Clinics in Oncology

Research Article
Published: 24 Jun, 2023

Bioinformatics-Based Identification of *CCNB2* as a Key Gene in the Progression of Chromophobe Cell Carcinoma of Kidney

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Abstract

Background: The present work aimed to screen biomarkers associated with Chromophobe cell carcinoma of kidney (Chrcc) by bioinformatics methods as key genes.

Methods: The GSE15641 data set was acquired from Gene Expression Omnibus (GEO) database, Differentially Expressed Genes (DEGs) were identified. Moreover, the Protein-Protein Interaction (PPI) networks were constructed and visualized using Cytoscape; we identified the significant core gene according to Kaplan-Meier (K-M) survival analysis. Their expression levels were verified in patients from The Cancer Genome Atlas (TCGA) database, and the relationships with clinical features and immune infiltration were analyzed. Finally, Gene Set Enrichment Analysis (GSEA) enrichment analysis was completed to detect significantly differential pathways.

Results: Totally 1153DEGs were identified, thereafter; the four overlapping hub genes (KRAS, EGFR, EHHADH, *CCNB2*) were obtained by four algorithms, with *CCNB2* identified as the only significant core gene according to Kaplan-Meier survival analysis. The high *CCNB2* expression in cancer was associated with adverse clinicopathological factors and had markedly poor Overall Survival (OS). Meanwhile, immune infiltration analysis demonstrated a positive correlation between *CCNB2* expression and Th2 cells enrichment levels in Chrcc, and a negative correlation with Cytotoxic and DC cells. At last, there was a significant difference in the liver cancer subclass and breast cancer grade by GSEA.

Conclusion: In the current work, *CCNB2* can be considered as a predictive molecular marker and a potential therapeutic target for Chrcc.

Keywords: Chrcc; Bioinformatics analysis; *CCNB2*; GSE15641

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Received Date: *07 Jun 2023*
Accepted Date: *20 Jun 2023*
Published Date: *24 Jun 2023*

Citation:
Xie Y, Li J, Yuan Y, Zhang Y, Zhang R. Bioinformatics-Based Identification of CCNB2 as a Key Gene in the Progression of Chromophobe Cell Carcinoma of Kidney. Clin Oncol. 2023; 8: 2004.

ISSN: 2474-1663

Introduction

Kidney cancer is one of the commonly seen malignant tumors of the urological system, and there are over 430,000 new cases and over 170,000 deaths because of kidney cancer each year in the world [1]. Although there are many pathological subtypes of kidney cancer, chrcc is the third most common subtype, occupying approximately 4% to 5% [2]. It is well known that chrcc is a low-grade malignant tumor that originates from the epithelium of the renal collecting duct, which has diverse clinical manifestations. Early stage patients (pathological stages I-III) can be cured by timely surgery, namely, partial or radical nephrectomy [3], and their prognosis is better than that of Renal Clear Cell Carcinoma (RCCC). However, for patients with recurrent tumors or those at the late stage (pathological stage IV), the prognosis is often dismal, and other adjuvant therapies, such as immunotherapy and targeted therapy, are still needed. Some studies prove that drugs like sunitinib and sorafenib have significant effects on patients with advanced kidney cancer [4]. Even so, the survival rate of patients with advanced tumors is unfortunately low, and the first-line treatment options are still unclear. As a result, it is urgently needed to further investigate the molecular mechanisms of chrcc, and to find the effective biomarkers and prognosis-related genes for the treatment of the disease, which can provide certain theoretical basis for the implementation of rational treatment.

Materials and Methods

Data collection

The high-throughput sequencing dataset GSE15641 was downloaded from GEO database, with

the gene microarray data type of expression profiling by array and species of *Homo sapiens*. The dataset annotation platform was GPL96, which contained 6 chrcc and 23 normal kidney tissue samples.

Identification of DEGs

In the above samples, the raw data were divided into chrcc and normal kidney tissue groups, and DEGs in the GSE15641 dataset were analyzed and screened using the GEO2R online analysis tool upon the thresholds of P<0.05 and |log(fold change, FC)| >1.0.

Functional enrichment analysis

Enrichment analysis contained GO analysis and KEGG pathway enrichment. GO enrichment is a common tool for histological data analysis, which can be categorized into three categories, containing Biological Process (BP), Cellular Component (CC) and Molecular Function (MF) for the comprehensive annotation of DEGs. Meanwhile, the KEGG database helps to comprehensively understand the enriched pathways. In the current work, GO enrichment analysis and KEGG pathway enrichment analysis were carried out separately on DEGs using the DAVID tool, then graphs were plotted for visualization, and the differences were regarded to be of statistical significance at P<0.05.

Construction of PPI networks and identification of hub genes

PPI networks based on DEGs were constructed by adopting the String online database tool and visualized with the Cytoscape software. Then, all DEGs on the PPI networks were scored by the CytoNCA plug-in, and sorted out according to four algorithms, namely, Betweenness, Degree, Network and Information.

Prognostic analysis of hub genes

TCGA, the Cancer Genome Atlas Project, is a database used to investigate genomic changes in cancer. The TPM formats of data on chrcc were selected, and then K-M survival analysis was conducted using Log-rank test for the obtained hub genes.

Validation of *CCNB2* in TCGA database

Altogether 90 cases of chrcc (TPM data) were selected from TCGA database, then R language ggplot2 package was utilized to detect whether the expression of *CCNB2* was different between cancerous and normal tissues in chrcc patients and also in paired tissues by the Wilcoxon rank sum test. Furthermore, Kruskal-Walli's test and Wilcoxon test were adopted to analyze the relationship between *CCNB2* expression and clinical characteristics in four aspects, namely, OS, Progression-Free Interval (PFI), Disease-Specific Survival (DSS), and pathological stage.

Immune infiltration analysis

With the aim of investigating the relationship between the *CCNB2* gene in chrcc and the infiltration levels of 24 immune cells in the immune microenvironment, the fractions of immune cells in tumor tissues were calculated by using the GSVA package in R language and the ssGSEA algorithm. Moreover, correlation analysis was completed by Spearman's test with a screening criterion of P<0.05.

GSEA Enrichment analysis

To search more deeply for *CCNB2* gene enrichment pathways, the R package "clusterProfiler" tool in the MSigDB collections collection (c2.all.v7.0) was utilized, with p<0.05 and FDR<0.25 being the screening criteria to identify significantly differential signaling

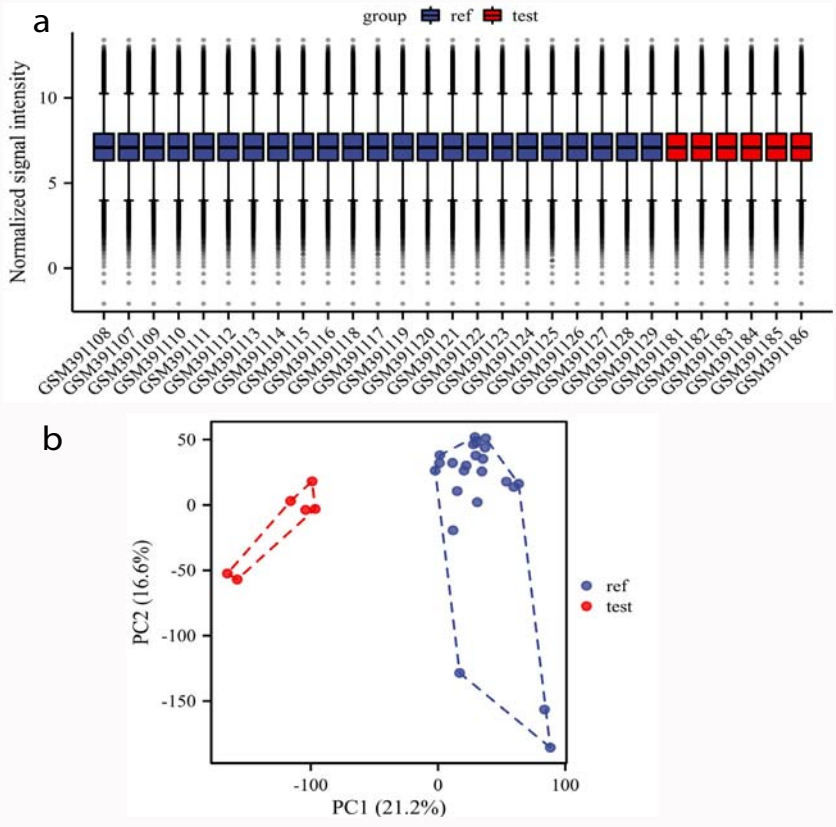


Figure 1a: Box plots for the samples of GSE15641 dataset.
Figure 1b: PCA plots for the samples of GSE15641 dataset.

pathways.

Results

Identification of DEGs

Totally, 1153 DEGs (containing 469 up-regulated genes and 684 down-regulated genes) were detected by analysis, which were visualized by plotting box plots and PCA plots, as shown in Figure 1. The median of each sample in the box plots was basically on a level, indicating a good degree of normalization between samples, while the samples of each group on the PCA plots were separated, indicating obvious differences between the two groups. Additionally, the heat map and volcano plot of DEGs are presented in Figure 2, with red color indicating up-regulated genes and blue color indicating down-regulated ones.

GO and KEGG enrichment analyses

According to the enrichment results (Figure 3), DEGs were mostly enriched into BP terms including organic anion transport, glycolysis/gluconeogenesis, response to metal ions, and response to renal phylogeny; CC terms such as the apical part of the cell, apical plasma membrane, extracellular matrix containing collagen, and lumen of secretory granules; whereas MF terms like receptor ligand activity, anion transmembrane transporter protein activity, coenzyme binding, and organic anion transmembrane transporter protein activity. KEGG pathway enrichment demonstrated that pathways like complement and coagulation cascades, glycolysis/gluconeogenesis, mineral uptake, arginine and proline metabolism, β-alanine metabolism, renin-angiotensin system, and steroid biosynthesis were significantly enriched.

PPI interactions and hub gene identification

The top 50 genes selected according to each criterion are shown in Figures 4A-4D, where the higher score is shown by the larger diameter of the circle and predicts the stronger association. The 15 DEGs with the highest scores were used to be hub genes (Table 1), then the interactions were analyzed and the four overlapping hub genes were obtained, namely, *KRAS*, *EGFR*, *EHHADH*, and *CCNB2* (Figure 4E).

Table 1: Top 15 hub genes sorted out according to betweenness, degree, network and information.

Betweenness	Degree	Network	Information
PPARGC1A	KRAS	EHHADH	KRAS
NCOA3	EGFR	KRAS	EGFR
FOS	FOS	EGFR	FOS
EHHADH	EHHADH	HIST1H2AJ	EHHADH
AOX1	HIST1H2AJ	GPI	HIST1H2AJ
CYP3A5	EGF	EGR1	EGF
HSD17B7	CCNB2	CCNB2	CCNB2
CCNB2	THBS1	HIST1H2AC	THBS1
LBR	HIST1H2AC	ADAMTS1	HIST1H2AC
EGFR	NOTCH1	SPON1	NOTCH1
KRAS	DNM1	SBSPON	DNM1
CENPA	AOX1	THSD7A	AOX1
NOTCH1	GPI	EGF	GPI
DHCR24	PPARGC1A	PIPOX	PPARGC1A
SC5D	DCN	THBS1	DCN

K-M survival analysis curve

As observed from the curve, the low expression of the *CCNB2* gene predicted the superior prognosis to the high expression, and the difference in survival time between the two groups was of statistical significance (Figure 5). Therefore, *CCNB2* was detected to be a core gene of clinical significance in chrcc.

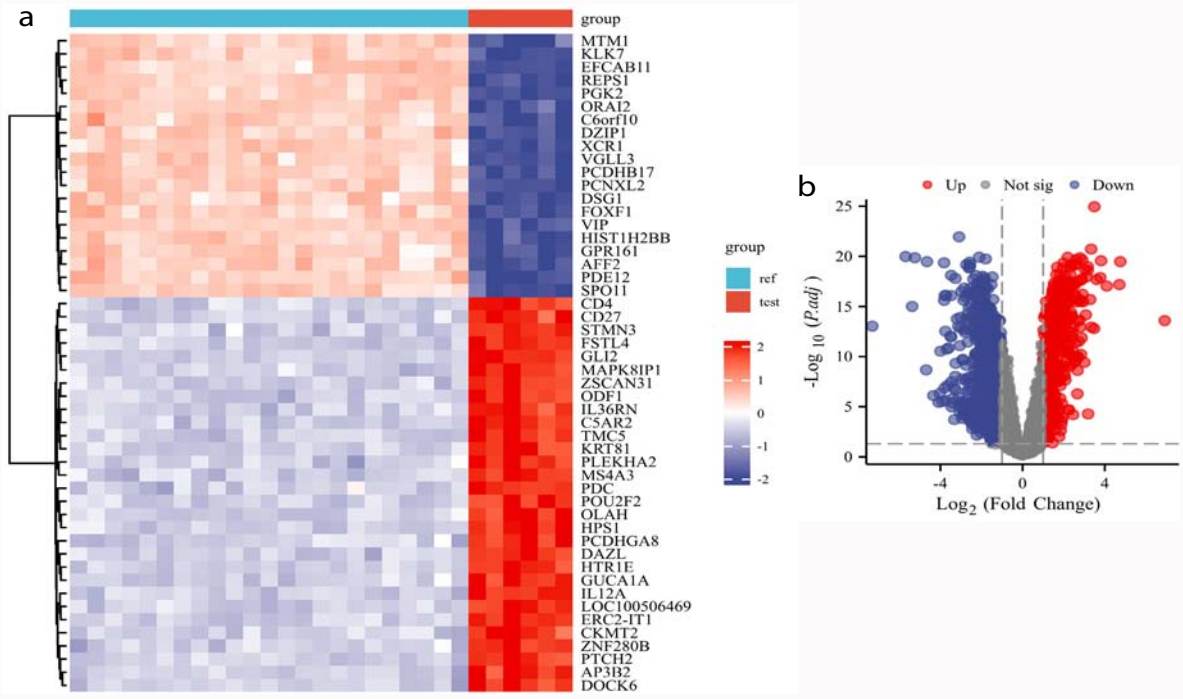


Figure 2a: Heat map for the samples of GSE15641 dataset.
Figure 2b: Volcano map for the samples of GSE15641 dataset.

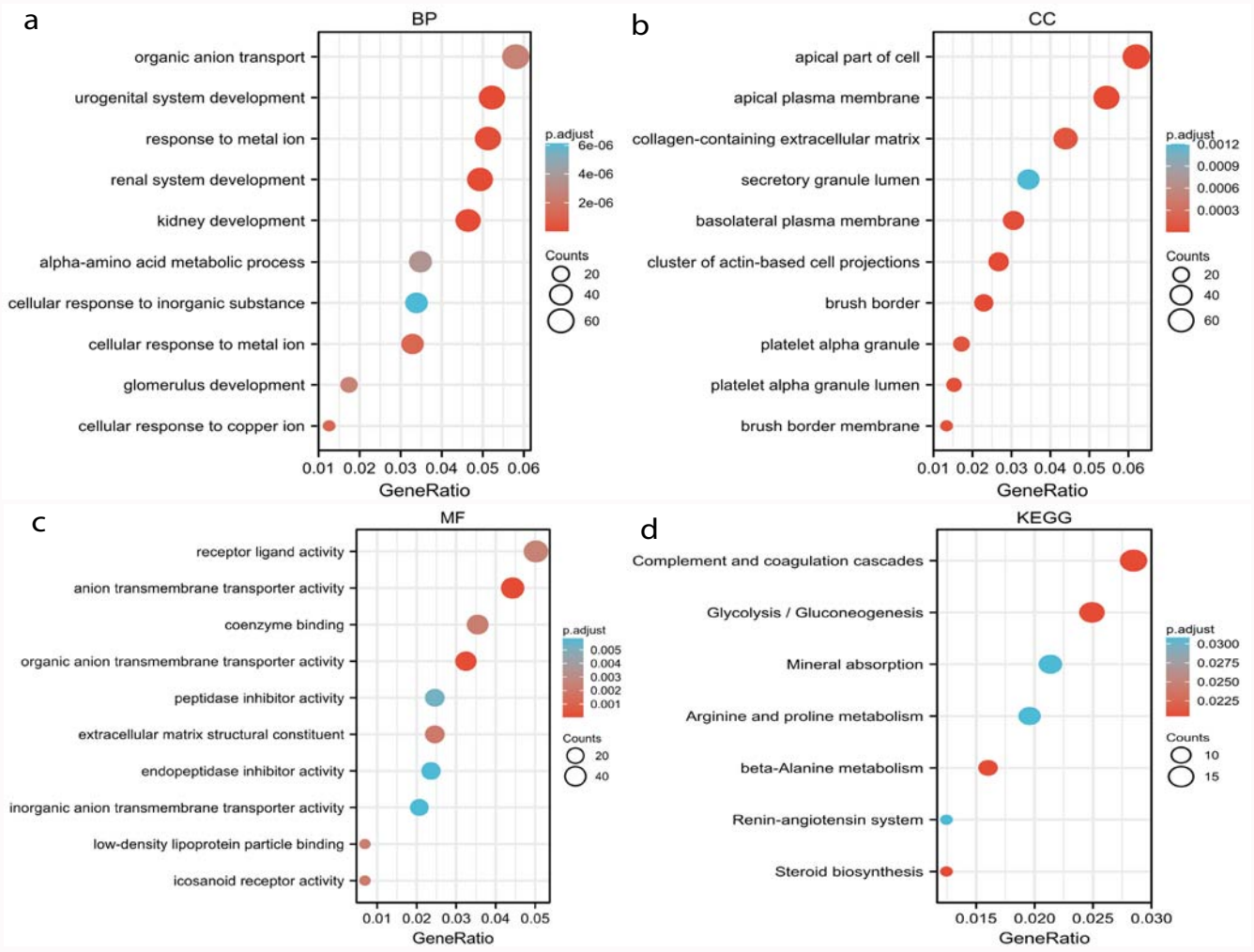


Figure 3a: BP enrichment results of DEGs.
Figure 3b: CC enrichment results of DEGs.
Figure 3c: MF enrichment results of DEGs.
Figure 3d: KEGG enrichment results of DEGs.

Data validation

According to our results, *CCNB2* was notably up-regulated in 65 chrcc tumor tissues in relative to 25 normal tissues (Figure 6A). Besides, it was also highly expressed in 24 paired tumor tissues (Figure 6B). According to statistical analysis, *CCNB2* was significantly associated with OS, PFI, DSS and pathological stage (Figures 6C-6F) (p-value <0.05).

Immune infiltration

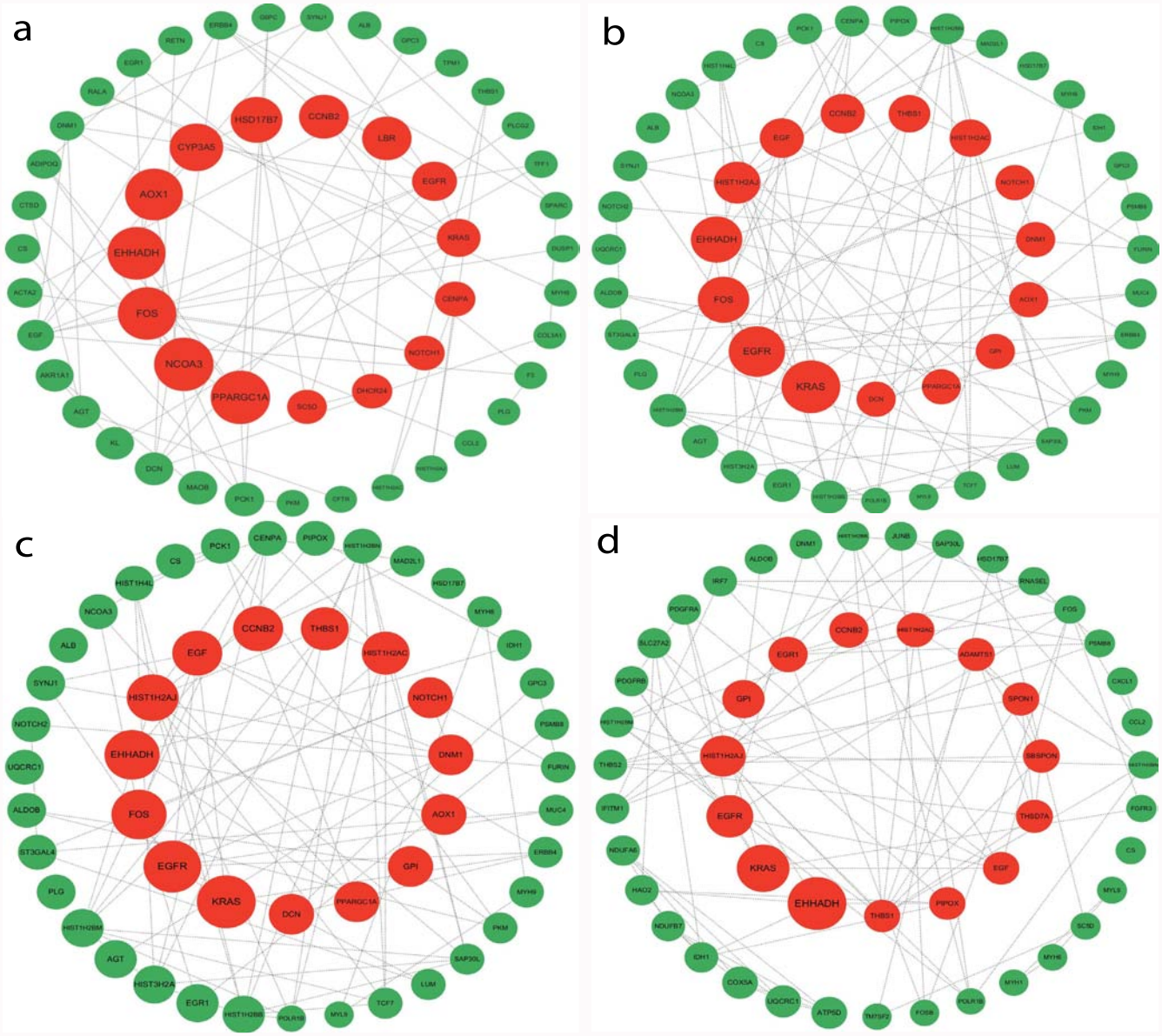
It was discovered that *CCNB2* expression showed positive relationship to the Th2 cell infiltration level, suggesting that there was statistical significance. Additionally, *CCNB2* expression was negatively related to cytotoxic cells, DC, iDC, Tgd, B cells, Th17 cells, and Neutrophils infiltration levels. The color shades of the circles in Figure 7A represent the size of the p-value.

GSEA enrichment

A total of 301 significant pathways were identified, of which, only 2 contained the *CCNB2* gene (Table 2), including liver cancer subclass and breast cancer grade (Figure 7B, 7C). This suggested an association between the *CCNB2* gene in chrcc pathogenesis and hepatocellular carcinoma and breast cancer.

Discussion

In the current study, the GSE15641 dataset was selected and altogether 1153 DEGs were identified. To investigate their interactions, GO enrichment analysis was conducted on the identified DEGs. According to our results, organic anion transport, glycolysis/gluconeogenesis, apical portion of the cell, apical plasma membrane, receptor ligand activity, and anion transmembrane transporter protein activity were significantly enriched. Additionally, KEGG pathway enrichment analysis identified seven significantly enriched pathways, including complement and coagulation cascade, glycolysis/gluconeogenesis, and mineral uptake. PPI networks were established based on DEGs, by using the CytoNCA plug-in, four hub genes, namely, *KRAS*, *EGFR*, *EHHADH*, and *CCNB2*, were obtained according to the Betweenness, Degree, Network, and Information algorithms. Moreover, through KM survival analysis, *CCNB2* was detected to be the only significant hub gene. Further, the expression of *CCNB2* was verified in tumor tissues derived from TCGA database, as a result, the expression of *CCNB2* in tumor tissues was significantly higher than that in normal samples. In addition, this study also discovered that the high expression of *CCNB2* in cancer tissues was closely related to unfavorable clinicopathological



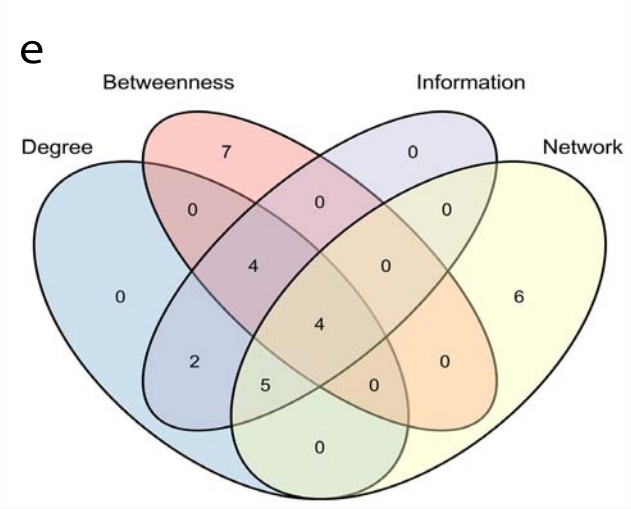


Figure 4a: The top 50 hub genes were screened according to the Betweenness.
Figure 4b: The top 50 hub genes were screened according to the Degree.
Figure 4c: The top 50 hub genes were screened according to the Information.
Figure 4d: The top 50 hub genes were screened according to the Network.
Figure 4e: Interaction according to the Betweenness, Degree, Information and Network.

Table 2: GSEA of the *CCNB2* gene.

ID	Set Size	Enrichment Score	NES	P val ue	Leading edge	Core enrichment
CHIANG_ LIVER_CANCER_ SUBCLASS_ UNANNOTATED_ DN	150	0.454657887	1.702744015	0.00255102	tags=41%, list=20%, signal=33%	MAD2L1/POLE2/MRPS12/ARPC1A/NUDT21/CCNB2/PSMD8/UQCRFS1/ TMEM251/ALDOA/MRPL42/SLC25A5/SLC29A1/NEU1/PSMC3/GLUD2/ SNRPD1/MANF/RAN/CDKN3/UXT/MRPL13/PGK1/STIP1/RNF141/PPP2R3C/ RCN2/ECT2/IMPA2/PSMD10/GTF2I/STT3A/CDK4/VBP1/PSMA2/CLNS1A/ E2F5/RHOA/SYPL1/MCTS1/NDUFA4/TPD52L1/PGD/HSP90AB1/ATP6V0D1/ CAPZA2/TM9SF2/DPM1/APOO/ATG3/PRDX4/WDR61/NUP37/PRKAB1/BAX/ RFC4/CNN3/MAPRE1/FEN1/SNRPD2/TPX2
SOTIRIOU_ BREAST_ CANCER_ GRADE_1_VS_3_ UP	142	0.462162007	1.724342232	0.00257732	tags=36%, list=20%, signal=29%	RACGAP1/MAD2L1/GTSE1/CEP55/STIL/CENPA/E2F1/CKS2/HMGB3/ UBE2S/HCCS/CCNB2/MRPL15/XPOT/POLR2K/CMC2/COX7B/PSMA7/ TUBA1C/CDCA8/CDKN3/ORMDL2/GMPS/DSN1/MRPL12/MRPS17/AURKA/ STIP1/ECT2/TIMM10/TOP2A/SLC52A2/TUBA1B/APOBEC3B/PRC1/DNAJC9/ NUDT1/NME1/CCNB1/MIS18A/MELK/NUP93/LAGE3/AURKB/SNRPF/E2F8/ CDC20/RFC4/FEN1/TPX2/VRK1

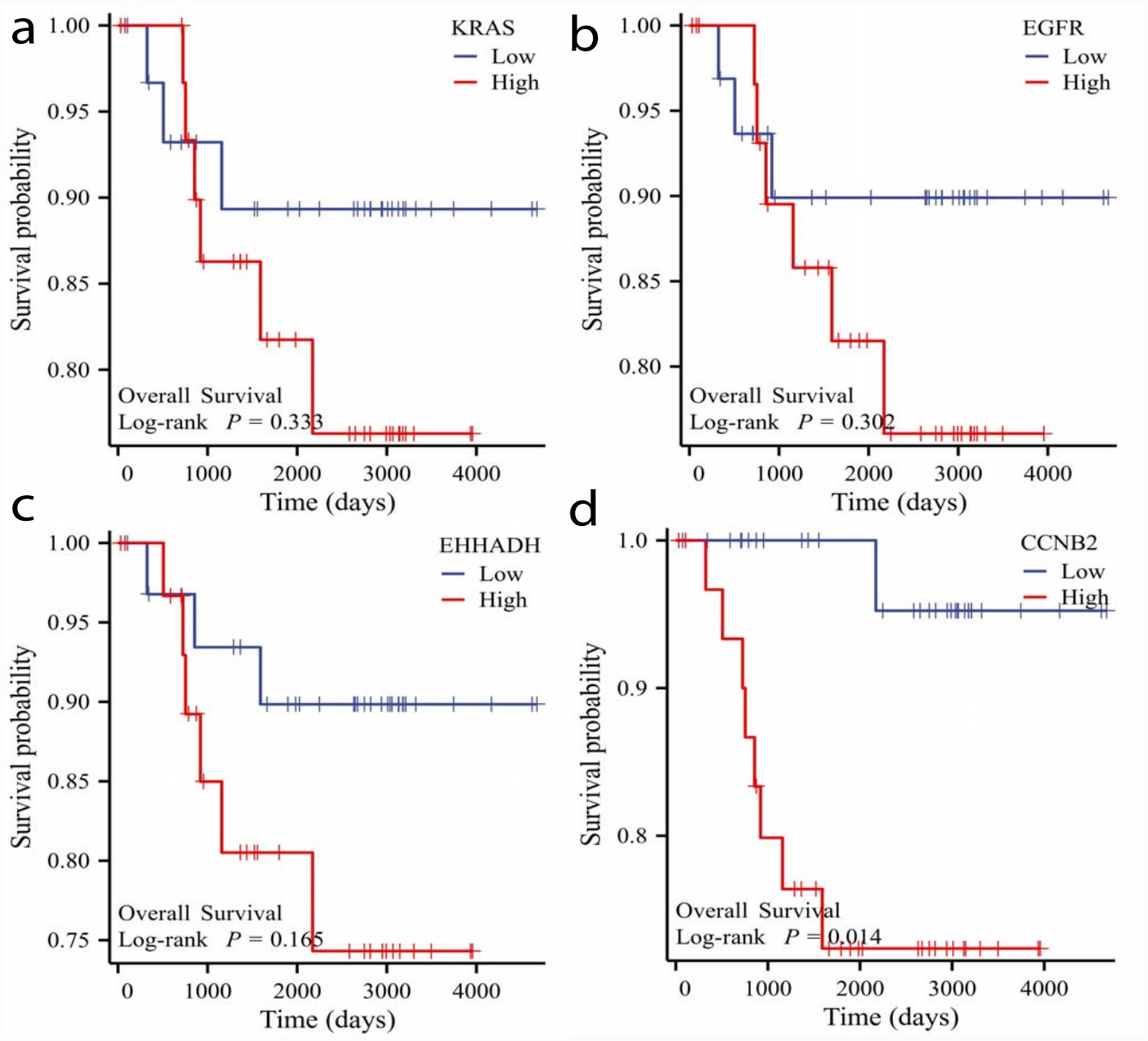


Figure 5a: Survival analysis curve of the KRAS.
Figure 5b: Survival analysis curve of the EGFR.
Figure 5c: Survival analysis curve of the EHHADH.
Figure 5d: Survival analysis curve of the *CCNB2*.

factors, which indicated that *CCNB2* might play an important role in tumor progression. Therefore, in line with the above study, it can be reasonably speculated that *CCNB2* is an effective biomarker for chrcc.

Cell cycle proteins, including cell cycle proteins A, B, C, D, and E, can not only bind to Cyclin-Dependent Kinase (CDK) to form a complex in the G1-S phase of mitosis, but also activate the corresponding protein kinase, thereby controlling the orderly growth and division of cells. According to previous studies, *CDK1*/*CCNB* form a complex that jointly controls the G2/M phase transition during the cell cycle, and *CCNB2* regulates the G2/M phase in the cell cycle, thus inhibiting apoptosis of nasopharyngeal carcinoma cells [5,6]. Consequently, it is speculated that the expression level of CCNB affects cell proliferation and division, and when cell division is not controlled autonomously and cells divide indefinitely, they tend to transform into cancer cells. In this view, the *CCNB* gene family is considered to make a vital role in the progression of tumors. In addition, Thomas K Hoffmann argued that *CCNB1* overexpression in head and neck squamous cell carcinoma showed significant relationship to high tumor grade [7]. Further, *CCNB2* is highly expressed in various tumors containing lung, breast, ovarian, and liver cancers [8-11], which fully suggests the possibility that *CCNB2* is an oncogene. Based on our results in this study, we can tentatively predict that *CCNB2* is a biomarker for the poor prognosis of chrcc, but its mechanism of action in chrcc still remains unknown, which should be explored in more experiments using a large number of specimens.

Tumor Microenvironment (TME) refers to the tumorigenesis, growth, metastasis, as well as internal and external environment in which tumor cells live, including not only tumor cells but also inflammatory and immune cells closely related to tumor cells. As we know, tumor development depends on the disturbance of TME, which eventually impairs the metabolic function of immune cells, thus increasing the risk of distant metastasis [12]. Tumor cells and TME will constantly interact with each other during the development process, and TME affects tumorigenesis and development through the tumor infiltration of immune cells [13,14]. TME has long been a hot spot in the field of tumor research, which has important implications for tumor understanding and treatment. For example, the high expression levels of *CCNB1* and *CCNB2* can affect the immune activity of TME in hepatocellular carcinoma, which are associated with poor prognosis [15]. According to our results, the increased *CCNB2* expression was positively correlated with the higher Th2 cells concentration. Moreover, some studies show that IL-4 binding to receptors can lead to TH2 cytokine secretion, which ultimately induces tumor growth and metastasis [16,17]; while others suggest that the presence of TH2 cells predicts the poor prognosis [18,19]. Besides, the increased expression of *CCNB2* was negatively related to Cytotoxic cells and DC cells. Cytotoxic CD4 T cells and DC cells, the key cells in the immune system, also constantly monitor the progression of tumor cells, where CD4 T cells produce IFN-γ in

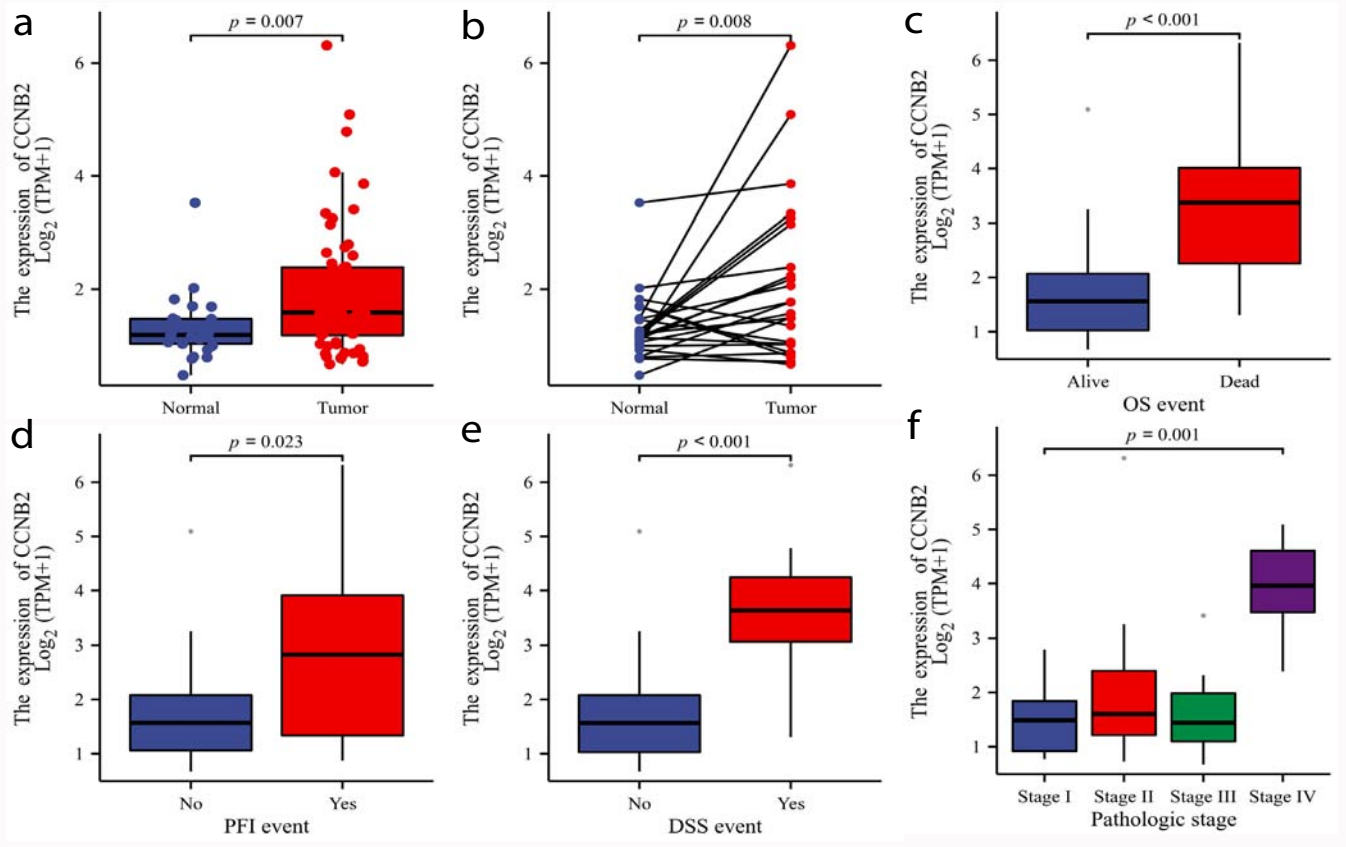


Figure 6a: *CCNB2* expression levels in chrcc and normal tissues.
Figure 6b: *CCNB2* expression levels in chrcc patients and matched normal tissues.
Figure 6c: Association of *CCNB2* expression with OS.
Figure 6d: Association of *CCNB2* expression with PFI.
Figure 6e: Association of *CCNB2* expression with DSS.
Figure 6f: Association of *CCNB2* expression with pathological stage.

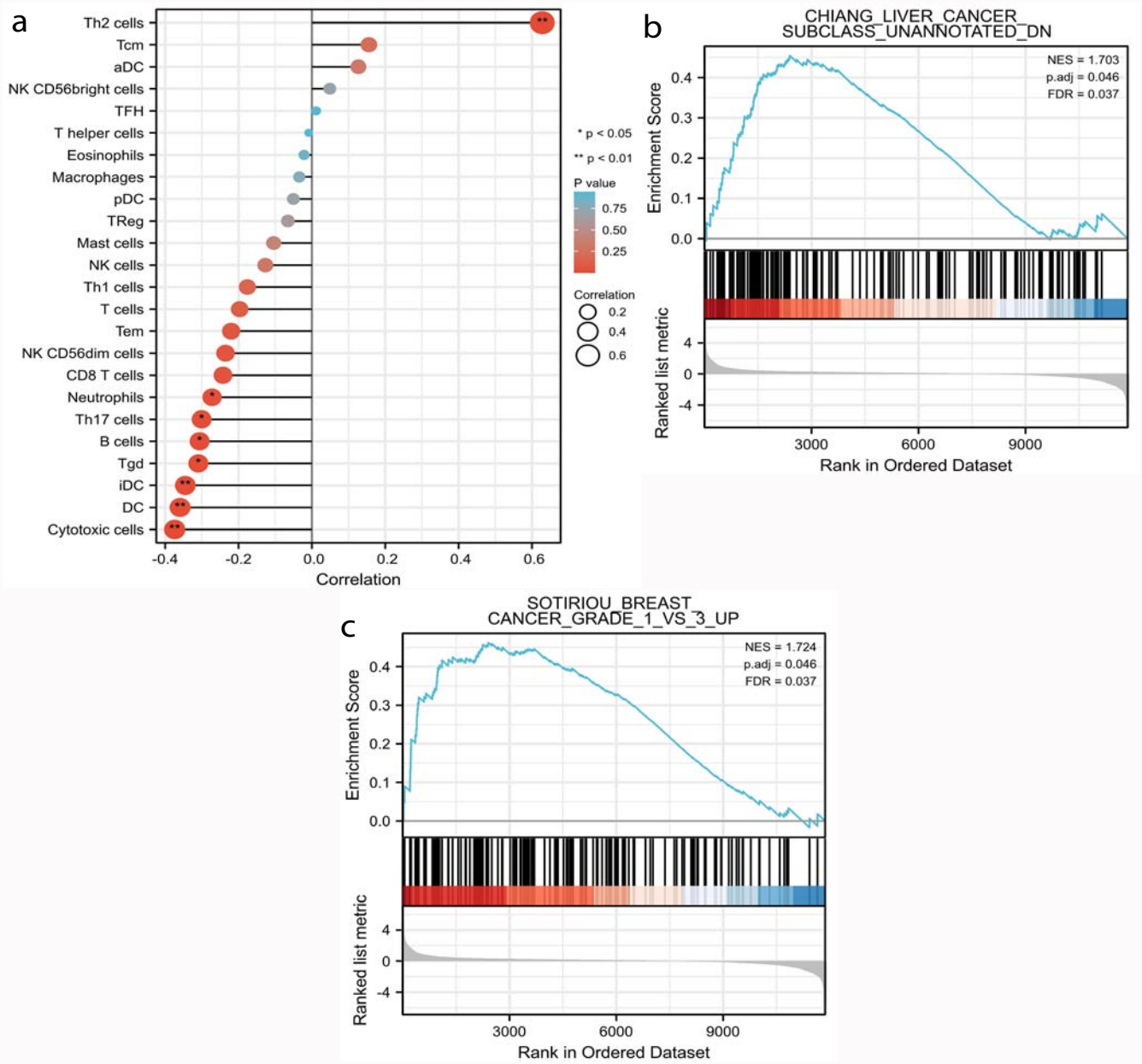


Figure 7a: Relationship between the *CCNB2* gene and infiltration levels of 24 kinds of immune cells.
Figure 7b: GSEA of the *CCNB2* gene.
Figure 7c: GSEA of the *CCNB2* gene.

the form of GZMB and PRF1 to directly kill tumor cells [20]. On the other hand, cancer therapies such as chemotherapy, radiotherapy, small molecule inhibitors, immunotherapy and intestinal flora function depending on DC cells [21]. Consequently, we hypothesize that the *CCNB2* gene affects the prognosis of chrcc by affecting the immune microenvironment of tumors.

Based on GSEA results, two pathways involving the *CCNB2* gene were obtained, namely, liver cancer subclass and breast cancer grade. Prior to this, it is shown that the *CCNB2* gene may promote the proliferation and metastasis of hepatocellular carcinoma by increasing JAG1 expression [22], and it is also shown to be closely correlated with poor prognosis of hepatocellular carcinoma and breast cancer, which to some extent suggests that *CCNB2* is related to the pathogenesis of liver cancer and breast cancer. Through this study, it is found that the high *CCNB2* gene expression predicts a poor clinical outcome of chrcc patients, but there is no study revealing their correlation and the mechanism of action. In short, a large number of experimental studies are still warranted to demonstrate the reliability of our results.

Conclusion

In patients with chrcc, the high expression of *CCNB2* may influence the poor prognosis through regulating the immune microenvironment and the expression levels of immune cells. Meanwhile, there is an association of *CCNB2* with hepatocellular carcinoma and breast cancer in terms of pathogenesis. Therefore, *CCNB2* can be considered as a predictive molecular marker and a potential therapeutic target for chrcc.

Funding

This work was supported by the Research Program of Natural Science Foundation in Chongqing (cstc2021jcyj-msxmX0484), National Natural Science Foundation of China (No.81801507) and Kuanren Talent Program of Second Affiliated Hospital of Chongqing Medical University (KY2019Y004).

Author Contribution

Yiteng Xie, Yuanfeng Zhang, Ronggui Zhang designed the study. Yiteng Xie, Junwu Li prepared the draft. Yiteng Xie, Yang Yuan prepared pictures and tables. All authors participated in the writing and revision of the manuscript and read the final manuscript.

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