002020~protease binding	8	5.031446541	2.73E-05	0.002516149
	GOTERM_MF_DIRECT	GO:0004872~receptor activity	10	6.289308176	1.15E-04	0.00843084
<b>Down-regulated genes</b>	GOTERM_CC_DIRECT	GO:0070062~extracellular exosome	107	48.1981982	1.60E-31	3.23E-29
	GOTERM_CC_DIRECT	GO:0016323~basolateral plasma membrane	18	8.108108108	4.91E-11	4.96E-09
	GOTERM_CC_DIRECT	GO:0016324~apical plasma membrane	21	9.459459459	2.94E-10	1.98E-08
	GOTERM_CC_DIRECT	GO:0005887~integral component of plasma membrane	40	18.01801802	5.81E-07	2.93E-05
	GOTERM_CC_DIRECT	GO:0005615~extracellular space	37	16.66666667	3.45E-06	1.39E-04
	GOTERM_BP_DIRECT	GO:0007588~excretion	10	4.504504505	6.84E-10	9.83E-07
	GOTERM_BP_DIRECT	GO:0006094~gluconeogenesis	8	3.603603604	1.27E-06	7.06E-04
	GOTERM_BP_DIRECT	GO:0001822~kidney development	10	4.504504505	1.47E-06	7.06E-04
	GOTERM_BP_DIRECT	GO:0001657~ureteric bud development	7	3.153153153	7.84E-06	0.002816272
	GOTERM_BP_DIRECT	GO:0055114~oxidation-reduction process	21	9.459459459	7.29E-05	0.01588296
	GOTERM_MF_DIRECT	GO:0005215~transporter activity	13	5.855855856	9.23E-06	0.004585068
	GOTERM_MF_DIRECT	GO:0008201~heparin binding	11	4.954954955	3.37E-05	0.008371823
	GOTERM_MF_DIRECT	GO:0004024~alcohol dehydrogenase activity, zinc-dependent	3	1.351351351	0.002256547	0.281792451
	GOTERM_MF_DIRECT	GO:0042802~identical protein binding	20	9.009009009	0.002755314	0.281792451
	GOTERM_MF_DIRECT	GO:0015301~anion:anion antiporter activity	4	1.801801802	0.002834934	0.281792451

**Table2** The KEGG pathway analysis of differentially expressed genes in the ccRCC.

Category	Term	Count	%	PValue	FDR
<b>Up-regulated genes</b>	hsa04510:Focal adhesion	14	8.805031447	5.42E-06	5.66E-04
	hsa04145:Phagosome	12	7.547169811	7.30E-06	5.66E-04
	hsa04066:HIF-1 signaling pathway	9	5.660377358	5.59E-05	0.002888511
	hsa04620:Toll-like receptor signaling pathway	9	5.660377358	1.13E-04	0.004389804
	hsa05323:Rheumatoid arthritis	8	5.031446541	2.23E-04	0.006910849
<b>Down-regulated genes</b>	hsa01100:Metabolic pathways	53	23.87387387	4.88E-09	8.00E-07
	hsa00010:Glycolysis / Gluconeogenesis	9	4.054054054	4.17E-05	0.003420768
	hsa01130:Biosynthesis of antibiotics	14	6.306306306	2.30E-04	0.012594775
	hsa01200:Carbon metabolism	10	4.504504505	3.32E-04	0.013626947
	hsa00350:Tyrosine metabolism	6	2.702702703	5.28E-04	0.017325663

**Table3** The candidates of hub genes with multiple database screenings.

Hub gene	Full name	Degree	UALCAN P value	GEPIA P value		Prognostic value in HPA	Expression level in ccRCC tissues	Expression level in renal cancer cell lines
				OS	DS			
TIMP1	Tissue Inhibitor Of Metalloproteinases	36	<0.0001	6.80E-07	3.70E-05	Positive	Up	Up
CASR	Calcium Sensing Receptor	25	0.0066	0.027	0.009	Negative	Down	Down
PCK1	Phosphoenolpyruvate Carboxykinase 1	20	0.00062	1.10E-06	0.00091	Positive	Down	Down
HMGCS2	3-Hydroxy-3-Methylglutaryl-CoA Synthase 2	20	<0.0001	3.90E-05	0.00021	Positive	Down	Down
SLC34A1	Solute Carrier Family 34 Member 1	17	0.0025	9.30E-06	0.00011	Negative	Down	Down
ALDOB	Fructose-Bisphosphate Aldolase B	17	0.00039	3.70E-06	0.00021	Negative	Down	Down
FABP1	Fatty Acid Binding Protein 1	17	0.045	0.00057	0.036	Negative	Down	Down
G6PC	Glucose-6-Phosphatase Catalytic Subunit 1	16	<0.0001	7.50E-11	3.20E-08	Positive	Down	Down
FBP1	Fructose-Bisphosphatase 1	15	<0.0001	1.90E-11	7.90E-09	Positive	Down	Down
ACAA1	Acetyl-CoA Acyltransferase 1	14	0.01	7.00E-04	0.0015	Positive	Down	Up
HADH	Hydroxyacyl-CoA Dehydrogenase	13	0.00012	5.30E-08	9.30E-06	Positive	Down	Up
HAO2	Hydroxyacid Oxidase 2	13	<0.0001	2.00E-07	5.00E-07	Positive	Down	Down
TGFB1	Transforming Growth Factor Beta Induced	11	0.0025	0.0019	0.0013	Positive	Up	Up
MYCN	MYCN Proto-Oncogene, BHLH Transcription Factor	11	0.0001	1.50E-05	0.035	Negative	Down	Down
RRM2	Ribonucleotide Reductase Regulatory Subunit M2	11	<0.0001	0.021	0.047	Positive	Up	Up
SUCLG1	Succinate-CoA Ligase GDP/ADP-Forming Subunit Alpha	11	0.00051	2.60E-05	6.00E-04	Positive	Down	Up

## Figures

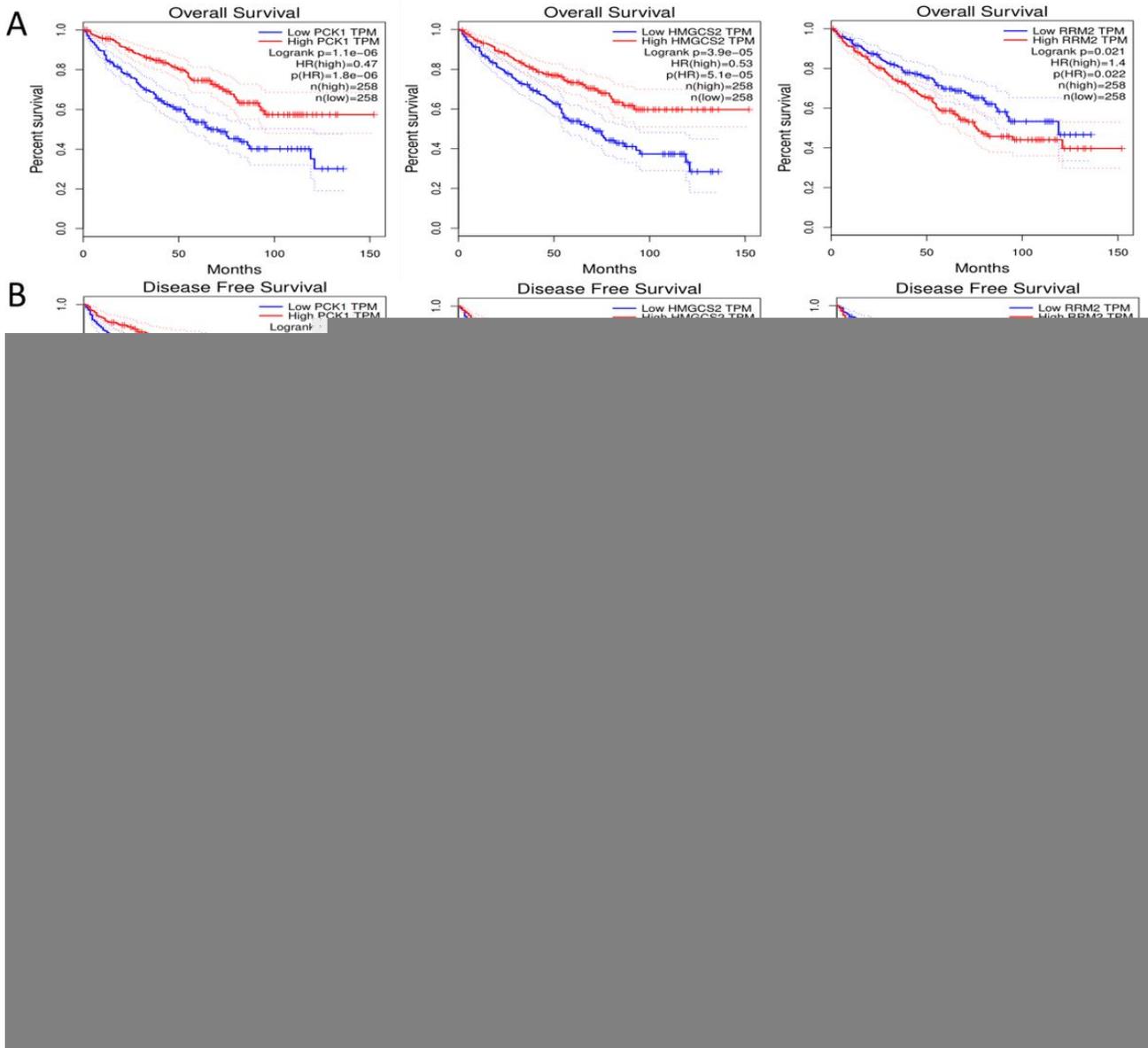
**Figure 1**

Volcano plots, Venn diagram and PPI network of the DEGs. (A) The volcano plots of 4 selected gene expression datasets with calculation of GEO2R. (B) In total, 382 overlapped genes in the four datasets were found dysregulated in ccRCC. (C) The PPI network of the DEGs was constructed with STING database and Cytoscape. Upregulated genes are marked in red; downregulated genes are marked in blue.

**Figure 2**

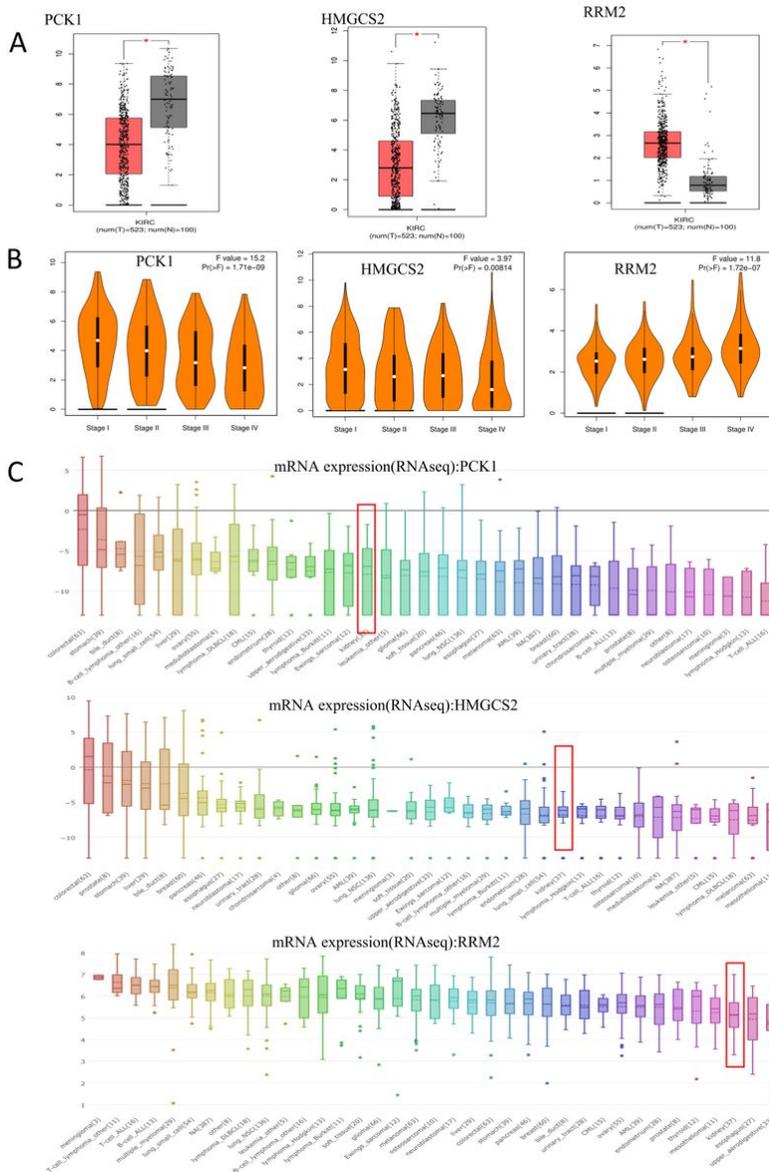
The most significant module of DEGs and function annotations of the genes in this module. (A) The most significant module was obtained with MCODE in Cytoscape software. (B) Cellular Component of the genes. (C) Biological Process of the genes. (D) Molecular

Function of the genes. (E) KEGG pathway analysis of the genes.



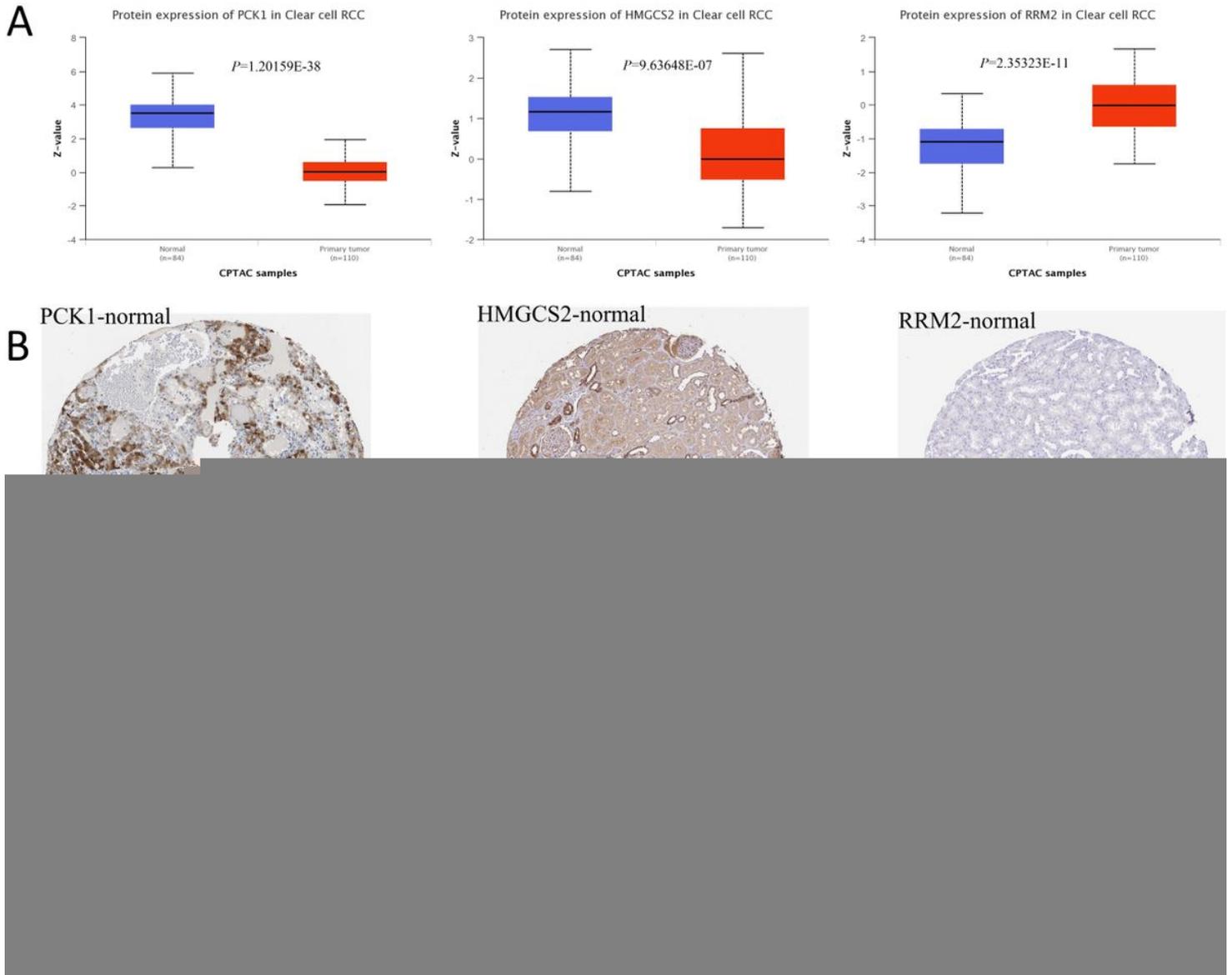
**Figure 3**

Survival analyses of PCK1, HMGCS2 and RRM2 in ccRCC with three websites. (A) Overall survival these genes with GEPIA. (B) Disease free survival of these genes with GEPIA. (C) Survival curves of these genes with UALCAN. (D) Survival curves of these genes with HPA.



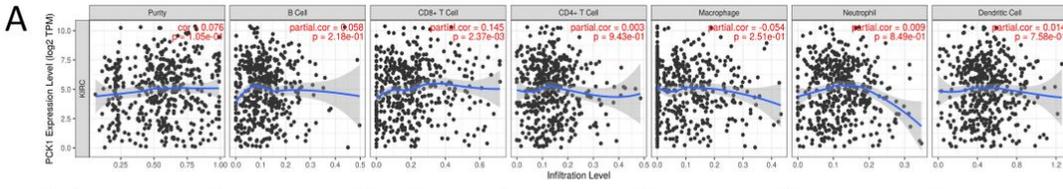
**Figure 4**

Verification of the mRNA and protein expression of PCK1, HMGCS2 and RRM2 in ccRCC with GEPIA and CCL. (A) mRNA expression of these genes in ccRCC and normal tissues with GEPIA. The red box represents tumor tissues and the gray box normal tissues. The red asterisk indicates  $P < 0.01$ . (B) mRNA expression of these genes in different pathologic stage of ccRCC with GEPIA. (C) mRNA expression of these genes in renal cancer cell lines using CCL.



**Figure 5**

Protein expression of PCK1, HMGCS2 and RRM2 in ccRCC and normal kidney tissues. (A) Protein expression of these genes with CPTAC datasets. (B) Protein expression of these genes with HPA immunohistochemical staining.



**Figure 6**

Correlation between candidate genes and tumor immune infiltration with TIMER analysis. (A) The correlation between these genes and infiltration levels of immune cells in ccRCC. (B) The relevance between candidate genes and PD-1(CD274), PDL-1(PDCD1) and CTLA4. (C) Drugs targeting RRM2 collected from DrugBank database.

**Figure 7**

Correlation between RRM2 and tumor immune infiltration using TISIDB. (A) The correlation between RRM2 expression and tumor-infiltrating lymphocytes (TILs) across 28 types of human heterogeneous cancers. (B) The correlation between RRM2 expression and TILs in ccRCC. (C) The correlation between RRM2 expression and immunoinhibitors across human heterogeneous cancers. (D) The correlation between RRM2 expression and immunoinhibitors in ccRCC.