Bioinformatic identification of key genes and analysis of prognostic values in clear cell renal cell carcinoma

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Abstract. The present study aimed to identify new key genes as potential biomarkers for the diagnosis, prognosis or targeted therapy of clear cell renal cell carcinoma (ccRCC). Three expression profiles (GSE36895, GSE46699 and GSE71963) were collected from Gene Expression Omnibus. GEO2R was used to identify differentially expressed genes (DEGs) in ccRCC tissues and normal samples. The Database for Annotation, Visualization and Integrated Discovery was utilized for functional and pathway enrichment analysis. STRING v10.5 and Molecular Complex Detection were used for protein-protein interaction (PPI) network construction and module analysis, respectively. Regulation network analyses were performed with the WebGestal tool. UALCAN web-portal was used for expression validation and survival analysis of hub genes in ccRCC patients from The Cancer Genome Atlas (TCGA). A total of 65 up- and 164 downregulated genes were identified as DEGs. DEGs were enriched with functional terms and pathways compactly related to ccRCC pathogenesis. Seventeen hub genes and one significant module were filtered out and selected from the PPI network. The differential expression of hub genes was verified in TCGA patients. Kaplan-Meier plot showed that high mRNA expression of enolase 2 (ENO2) was associated with short overall survival in ccRCC patients (P=0.023). High mRNA expression of cyclin D1 (CCND1) (P<0.001), fms related tyrosine kinase 1 (FLT1) (P=0.004), plasminogen (PLG) (P<0.001) and von Willebrand factor (VWF) (P=0.008) appeared to serve as favorable factors in survival. These findings indicate that the DEGs may be key genes in ccRCC pathogenesis and five genes, including ENO2, CCND1, FLT1, PLG and VWF, may serve as potential prognostic biomarkers in ccRCC.

Introduction

Renal cell carcinoma (RCC) accounts for 2-3% of all human malignancies (1). It is estimated that more than 338,000 people are diagnosed with RCC each year, with a 22% increase projected by 2020; there are more than 140,000 RCC-related deaths per year (2). Clear cell renal cell carcinoma (ccRCC) is the most common (~75%), lethal subtype of RCC (3). Over the past decade, with improved surgical procedures and the application of specific targeted drugs, the survival of RCC patient has markedly improved (4). However, early accurate diagnosis of ccRCC is still a great challenge and chemotherapeutic or radiotherapeutic resistance remains (4).

A comprehensive understanding of ccRCC initiation, progression and metastasis contributes to early diagnosis and precise treatment. Previous studies have demonstrated that mutations of VHL are significant drivers of ccRCC by regulating various biological processes, and VHL alterations are considered as prognostic markers in ccRCC (5). Moreover, targeted therapies associated with the pVHL/HIF pathway have been tested in phase 3 trials (4). VHL alterations alone are insufficient to cause the cancer, as ccRCC is a systemic biological disease. Sequencing studies have identified some other specific molecular genetic alterations of ccRCC, such as mutations of TCEB1 (6), PBRM1 (7) and abnormal expression of miR-92 (8), miR-210 (9). Further insights into the molecular biology of ccRCC could help us find some novel molecular biomarkers and potential targets for early diagnosis and precise treatment.

Gene expression profiling arrays make it possible to identify numerous differentially expressed genes in tumor samples compared to non-tumor samples at the same time. In this study, we performed an integrated bioinformatics analysis
Results

Identification of DEGs in ccRCC. A total of 591, 325 and 1118 genes were extracted from the GSE36895, GSE46699 and GSE71963 datasets, respectively. There were 229 genes consistently differentially expressed in all three datasets (Fig. 1), including 65 upregulated DEGs and 164 downregulated DEGs in ccRCC tissues compared with normal kidney tissues (Table I).

GO analysis of DEGs in ccRCC. After performing GO analysis of DEGs with DAVID online, the DEGs were classified into three groups: biological process group, molecular function group and cellular component group. We found that the upregulated genes were mainly enriched in biological processes related to hypoxia, blood vessel morphogenesis and angiogenesis. The downregulated genes were commonly involved in functional terms associated with cellular components, metabolism and homeostasis.

Pathway enrichment analysis of DEGs in ccRCC. KEGG pathway enrichment analysis of DEGs was also conducted with DAVID online. KEGG results of the up- and downregulated genes were displayed in Tables II and III, respectively. The upregulated genes were mostly enriched in HIF-1 signaling pathway, PPAR signaling pathway, focal adhesion, coagulation cascades and AMPK signaling pathway. The downregulated genes were mainly enriched in metabolic pathways, collecting duct acid secretion, aldosterone-regulated sodium reabsorption, carbon metabolism and biosynthesis of antibiotics.

Discussion

The current study successfully revealed the potential targets and molecular mechanisms of ccRCC for future therapy. We first identified differentially expressed genes (DEGs) using three datasets. Network analysis, miRNA target prediction and functional enrichment analysis were conducted to explore the function and mechanisms of DEGs. The results showed that the upregulated genes were mainly enriched in proliferative pathways and the downregulated genes were mainly involved in metabolic pathways. We further performed survival analysis and found that the DEGs were closely related to the survival of ccRCC patients. These results provided new insights into the molecular mechanisms of ccRCC and provided potential therapeutic targets for the treatment of ccRCC.

Figure 1. Identification of DEGs in three mRNA expression profiles (GSE36895, GSE46699 and GSE71963). DEGs, differentially expressed genes.

Materials and methods

Data collection. Three gene expression profiles (GSE36895, GSE46699 and GSE71963) were acquired from Gene Expression Omnibus (GEO) database, a free public genomics data repository for array- and sequence-based data.

The array data of GSE36895 included 29 ccRCC tumor samples and 23 matched adjacent normal kidney cortices (10). GSE46699 was comprised of 126 samples including 65 ccRCC tumors and 61 patient-matched adjacent-normal tissues (11). GSE71963 contained 32 ccRCC tumor samples and 16 normal kidney samples (12).

Data processing. GEO2R, a tool for online analysis of GEO series based on the R programming language (13), was used to screen DEGs between the normal kidneys and ccRCC samples. Adjusted P-value (adj. P) and log Fold Change (llog FCI) were used to select significant DEGs. adj. P<0.05 and llog FCI >2 were chosen as the cutoff criteria.

Functional and pathway enrichment analysis. Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of DEGs was carried out using The Database for Annotation, Visualization and Integrated Discovery (DAVID) online (14,15). P<0.05 was selected as the cutoff value.

PPI network construction and significant module analysis. STRING v10.5 was utilized for functional interaction analysis to construct a PPI network (16). Confidence scores >0.7 were considered significant. Genes with degrees >10 were selected as hub genes. The PPI network was visualized by Cytoscape software, and module of PPI network was screened by WebGestal software (18).

Regulation network analyses. The miRNAs and transcription factors (TFs) that potentially regulated the DEGs were predicted using Overrepresentation Enrichment Analysis (ORA) in WebGestal software (18). Then miRNA-target network and TF-target network were also visualized using Cytoscape software.

TCGA verification and survival analysis of hub genes. UALCAN, a tool for in-depth analyses of The Cancer Genome Atlas (TCGA) data, was utilized to verify the differences in expression levels of hub genes (19). The correlation of hub genes with overall survival (OS) of ccRCC patients was examined by recruiting UALCAN as well. Patient data were categorized into two groups based on transcripts per million (TPM) value. The data with TPM greater than upper quartile were assigned to a high expression group and the others with TPM below upper quartile belonged to low/medium expression group. Survival analysis was performed by Kaplan-Meier method, and the log-rank test was carried out. P<0.05 was selected as the cutoff value.

Pathway enrichment analysis of DEGs in ccRCC. KEGG pathway enrichment analysis of DEGs was also conducted with DAVID online. KEGG results of the up- and downregulated genes were displayed in Tables II and III, respectively. The upregulated genes were mostly enriched in HIF-1 signaling pathway, PPAR signaling pathway, focal adhesion, coagulation cascades and AMPK signaling pathway. The downregulated genes were mainly enriched in metabolic pathways, collecting duct acid secretion, aldosterone-regulated sodium reabsorption, carbon metabolism and biosynthesis of antibiotics.

Conclusion

In conclusion, the present study revealed potential targets and molecular mechanisms of ccRCC for future therapy. The DEGs were closely related to the survival of ccRCC patients. The results provided new insights into the molecular mechanisms of ccRCC and provided potential therapeutic targets for the treatment of ccRCC.
A total of 169 genes of the 229 DEGs in all three datasets were filtered into the PPI network complex, containing 169 nodes and 432 edges (Fig. 2A). There were 44 upregulated genes and 125 downregulated genes among the 169 DEGs. Seventeen nodes with a degree >10 were identified as hub genes, such as ALB, VEGFA, EGF, AQP2, ENO2, PLG, FLT1, etc. (bold in Table I). The characteristic properties of the hub nodes based on analysis of the PPI network were tabulated in Table IV. These properties included degree, betweenness, closeness, stress and average shortest path length. After performing module analysis by MCODE, the most significant module was screened out from the PPI network of DEGs, composed of 15 nodes and 54 edges (Fig. 2B). Functional and pathway enrichment analysis of nodes in the module was displayed in Table V. Most of these nodes were enriched in the functional terms related to substance transport and the pathways associated with cancer.

### TF-DEG regulatory network

The DEG-associated transcriptional regulatory network was shown in Fig. 3A. A total of 65 upregulated DEGs and 164 downregulated DEGs were identified in ccRCC tissues, compared with normal kidney tissues. The hub genes were shown in boldface. DEGs, differentially expressed genes; ccRCC, clear cell renal cell carcinoma.

### Table I. DEGs in ccRCC tissues compared with normal controls.

| DEGs       | Gene name                                                                 |
|------------|---------------------------------------------------------------------------|
| Upregulated| TNFAIP6, PFKP, NDUFA4L2, CXCR4, NPTX2, C1QC, FLT1, LOX, PDK1, COL23A1,    |
|            | CDC2A2, GAS2L3, KCNK3, NETO2, FABP7, RNASET2, ANGPTL4, GIC1, SCID,         |
|            | HILPDA, LOXL2, DGCR5, EGF, AQP2, EGF, ENO2, PLG, FLT1, COLA1, MPPED2,     |
|            | EHF, HMGCS2, HPD, GGACT, SLC7A13, HRG, UGT3A1, GATA3, TMEM174, SLC13A1,   |
|            | PROM2, CALB1, SUSD2, KCNJ1, SLC12A3, CRYAA, HSD1B2, DEFB1, GPC5, C1QC,    |
|            | UCHL1, FABP1, TMEM30B, CYP4F2, NELL1, MTURN, FGF9, NPHS2, PAK1, SLC4A9,   |
|            | TFCP2L1, ALDH4A1, SLC12A1, ERF2, ASS1, TACSTD2, PVALB, FOXI1, ABAT, ALB,  |
|            | DMR12, TFAP2B, GLDC, FB1, RASD1, PLPPI, CYP4F3, GSTM3, ESR, SLC47A2,      |
|            | KCNJ1, SLC34A1, MUC15, PTPRO, DPEP1, MECON, ACSF2, CYP17A1, MT1G, PLG,    |
|            | UPP2, MFSDF4A, SLC22A8, HAO2, ALDHEA1, MT1F, TMEM13B, C1L1, EGF, DCXR,     |
|            | UMOD, ATP6V0D2, ANK2, HOGA1, DI1, EFL5, SCNN1A, HSPA2, SOSTDC1, TYP1,     |
|            | ENPP1, PCP4, GPC3, H565ST2, CLDN8, PCK1, LSC5A2, NOX4, BMP1B, G6PC,       |
|            | WNK4, ADH6, HEPA, CAM2, SOST, SH3GL2, SCNN1B, ALB, ALDOB, DCN, SCNN1G,    |
|            | KCNJ10, SLC13A3, SUCNR1, AFM, RAB25, ACPP, HPDG, FXYD4, DNER, RHCG, CYP4A11, |
|            | CTXN3, KCNJ15, GRB14, PTH1R, GGT6, SLC26A7, C7, TMEM178A, OGDHL,          |
|            | ATP6V1B1, DUSP9, SERPINA5, SFRP1, CLCNKB, SLC7A8, SLC7A8, PIPOX, MAL2,    |
|            | PDE1A, TMPRSS2, GPAT3, PRODH2, FAM151A, EPCAM, MRO, ATP6V0A4               |

A total of 65 upregulated DEGs and 164 downregulated DEGs were identified in ccRCC tissues, compared with normal kidney tissues. The hub genes were shown in boldface. DEGs, differentially expressed genes; ccRCC, clear cell renal cell carcinoma.

### Table II. KEGG pathway enrichment analysis of 65 upregulated DEGs.

| Pathway                  | Name                        | P-value     | Genes                                      |
|--------------------------|-----------------------------|-------------|--------------------------------------------|
| hsa04066 HIF-1 signaling | PDK1, FLT1, VEGFA, EGLN3,   | 1.14x10^-3  |
|                          | ENO2, HK2, ANGPT2            |             |                                            |
| hsa03320 PPAR signaling  | CD36, SCD, FABP7, FABP5,    | 4.19x10^-4  |
|                          | ANGPTL4                      |             |                                            |
| hsa04510 Focal adhesion  | CAV2, VWF, LAMA4, CAV1,     | 7.01x10^-4  |
|                          | CCND1, FLT1, VEGFA           |             |                                            |
| hsa04610 Complement and  | C1QB, VWF, C3, C1QC          | 5.81x10^-3  |
| coagulation cascades     |                             |             |                                            |
| hsa04152 AMPK signaling  | CCND1, CD36, SCD, PFKP       | 2.70x10^-2  |
|                          |                             |             |                                            |
| hsa05150 Staphylococcus  | C1QB, C3, C1QC               | 3.35x10^-2  |
| aureus infection         |                             |             |                                            |
|                          |                             |             |                                            |
| hsa05151 P3K-Akt signaling| VWF, LAMA4, CCND1, FLT1,    | 3.53x10^-2  |
|                          | VEGFA, ANGPT2                |             |                                            |
| hsa05230 Central carbon | PDK1, PFKP, HK2              | 4.57x10^-2  |
| metabolism in cancer     |                             |             |                                            |
| hsa00010 Glycolysis/Gluconeogenesis | ENO2, PFKP, HK2 | 4.96x10^-2 |

The pathways were ranked by P-value. KEGG, Kyoto Encyclopedia of Genes and Genomes; DEGs, differentially expressed genes.
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90 nodes with 135 edges were contained in this regulation network, including 61 downregulated genes, 19 upregulated genes and 10 TFs.

miRNA‑DEG regulatory network. In total, 6 miRNAs were filtered out (miR‑144, miR‑96, miR‑503, miR‑150, miR‑383 and miR‑338) (Fig. 3B). A total of 31 nodes and 28 edges were included in this regulatory network.

TCGA validation and the Kaplan‑Meier plot. TCGA data of ccRCC patients were used via the UALCAN data portal. The hub genes identified from the PPI network were differentially expressed between ccRCC tissues and normal tissues (Fig. 4). The expression trends were identical within the three GEO datasets. Kaplan‑Meier curve for overall survival of TCGA patients with ccRCC was obtained according to the low and high expression of each gene. The results showed that patients in the high mRNA expression group for ENO2 had significantly worse OS than those in the low/medium expression group (P=0.023) (Fig. 5A). While high mRNA expression level of CCND1 was associated with longer OS for ccRCC patients (P=0.000), as well as FLT1 (P=0.004), PLG (P=0.000), and VWF (P=0.008) (Fig. 5B‑E).

**Discussion**

The prognosis remains uncertain in ccRCC patients. Identifying novel potential biomarkers for early diagnosis, prognostic evaluation or targeted therapy may improve patient outcomes. Here we performed an in‑depth analysis of three expression profiles (with 126 ccRCC tissues and 100 normal controls) using bioinformatics method and identified 65 up- and 164 downregulated genes. Then we constructed a PPI network of DEGs and extracted 17 hub genes and one significant module from the PPI network. GO and KEGG pathway analysis revealed that the DEGs were commonly involved in functional terms and pathways related to the progression and prognosis of ccRCC. For example, hypoxia and HIF‑1 pathway alterations are critical for the initiation and metastasis of ccRCC (20). Hypoxia could induce a series of tumor‑related aberrations within cellular metabolism, apoptosis, migration and angiogenesis through dysregulation of HIF target genes (20). Drugs targeting the HIF‑1 pathway have proven to be effective in treating ccRCC patients (21). In addition, metabolic pathways play a critical role in ccRCC progression according to previous studies, as well as glycolysis/gluconeogenesis, AMPK signaling pathway, and PI3K‑Akt signaling pathway (22).

Interestingly, the *Staphylococcus aureus* infection pathway was found to be significant in our study. Growing evidence has indicated that bacterial infection is highly associated with certain human malignancies (23). It has been reported that lipoteichoic acids from *S. aureus* induce proliferation of two human non‑small‑cell lung cancer cell lines, A549 and H226 (24). However, the role of *S. aureus* infection in ccRCC still remains to be detected.

Using a Kaplan‑Meier plot for survival analysis, the mRNA expression levels of ENO2, CCND1, PLT1, PLG and VWF were found to be significantly correlated with OS in ccRCC.

### Table III. KEGG pathway enrichment analysis of 164 downregulated DEGs.

| Pathway Name                                      | Pathway Name                                      | Genes                                                                 |
|--------------------------------------------------|--------------------------------------------------|-----------------------------------------------------------------------|
| Metabolic pathways                               | Metabolic pathways                               | TYRP1, SORD, ASS1, OGDHL, ALDOB, UPP2, ADH6, ATP6V1B1, GPAT3, PIPOX, GLDC, CYP27B1, ALDH4A1, ATP6V0D2, HPD, ALDH6A1, KL, HOAG1, FBPI, PCK1, CYP4A11, CYP17A1, GGT6, G6PC, HMC5S2, HAO2, ABAT, PRODH2, CYP4F3, CYP4F2, ATP6V1G3, PSAT1, ATP6V0A4, DCXR |
| Collecting duct acid secretion                    | Collecting duct acid secretion                    | CLCNKB, SLC4A1, ATP6V1G3, ATP6V1B1, ATP6V0A4, ATP6V0D2               |
| Aldosterone‑regulated sodium reabsorption        | Aldosterone‑regulated sodium reabsorption        | FXYD4, HSD11B2, SCNN1G, SCNN1B, SCNN1A, KCNJ1                         |
| Carbon metabolism                                | Carbon metabolism                                | ALDH6A1, OGDHL, ALDOB, HAO2, FBPI, PSAT1, GLDC                        |
| Biosynthesis of antibiotics                      | Biosynthesis of antibiotics                      | HMCGS2, ASS1, OGDHL, ALDOB, HAO2, FBPI, PSAT1, PCK1, PCK1             |
| Glycolysis/gluconeogenesis                       | Glycolysis/gluconeogenesis                       | G6PC, ALDOB, FBPI, ADH6, PCK1                                        |
| Taste transduction                               | Taste transduction                               | PDE1A, SCNN1G, SCNN1B, SCNN1A                                       |
| Vibrio cholerae infection                        | Vibrio cholerae infection                        | ATP6V1G3, ATP6V1B1, ATP6V0A4, ATP6V0D2                               |
| Glyoxylate and dicarboxylate metabolism          | Glyoxylate and dicarboxylate metabolism          | HAO2, HOAG1, GLDC                                                    |

The pathways were ranked by P‑value. KEGG, Kyoto Encyclopedia of Genes and Genomes; DEGs, differentially expressed genes.
Enolase 2 (ENO2) encodes an enolase isoenzyme which is considered as a sensitive and specific biomarker for small-cell lung cancer (25,26). According to our KEGG results, ENO2 was involved in several pathways compactly related to ccRCC pathogenesis such as glycolysis/gluconeogenesis, HIF-1 signaling pathway and metabolic pathways. In addition, ENO2 is found to be induced by HIF-2a although suppression of its mRNA expression alone does not significantly inhibit the growth of the ccRCC cell line 786-O (27). Combining our survival analysis, we infer that ENO2 may be an indicator in the diagnosis and prognosis rather than a potential target for therapy.

Cyclin D1 (CCND1) encodes an essential protein in the cell cycle which shows dual functions in cell growth. It is well-established that CCND1 regulates the cell cycle transition from G1 to S phase by binding to CK4 and CDK6 (28,29). Previous studies suggest that the overexpression of CCND1 promotes cell growth in many malignancies (30-34). Other studies have shown an apoptotic induction effect of CCND1. Consistent expression of an exogenous CCND1 significantly inhibits cell proliferation (35) and induces apoptosis in mammary epithelial cell lines (36). Upregulated CCND1 induces apoptosis of fibroblasts (37) and has a positive

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**Figure 2.** DEGs protein-protein interaction (PPI) network complex and one significant module obtained from PPI network. (A) DEGs PPI network containing 169 nodes and 432 edges. (B) One significant module composed of 15 nodes and 54 edges. Red nodes and green nodes stand for upregulated genes and downregulated genes, respectively. Lines represent the interaction between nodes. DEG, differentially expressed genes.
Table IV. Topology properties of 17 hub genes.

| Genes name | Degree | Betweenness centrality | Closeness centrality | Clustering coefficient | Stress | Average shortest path length |
|------------|--------|------------------------|----------------------|------------------------|--------|----------------------------|
| ALB        | 50     | 0.42                   | 0.50                 | 0.10                   | 30,746 | 2.00                       |
| VEGFA      | 35     | 0.14                   | 0.42                 | 0.15                   | 11,030 | 2.40                       |
| EGF        | 26     | 0.14                   | 0.45                 | 0.25                   | 11,990 | 2.23                       |
| AQP2       | 19     | 0.20                   | 0.41                 | 0.23                   | 15,956 | 2.44                       |
| ENO2       | 17     | 0.08                   | 0.39                 | 0.13                   | 6,610  | 2.60                       |
| PLG        | 16     | 0.01                   | 0.38                 | 0.45                   | 1,644  | 2.62                       |
| CAV1       | 15     | 0.05                   | 0.39                 | 0.29                   | 4,140  | 2.57                       |
| KNG1       | 15     | 0.04                   | 0.38                 | 0.45                   | 3,414  | 2.62                       |
| CXCR4      | 15     | 0.02                   | 0.38                 | 0.45                   | 3,020  | 2.62                       |
| FLT1       | 15     | 0.01                   | 0.39                 | 0.51                   | 1,474  | 2.58                       |
| VWF        | 14     | 0.00                   | 0.37                 | 0.52                   | 582    | 2.67                       |
| GLDC       | 13     | 0.06                   | 0.34                 | 0.15                   | 5,708  | 2.96                       |
| DCN        | 12     | 0.09                   | 0.37                 | 0.26                   | 6,442  | 2.69                       |
| CCND1      | 12     | 0.04                   | 0.38                 | 0.47                   | 2,944  | 2.65                       |
| SLC12A1    | 12     | 0.03                   | 0.38                 | 0.42                   | 3,942  | 2.62                       |
| ALDH4A1    | 12     | 0.03                   | 0.31                 | 0.21                   | 3,776  | 3.20                       |
| FGF1       | 11     | 0.02                   | 0.37                 | 0.53                   | 1,592  | 2.67                       |

The genes were ranked by degree.

Table V. Functional and pathway enrichment analyses of nodes in the significant module.

| Term          | Description                                      | Count | P-value          |
|---------------|--------------------------------------------------|-------|-----------------|
| GO:0006811    | Ion transport                                    | 12    | 6.36x10^-10     |
| GO:0034220    | Ion transmembrane transport                      | 10    | 1.07 x10^-08    |
| GO:0007588    | Excretion                                        | 5     | 1.97 x10^-08    |
| GO:0016324    | Apical plasma membrane                           | 7     | 4.25 x10^-08    |
| GO:0015672    | Monovalent inorganic cation transport            | 8     | 4.97 x10^-08    |
| GO:0050878    | Regulation of body fluid levels                  | 8     | 6.29 x10^-08    |
| GO:0030001    | Metal ion transport                              | 9     | 7.29 x10^-08    |
| GO:0016324    | Apical plasma membrane                           | 7     | 1.68 x10^-07    |
| GO:0055085    | Transmembrane transport                          | 10    | 1.70 x10^-07    |
| GO:0006812    | Cation transport                                 | 9     | 2.94 x10^-07    |
| KEGG:hsa04960 | Aldosterone-regulated sodium reabsorption        | 4     | 1.94 x10^-05    |
| KEGG:hsa04510 | Focal adhesion                                    | 5     | 1.40 x10^-04    |
| KEGG:hsa05219 | Bladder cancer                                    | 3     | 1.50 x10^-03    |
| KEGG:hsa04742 | Taste transduction                               | 3     | 1.81 x10^-03    |
| KEGG:hsa05212 | Pancreatic cancer                                 | 3     | 3.73 x10^-03    |
| KEGG:hsa04066 | HIF-1 signaling pathway                          | 3     | 8.32 x10^-03    |
| KEGG:hsa04151 | PI3K-Akt signaling pathway                        | 4     | 1.14 x10^-02    |
| KEGG:hsa05205 | Proteoglycans in cancer                          | 3     | 3.22 x10^-02    |
| KEGG:hsa04015 | Rap1 signaling pathway                           | 3     | 3.52 x10^-02    |
| KEGG:hsa04014 | Ras signaling pathway                            | 3     | 4.03 x10^-02    |
| KEGG:hsa04060 | Cytokine-cytokine receptor interaction            | 3     | 4.16 x10^-02    |

Two GO categories including GO FAT and GO Direct was used for GO analysis. The top 10 GO terms were selected by P-value. If the term was filtered out by GO DIRECT and GO FAT at the same time, the more significant one would be selected. The GO terms and pathways were ranked by P-value. GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.
correlation with a high apoptotic index in squamous cell carcinomas (38). Our analysis and previous studies show that \(\textit{CCND1}\) is upregulated in ccRCC patients (39). Furthermore, it has been reported that reducing \(\textit{CCND1}\) expression leads to a suppression of tumor growth in ccRCC (27). \(\textit{CCND1}\) is considered as an oncogene in ccRCC. Interestingly, our results showed that high expression of \(\textit{CCND1}\) was associated with favorable prognosis in ccRCC. Similarly, \(\textit{CCND1}\) is elevated and has a favorable effect on disease-free survival in papillary superficial bladder cancer (40). Two independent studies have shown that colon cancer patients with higher \(\textit{CCND1}\) expression have better outcomes (41,42). The molecular mechanism of \(\textit{CCND1}\) in cancer awaits further investigation.

Figure 3. Regulatory network complex. (A) TF-DEG regulatory network containing 61 downregulated genes, 19 upregulated genes and 10 TFs. (B) miRNA-DEG regulatory network containing 31 nodes and 28 edges. Red nodes, green nodes, blue nodes and yellow nodes stand for upregulated genes, downregulated genes, TFs and miRNAs respectively. TF, transcription factor; miR, miRNA; DEG, differentially expressed genes.
The importance of VEGF in RCC progression is well established and several VEGFR inhibitors such as sunitinib and sorafenib have proven to be significantly beneficial for progression-free survival (PFS) and OS in phase 3 trials (43,44). Recent research has demonstrated that FLT1 (also known as VEGFR‑1) protein expression in the tumor epithelium of localized ccRCC patients has a negative effect on prognosis (45). Other studies have found that high mRNA expression level of FLT1 in ccRCC tissue were associated with longer OS. The implication of FLT1 in ccRCC remains unclear. It should be noted that FLT1 can be generated as a transmembrane form and a soluble form. Soluble FLT1 (sFlt1) lacks transmembrane and intracellular domains in contrast to the primary form, a full-length transmembrane receptor (47). Additionally, sFlt1 is thought to be a natural antagonist of VEGF. Recent studies have found that sFlt1 has an antitumor effect on several cancer cells (48-50). Enhanced sFlt1 expression in the serum of breast cancer patients inhibits circulating tumor cells entering the peripheral blood, which may contribute to favorable outcomes (51). Herein we hypothesize that not transmembrane FLT1 but sFLT1 may have an antitumor effect on ccRCC and the value of sFlt1 in patient serum or urine may be worthy of further evaluation.

More and more evidence has demonstrated that plasminogen-plasmin system components are involved in tumor growth, invasion and metastasis by regulating angiogenesis and cell migration (52). The high levels of uPA, uPAR or PAI‑1 expression have proven to be prognostic biomarkers of poor outcome in many cancers, such as ovary cancer, breast cancer and renal cancer (53). The mRNA expression level of PLG in ccRCC patients was found to be downregulated in our analysis and other studies (54). Our results revealed that the ccRCC patients with a higher PLG mRNA expression had longer OS. Similar results have been reported in advanced ovarian cancer recently, and PLG was identified to be a favorable prognostic biomarker in this disease (55).

Another favorable biomarker in our analysis is Von Willebrand Factor (VWF), which shows dual functions in angiogenesis and cancer metastasis according to previous data (56). VWF exhibits a pro-apoptotic effect on 769P, a ccRCC-derived cell line (57). While others have found that serum VWF levels are notably higher in progressive RCC patients compared with stable RCC patients (58). More studies should be done to clarify the link between VWF and ccRCC.
The main limitation of our study is that exploration is done at a bioinformatics level, in silico. Future studies, especially biological experiments in vitro and in vivo are needed to validate the function of these DEGs in ccRCC.

In conclusion, through an integrated bioinformatics analysis of three gene profiles, we identified 229 DEGs, which may contain key genes in ccRCC pathogenesis. Five of the 17 hub genes including ENO2, CCND1, FLT1, PLG and VWF were filtered out through our analysis and may be potential prognostic biomarkers in ccRCC.

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

Figure 5. Kaplan-Meier survival curve for (A) ENO2, (B) CCND1, (C) FLT1, (D) PLG and (E) VWF expression levels in TCGA patients with ccRCC. The log-rank test was carried out on the relevant results. ccRCC, clear cell renal cell carcinoma; TCGA, The Cancer Genome Atlas.
Authors’ contributions
BH, LY and TL designed the study; TL, XC, SZ, BG, BH, YL and CY performed microarray data analysis; YM, FL and TW performed literature research and data acquisition and participated in the data analysis; TL and XC wrote the manuscript; BH and LY revised the manuscript; YL and CY edited the manuscript and approved the final version of the manuscript; LY obtained the funding. All authors read and approved the final manuscript.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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