ORIGINAL ARTICLE

Identification of Prognostic Related Hub Genes in Clear-cell Renal Cell Carcinoma via Bioinformatical Analysis

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Key words: clear-cell renal cell carcinoma; bioinformatical analysis; differentially expressed genes

Objective To identify new genes that correlate with prognosis of clear-cell renal cell carcinoma (ccRCC) via bioinformatics analysis.

Methods The gene expression profiles of 62 ccRCC and 54 normal kidney tissues were available from the Gene Expression Omnibus database: GSE12606, GSE36895 and GSE66272. The differentially expressed genes were screened with GEO2R and J Venn online tools. Functional annotation including Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) was applied to identify the possible function of the hub genes involved in prognosis of ccRCC. In protein protein interaction network (PPI network), the STRING online tool was used to visualize the network of the differentially expressed genes, and the core gene was selected by MCODE App in Cytoscape software. Finally, GEPIA Survival Plot was performed to assess genes

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associated with worse survival.

**Results** We totally found 648 differentially expressed genes, including 222 up-regulated genes and 426 down-regulated genes. PPI network showed that in 28 up-regulated genes 7 (CCNE2, CDK1, CDC6, CCNB2, BUB1, TTK and PTTG1) enriched in cell cycle and 4 (CCNE2, CDK1, CCNB2 and RRM2) enriched in p53 signaling pathway. GEPIA Survival Plot assay revealed that ccRCC patients carrying CDK1, CCNB2, RRM2, BUB1, PTTG1 had a worse survival. GEPIA Box Plot showed that BUB1, CCNB2, PTTG1, RRM2 were over expressed in the ccRCC tissues in contrast to the normal tissues (P<0.05).

**Conclusion** In ccRCC patients with the four up-regulated differentially expressed genes including BUB1, CCNB2, PTTG1, RRM2 might manifest a poor prognosis.

Clear-cell renal cell carcinoma (ccRCC) is one of the most common malignant lesion in renal cell carcinoma,[1, 2] with an estimation of 65 000 new cases per year in the USA.[3] Even ccRCC patients have improved survival rates through surgical techniques and specific targeted therapy, the predictability of outcome is still poor.[4] Therefore, to ameliorate the cure rate of ccRCC, more reliable and favorable prognostic factors should be sought as targets.

In this research, we selected the data of GSE12606, GSE36895 and GSE66272 from Gene Expression Omnibus (GEO) datasets to mine differentially expressed genes (DEGs) related to ccRCC prognosis from the whole expression profiling of transcriptome sequencing gene in ccRCC patients and normal individuals using bioinformatical analysis,[5, 6] to provide useful biomarkers related to survival and some effective target for ccRCC treatment.

**MATERIALS AND METHODS**

**Data downloading and DEG analysis**
Three datasets including GSE12606, GSE36895 and GSE66272 were collected from the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) and generated using
GPL570 Platforms. GSE12606 includes 6 ccRCC tumor tissues and 4 normal tissues; GSE36895 includes 29 ccRCC tumor tissues and 23 normal renal cortices, GSE66272 contains 27 primary ccRCC tumor tissues and 27 adjacent normal kidney sample. DEGs between the cancer and non-cancer tissues from the data of GSE12606, GSE36895 and GSE66272 were screened via GEO2R online tools (|logFC|>1.5 and adjust P value < 0.05), and then were imported to the J Venn online tools (http://jvenn.toulouse.inra.fr/app/example.html) to obtain commonly DEGs (c-DEGs) from the three database. DAVID (https://david.ncifcrf.gov) was used to detect the association of c-DEGs gene with biological annotation terms (Gene Ontology, GO and Kyoto Encyclopedia of Genes and Genomes, KEGG pathway). When P-value and false discovery rate (FDR) value are all less than 0.05 unless otherwise specified, it is considered as having statistical significance. The potential correlation was examined by STRING website (https://string-db.org) between these c-DEGs (maximum number of interactors=0 and confidence score≥0.4) to obtain the tsv format which was imported to Cytoscape software to analyze the current network. Then the modules of the protein protein interaction network (PPI network) were checked in the MCODE app (degree cutoff=2, max. Depth=100, k-core=2, and node score cutoff=0.2) to filter the core genes and perform re-KEGG pathway analysis. After that, the survival rate of patients having core genes was assessed by GEPIA Survival Plot and the hub genes expression was assayed by GEPIA Box Plot (http://gepia.cancer-pku.cn/).

RESULTS

Identification of c-DEGs in ccRCC
We extracted 2032, 1135 and 2826 DEGs from GSE12606, GSE36895 and GSE66272 respectively via GEO2R tools. And then we applied J Venn online tool to screen the DEGs in the three database mentioned above and found 648 c-DEGs including 222 up-regulated genes and 426 down-regulated genes in ccRCC tissues (Figure 1).
Figure 1. The Venn diagram of 648 commonly differentially expressed genes (c-DEGs) in the three datasets.
A. 222 up-regulated c-DEGs (logFC>1.5); B. 426 down-regulated c-DEGs (logFC<-1.5).

Results of GO and KEGG pathway analysis

As shown in Figure 2, GO analysis results indicated that ① for biological processes, up-regulated c-DEGs were particularly enriched in response to hypoxia, adaptive immune response, and down-regulated c-DEGs in excretion; ② for cell components, up-regulated c-DEGs were significantly enriched in membrane, extracellular space, extracellular region and cell surface, and down-regulated c-DEGs in extracellular exosome, apical plasma membrane, basolateral plasma membrane, integral component of plasma membrane and integral component of membrane; ③ for molecular function, up-regulated c-DEGs were enriched in protein binding and down-regulated in anion antiporter activity.

KEGG analysis demonstrated that up-regulated c-DEGs were particularly enriched in hypoxia inducible factor-1 (HIF-1) signaling pathway and phagosome, and down-regulated were significantly enriched in metabolic pathways (Figure 3).
Figure 2. GO analysis showing the biological processes, cell components, and molecular function of clear-cell renal cell carcinoma (ccRCC) that c-DEGs may take part in.

Figure 3. KEGG pathway analysis revealing the function of c-DEGs which can perform in ccRCC tissues.

The Function DEGs may fulfill in ccRCC
We used STRING website and Cytoscape software to structure a PPI network complex which composed of 549 nodes and 2571 edges, but the PPI network of c-DEGs had only 99 nodes. Next, we applied Cytotype MCODE to identify the core nodes of the c-DEGs PPI network and found 28 core genes (ANLN, ASPM, BIRC5, BUB1, CCNB2, CCNE2, CDC6, CDCA2, CDCA7, CDK1, CENPK, CENPU, CEP55, DLGAP5, DTL, KIAA0101, KIF14, KIF20A, NUF2, NUSAP1, PRC1, PTTG1, RAD51AP1, RRM2, TOP2A, TPX2, TTK, UHRF1) consisting of 28 nodes and 361 edges (Figure 4). At last, KEGG pathway
enrichment re-analysis showed that 28 nodes were enriched in cell cycle (CCNE2, CDK1, CDC6, CCNB2, BUB1, TTK and PTTG1) and p53 signaling pathway (CCNE2, CDK1, CCNB2 and RRM2), as shown in Figure 5.

Figure 4. PPI network of c-DEGs structured by STRING and Module analysis. The module consisted of 28 nodes and 361 edges. Purple represents up-regulated genes and green indicates down-regulated genes.
Figure 5. Re-analysis of 14 highly expressed genes by KEGG pathway enrichment.
A. Seven genes (CCNE2, CDK1, CDC6, CCNB2, BUB1, TTK and PTTG1) enriched in cell cycle. B. Four genes (CCNE2, CDK1, CCNB2 and RRM2) enriched in p53 signaling pathway.

DEGs involved in prognosis
We exploited GEPIA Survival Plot to determine poor prognosis related genes among eight core genes enriched by re-KEGG pathway enrichment and discovered and it included CDK1, CCNB2, RRM2, BUB1, PTTG1 were significantly related to worse survival and CCNE2, CDC6, TTK were not associated with poor survival (Figure 6). Furthermore, GEPIA Box Plot was used to perform expression analysis of the genes that may have an effect on survival of ccRCC. and then the significant worse survival genes were proceeded expression analysis on box plots of GEPIA and the results showed that BUB1, CCNB2, PTTG1, RRM2 were over expressed in the ccRCC tissues in contrast to the normal tissues (P<0.05, Figure 7).
Figure 6. The genes that participate in worse survival of ccRCC.
DISCUSSION

The ccRCC is a malignant tumor that originates from the urinary tubular epithelial system of renal parenchyma, and it accounts for 60%-85% of the renal cell carcinoma.\[^{7, 8}\] Because of accumulating glycogen and lipid in position of ccRCC cells, it is not sensitive to radio- or chemo-therapy.\[^{9}\] Fortunately, molecular targeted therapy for ccRCC has made certain progress, and 20%-40% ccRCC patients have been effectively treated. However, molecular targeted therapy for high-risk ccRCC patients is not effective, and the long-term survival rate is not satisfactory.\[^{10, 11}\] Therefore, we decided to compare the expression pattern of ccRCC tissues and normal tissues through data mining to investigate the commonly differentially expressed and prognostic genes in order to reveal the underlying mechanisms.

In this study, we utilized bioinformatic analysis to analyze the selected datasets GSE12606, GSE36895 and GSE66272 and identified four up-regulated gene such as \textit{CCNB2}, \textit{RRM2}, \textit{BUB1}, \textit{PTTG1} in ccRCC tissues that correlated with worse survival of ccRCC.
**BUB1** (Budding uninhibited by benzimidazoles-1) gene encodes an mitotic checkpoint serine/threonine kinase which is considered as a sensitive and specific biomarker for gastric cancer, breast cancer, colorectal cancer etc.[12-15] KEGG pathway re-analysis results showed that BUB1 was involved in cell cycle pathway compactly related to ccRCC pathogenesis. In addition, BUB1 as a key mediator of TGF-β signaling and interacted with TGFBR1 in the presence of TGF-β and promoted the heterodimerization of TGFBR1 and TGFBRII, and open the growth of invasive cancer cells.[16, 17] In subsequent experiments, it was found that in various normal and cancers, small molecule inhibitors of BUB1 kinase activity (2OH-BNPP1) and kinase-deficient mutants of BUB1 inhibited TGF-β and ternary complexes form. The cell line and administration of 2OH-BNPP1 to mice bearing lung carcinoma xenografts reduced the amount of phosphorylated SMAD2 in tumor tissue.[17] These studies suggest that BUB1 can regulate the diagnosis, prognosis or therapy of cancer cells by regulating TGF-β signaling. Combining our survival analysis, we infer that **BUB1** may be an indicator in the diagnosis and prognosis for ccRCC patients.

**CCNB2** (cyclin B2) which is a member of cyclin family proteins, regulates the activities of cyclin dependent kinases (CDKs). Different cyclins function spatially and temporally in specific phases of the cell cycle[18, 19] and its expression was increased in a variety of human cancers, such as colorectal adenocarcinoma, gastric cancer etc.[20, 21] Mo et al.[22] found that serum circulating **CCNB2** mRNA expression was increased in cancer patients and associated with cancer stage and metastasis status. After therapeutic treatment, its expression level was significantly decreased compared with before treatment. In order to explore the relationship between **CCNB2** and renal cell cancer, Yamamura et al.[23] discovered that the **CCNB2** gene was significantly increased in stably expressed sFRP2 renal cell lines compared with A498 renal cancer cell lines. Although there is no more evidence that **CCNB2** is closely linked to ccRCC, the cyclin family proteins play an important role in the diagnosis, prognosis and treatment of ccRCC patients and associate with cell cycle.[24, 25] And the molecular mechanism of **CCNB2** in ccRCC awaits further investigation.
PTTG1 (pituitary tumor-transforming gene-1) is a recently identified oncogene involved in the progression of malignant tumors, and it was first isolated from rat pituitary tumor cells in 1997.\textsuperscript{[26, 27]} In contrast to its restricted expression in normal tissues, PTTG1 is largely examined in various cancers and is adjoined with metastasis and poor clinical outcome.\textsuperscript{[28, 29]} Wei et al.\textsuperscript{[26]} demonstrated that PTTG1 mRNA and protein levels were significantly higher in ccRCC than normal tissues and its expression was significantly correlated with T stage, N classification, metastasis, recurrence and Fuhrman grade, which suggest that the PTTG1 over expression indicates a poor prognosis in ccRCC patients. And the other target gene is RRM2 (ribonucleotide reductase subunit M2) which encodes an essential protein for DNA synthesis and is responsible for the reduction of ribonucleotides to deoxyribonucleotides, providing a balanced supply of precursors for DNA synthesis and repair.\textsuperscript{[30]} Morikawa et al.\textsuperscript{[31]} found that the RRM2 siRNA inhibited the cell proliferation, migration and invasion and cell apoptosis was increased in primary ccRCC cell lines, indicating that RRM2 inhibitor may be used as an important potential target drug in the treatment of ccRCC.

In conclusion, we have identified several significant prognostic factors in ccRCC patients using integrated bioinformatic analysis. We have identified commonly changed 648 c-DEGs candidate genes, and finally found 4 mostly changed genes (BUB1, CCNB2, PTTG1, RRM2), which were significant enriched in cell cycle and p53 signaling pathway. These findings could provide useful biomarkers related to survival and some effective target for ccRCC treatment.

\textit{Conflict of Interests Statement}

\textit{The authors declare no conflict of interests.}

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