Identification of 8 Feature Genes Related to Clear Cell Renal Cell Carcinoma Progression Based on Co-Expression Analysis

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Keywords

Clear cell renal cell carcinoma · Weighted gene correlation network analysis · Biomarker · Prognosis

Abstract

Background: The aim of the study was to screen biomarkers related to clear cell renal cell carcinoma (ccRCC) progression and prognosis. **Methods:** 1,026 ccRCC-related genes were dug from 494 ccRCC samples in TCGA based on weighted gene co-expression network analysis, and 7 modules were identified. Afterward, Gene Ontology and Kyoto Encyclopedia of Genes and Genomes enrichment analyses were conducted on modules of interest. Genes in these modules were taken as the input to construct a protein-protein interaction network. Thereafter, 30 genes with the highest connectivity were taken as core genes. Univariate Cox regression, LASSO Cox regression, and multivariate Cox regression analyses were performed on core genes. Univariate and multivariate Cox regression analyses were performed on patients' clinical characteristics and risk scores. **Results:** Stage displayed significantly strong correlations with green module and red module ($p < 0.001$). Genes in modules participated in biological functions including T-cell proliferation and regula-

tion of lymphocyte activation. GSEA showed that high- and low-risk groups exhibited significant enrichment differences in pathways related to immunity, cell migration, and invasion. Immune infiltration analysis also presented a strong correlation between the expression of these 8 genes and immune cell infiltration in ccRCC samples. It was displayed that risk score could be an independent factor to assess patients' prognosis. **Conclusion:** We determined biomarkers relevant to ccRCC progression, offering candidate targets for ccRCC treatment.

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Published by S. Karger AG, Basel

Introduction

Clear cell renal cell carcinoma (ccRCC) is the most prevalent renal cell carcinoma, featuring abnormal changes of cell metabolism [1]. Cancer-driven abnormal metabolism alters fatty acid, including the alteration in ccRCC to excessive lipid storage [2]. Due to abundant lipids and glycogen deposits, ccRCC cells show as malignant epithelial cells with clear cytoplasm. In addition, allele gene deletion, gene mutation, or promoter hypermethylation is typical molecular alteration of ccRCC [3,

4]. All these complex pathogeneses lead to a huge challenge for ccRCC diagnosis and treatment.

So far, surgical resection has been the major treatment method for ccRCC, but this method is only suitable for patients in the early stage while not for those in the advanced stage or who developed tumor metastasis. Standard treatment method for ccRCC metastatic patients is a systemic therapy, with a small part of them can receive metastasectomy after rigorous screening [5]. In the past 15 years, the major treatment method for advanced ccRCC has been based on cytokines. However, this method has been gradually abandoned by doctors and patients due to adverse toxicity and low response rate [6]. In recent years, cancer treatment has been developed toward various newer and more effective targeted and immune treatments. Nevertheless, it is indispensable to accurately control genes related to ccRCC progression to find an effective targeted treatment.

With the popularization and gradual development of gene chips and high-throughput sequencing technology, methods based on big data integration and bioinformatics potentiate identifying genes relevant to tumor progression and prognosis. Weighted gene co-expression network analysis (WGCNA) is a systemic biological method for identifying highly correlated gene sets [7]. WGCNA can be applied to screen candidate biomarkers or therapeutic targets according to internal features of gene sets and correlation between gene sets and phenotypes [7]. Zhai et al. [8] obtained molecular index and prognostic index relevant to recurrence of colon carcinoma via WGCNA (SERP2, EFEMP2, FBN1, SPARC, and LINC0219). Ding et al. [9] searched lung cancer-related key pathways through pathway enrichment analysis and WGCNA based on transcripts. They found that glucose metabolism especially glycolytic pathway in lung cancer is interfered. That is to say, glucose metabolism disorder may be associated with canceration of lung cancer and a potential therapeutic target of the disease [9]. Liu et al. [10] used WGCNA to analyze genes associated with breast cancer in Gene Expression Omnibus (GEO) database and identified 5 modules related to breast cancer. lncRNAs identified in these modules were screened by univariate Cox regression, LASSO regression, and multivariate regression analyses, to obtain lncRNAs with prognostic value [10]. Therefore, it is of great significance to screen cancer-related biomarkers via WGCNA for relevant research of cancer progression and prognosis.

In this study, relevant data in TCGA, GEO, and National Center for Biotechnology Information (NCBI) databases were dug given the decisive impact of sample size

and tissue origin on the accuracy of model. A model that could be generally applied in early diagnosis and prognosis of ccRCC was established. Hence, researching the correlation between changes of transcript overview and carcinogenic risk score of ccRCC is conducive to clarifying underlying molecular mechanism of ccRCC development and progression.

Materials and Methods

Data Download and Differential Analysis

Original data were downloaded from GEO database (<https://www.ncbi.nlm.nih.gov/geo/>). Differential expression analysis was performed on 4 groups of chip data (GSE15641, GSE40435, GSE46699, and GSE53757) by using R package “limma” in the normal group and tumor group. RANK analysis ($|\log FC| > 1$, FDR < 0.05) was conducted on the result of differential expression analysis by using R package “RobustRankAggreg” to acquire significantly differentially expressed genes in 4 groups of chips. ccRCC-related 622 genes were accessed from NCBI (<https://www.ncbi.nlm.nih.gov/>) database (online suppl. Table 1; for all online suppl. material, see www.karger.com/doi/10.1159/000520832). The 622 genes were intersected with the obtained differential genes from multichip conjoint analysis, and 1,026 mRNAs were obtained. Expression data of mRNAs and relevant clinical information of ccRCC were downloaded from TCGA database (<https://portal.gdc.cancer.gov/>). Samples with incomplete clinical information were deleted, and the remained samples were used for the following WGCNA (online suppl. Table 2). Moreover, gene expression data and clinical data of ccRCC in GSE29609 were downloaded from GEO database as the test set (online suppl. Table 3). Detailed information is shown in Table 1.

Construction of WGCNA and Identification of Important Clinical Modules

“WGCNA” package was used to construct weighted gene co-expression network in R language environment. First, 7 outlier samples were removed by establishing a sample clustering tree. Afterward, Pearson correlation analysis was used to calculate the correlation between all genes, and similar matrix was established. Next, Pearson similar matrix was transformed into an adjacency matrix (scale-free network) to calculate topological overlap matrix. In this way, indirect correlation was considered, and noise and pseudo-correlation were reduced. Thereafter, modules were stratified and clustered by dissimilarity degree (1-topological overlap matrix). Highly similar gene modules were merged (the minimum module was set to contain 30 genes; module dissimilarity < 0.25 was taken as the threshold value). To determine modules with clinical significance, the correlation between module feature vector and clinical features was calculated. Finally, modules of interest were screened for the following analysis.

Functional Annotation and Pathway Enrichment Analyses

Gene ontology (GO) functional annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were performed on intra-modular genes by using R package “clusterProfiler.” Screening criteria of significant enrichment were p value < 0.05 and q value < 0.05 .

Fig. 1. Screening results of ccRCC-related genes. **a** Heat map of differential genes. **b** Venn diagram of intersection of genes related to ccRCC from GEO and NCBI databases.

Table 1. Downloaded results of data

Accession	Samples, <i>n</i>			
	platform	total	normal	tumor
Study				
GSE15641	GPL96	55	23	32
GSE40435	GPL10558	202	101	101
GSE46699	GPL570	130	65	65
GSE53757	GPL570	144	72	72
Train set				
TCGA-KIRC	Illumina	611	72	539
Test set				
GSE29609	GPL1708	39	–	39

Identification of Core Genes and Establishment of the Prognostic Model

STTRING (<http://string-db.org/cgi/input.pl>) database was used to analyze protein-protein interactions (PPIs) of mRNA-encoding proteins. Top 30 genes in connectivity were defined as core genes. Univariate regression analysis ($p < 0.05$) was conducted on the obtained core genes by using “survival” package to acquire genes significantly associated with patients’ prognosis. Afterward, R package “glmnet” was applied to conduct LASSO regression analysis on the obtained genes that were significantly related to prognosis, so as to further choose genes. A prognostic model with genes selected by LASSO was established through multivariate Cox regression analysis. Each patient’s risk score was calculated by mRNA expression and risk coefficient of each gene. Risk score = $\sum_{i=1}^n (\text{Coef}_i \times X_i)$ (Coef_{*i*}: risk coefficient, *X_i*: gene expression level

after standardized by z-score). Risk score was used for predicting patients’ prognosis. Median risk score was the critical value to divide patients into high-risk and low-risk groups. Survival analysis was performed in high-risk and low-risk groups by using R package “survival.” Receiver-operating characteristic (ROC) curves were drawn, and area under the curve (AUC) values of 3-year and 5-year overall survival (OS) were calculated to detect the predictive accuracy of the model. The prognostic model was validated with the obtained test set GSE29606 from GEO database.

Gene Set Enrichment Analysis

In gene set enrichment analysis (GSEA), samples were divided into high-risk and low-risk groups according to the median value of risk score of samples. GSEA software was used, and KEGG gene set was taken as the reference. According to the default weighted

Fig. 2. Establishment of weighted gene co-expression network and interaction between the obtained key modules. **a** Analysis of scale-free fitting coefficient of different soft threshold powers (β). **b** Analysis of average connectivity of each soft threshold power. **c** Histogram of connectivity distribution when $\beta = 5$. **d** Checking scale-free

topology network when $\beta = 5$. **e** Gene dendrogram was obtained by clustering differences based on same topology overlap and corresponding modules. Each colored row represents a color-coded module that includes a group of highly connected genes. **f** Heat map of the correlation between modules and clinical features.

enrichment statistic method, the number of permutations was set to repeat 1,000 times. Significantly enriched biological pathways were screened with FDR < 0.25 as the threshold value.

Correlation Analysis between Feature Genes and Immune Cell Infiltration

TIMER is a database (<https://cistrome.shinyapps.io/timer/>) specially designed for analysis of immune cell infiltration in various cancers. Feature genes were taken as the input to analyze the correlation between their expression in ccRCC and the infiltration degree of B cells, CD8+ T cells, CD4+ T cells, macrophage cells, neutrophil cells, and dendritic cells.

Survival Analysis of the Prognostic Model

Univariate and multivariate analyses were conducted combining patient's risk score and clinical data to evaluate the independence of the prognostic model. Nomogram was generated by using

R package "rms" combining clinical data and risk score to predict the 3-year and 5-year OS possibility. R package "foreign" was applied to generate the calibration curves for 3 years and 5 years to validate the predictive efficiency of the nomogram.

Results

Screening of Differentially Expressed Genes in ccRCC

To visualize the differentially expressed genes in ccRCC, a heat map was drawn with all differential genes (Fig. 1a). ccRCC-related 622 genes were downloaded from NCBI and then intersected with 477 differential genes obtained from GEO. Last, a total of 1,026 mRNAs were acquired for the subsequent analysis (Fig. 1b).

Fig. 3. GO and KEGG enrichment analyses of genes in the green and red modules. **a** Result of GO enrichment analysis. **b** Result of KEGG enrichment analysis. CAMs, cell adhesion molecules; IBD, inflammatory bowel disease.

Construction of Weighted Gene Co-Expression Network

First, 494 samples and 1,026 mRNAs in TCGA database were taken as input. We ascertained 5 as the suitable β value (Fig. 2a–d) to guarantee scale-free network. A total of 7 modules were determined after similar modules were merged. Turquoise, blue, brown, gray, red, yellow, and green modules contained 228, 214, 142, 215, 48, 111, and 68 genes, respectively (Fig. 2e). We calculated the correlation coefficient and significance between module feature vectors and clinical features (Fig. 2f). It was found that stage showed a significantly strong correlation with the green module and red module ($p < 0.001$). That is to say, the expression trend of genes in the 2 modules was

relevant to ccRCC progression. Hence, green and red modules were chosen as the target module for further research.

GO and KEGG Enrichment Analyses

GO and KEGG enrichment analyses were performed on the green module and red module to find mainly biological processes and signaling pathways enriched by genes. GO enrichment analysis displayed that genes in these 2 modules were most relevant to biological functions, such as regulation of T-cell proliferation and lymphocyte proliferation and phagocytic vesicle (Fig. 3a). KEGG analysis showed that genes in the 2 modules were mainly enriched in immune-related signaling pathways

Fig. 4. Identification and validation of feature genes. **a** Top 30 connected genes in PPI network in the red and green modules. **b** LASSO coefficient profiles of 17 genes obtained by LASSO regression. **c** Cross-validation was used to tune parameter selections in the LASSO model. **d** Forest plot of 8 genes obtained by multivariate Cox regression analysis. **e** Kaplan-Meier curves of patients in the high-risk and low-risk groups in the train set. **f** ROC curves of 3-year and 5-year ROC curves in the train set. **g** Kaplan-Meier curves of the high-risk and low-risk groups based on patient's risk score in the test set. **h** ROC curves of 3 years and 5 years in the test set.

Fig. 5. Results of GSEA. **a** ENDOCYTOSIS signaling pathway. **b** PEROXISOME signaling pathway. **c** WNT signaling pathway. **d** TGF-BETA signaling pathway. **e** MAPK signaling pathway. **f** MTOR signaling pathway. ES, enrichment score.

(For figure see next page.)

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like allograft rejection, antigen processing and presentation, and phagosome (Fig. 3b). The above results indicated that genes in the green module and red module may affect biological functions including T-cell proliferation, lymphocyte proliferation, and phagocytic vesicle, and affect tumor progression via immune-related pathways.

Identification and Validation of Feature Genes

To identify core genes, PPI network was constructed with all genes in the green and red modules. The top 30 connected genes were selected as core genes in the module (Fig. 4a). Univariate Cox regression analysis was performed on these 30 genes, and 17 genes significantly related to patients' prognosis were obtained (online suppl. Table 4). These genes were further screened to 11 genes by LASSO regression analysis (Fig. 4b, c). An 8-gene-based prognostic model was established on genes selected by LASSO via multivariate COX regression analysis. The forest plot of 8 genes is shown in Figure 4d. Kaplan-Meier curves presented that the survival of patients with low risk was significantly longer than those with high risk in the train set (Fig. 4e). AUC of ROC of 3-year and 5-year OS in the train set was 0.73 and 0.76, respectively (Fig. 4f). The prognostic model was testified by the test set. The result showed that the survival of patients with low-risk score was remarkably longer than those with high-risk score (Fig. 4g). AUC of 3-year and 5-year OS in the test set was 0.73 and 0.77, respectively (Fig. 4h), suggesting that the established model could accurately predict patients' prognosis. In conclusion, 8 genes constituting the prognostic prediction model of ccRCC (CTLA4, DLGAP5, PLK1, CD44, BIRC5, MAD2L1, HLA-DRA, and HLA-G) could effectively predict patient's prognosis.

Results of GSEA

To determine differences in signaling pathways between the high-risk and low-risk groups in ccRCC, GSEA was performed on gene expression data in TCGA-KIRC samples. It was displayed that high-risk and low-risk groups presented significant differences in signaling pathways relevant to cell migration and invasion, such as ENDOCYTOSIS, PEROXISOME, MAPK, MTOR, TGF-BETA, and WNT (Fig. 5a–f). In sum, prognostic differences in the high-risk and low-risk groups may be associated with cell immunity, migration, and invasion.

Correlation Analysis of Feature Genes and Immune Cell Infiltration

Immune infiltration plays an essential role in tumor survival and development; thus, the relationship between

these 8 genes and immune cell infiltration was explored by using TIMER database. It was exhibited that increased tumor purity (i.e., the percentage of cancer cells in solid tumor samples) was negatively correlated with the expression of these 8 genes in ccRCC. This may result from the increased number of infiltrating B cells, CD8+ T cells, CD4+ T cells, macrophage cells, neutrophil cells, and dendritic cells (Fig. 6).

The Predictive Performance of the Prognostic Model on the Prognosis of ccRCC

Thereafter, we investigated whether this 8-gene-based risk model could be an independent factor for predicting ccRCC patient's prognosis. Univariate Cox regression analysis revealed that age, T stage, M stage, clinical stages, and risk score all significantly affected patients' prognosis ($p < 0.001$) (Fig. 7a). Multivariate Cox regression analysis showed that risk score alone still significantly affected patients' prognosis ($p < 0.001$) (Fig. 7b). The 3-year and 5-year OS possibility of patients was predicted by the nomogram generated based on patients' clinical features (Fig. 7c). The calibration curves presented a relatively good fitting between the predicted rate and actual rate of 3-year and 5-year survival (Fig. 7d). The above results presented that our established model was expected to be a prognostic predicting index superior to patient's clinical features. Moreover, the model is not influenced by patients' clinical features to be an independent factor to evaluate their prognosis.

Discussion

This article screened modules of interest using WGCNA. Eight feature genes relevant to ccRCC patient's prognosis were acquired combining PPI and COX analyses: CTLA-4, BIRC5, HLA-G, DLGAP5, PLK1, CD44, MAD2L1, and HLA-DRA. Our research offers novel biomarkers for ccRCC prognosis along with reliable bases for clinical application of these biomarkers.

Analysis of ccRCC through bioinformatics and screening of biomarkers have been enjoying the limelight over the recent years. For instance, Vastrad et al. [11] analyzed differential expression of genes in ccRCC in GEO. Then, they found ccRCC-associated key genes using PPI network and validated by experiments. Last, they gained 10 feature genes associated with ccRCC progression or prognosis. Lv et al. [12] screened differentially expressed genes of ccRCC with the robust rank aggregation method. They gained 4 biomarkers related to prognosis and biomarkers

Fig. 6. Correlation analysis of 8 gene expressions and immune cell infiltration. Correlation analysis of the expression of 8 feature genes and immune cell infiltration was performed via TIMER Web site.

combining WGCNA. Compared to the studies above, our study proposed a novel ccRCC prognostic model based on databases (GEO, NCBI, and TCGA), where a wider range of data were applied.

Feature genes screened here pertain to immune process of cancers. Some immunosuppressive proteins stimulate cancer progression by restraining immune response [13]. Out of them, CTLA-4 is able to downregulate T-cell activation [14]. Omura et al. [15] elaborated PD-L1 and CTLA-4 levels in patient's blood capable of predicting the prognosis of colorectal cancer patients. In addition, combination of PD-1 and CTLA-4 improves young ccRCC patient's prognosis [16]. BIRC5, appertaining to inhibitor of apoptosis family, inactivates caspase-3 and caspase-7 to restrain cell apoptosis while stimulating cell proliferation [17, 18]. Wan et al. [19] analyzed the role of differentially expressed autophagy-related genes in ccRCC, in which BIRC5 was screened out as a ccRCC prognosis-related gene. In non-typical HLA-I-type molecules, HLA-G plays an important part in immune inhibition and interacts with multiple immune cells to suppress their functions [20]. Similarly, Yang et al. [21] comprehensively analyzed 44 immune checkpoints abnormally expressed in ccRCC constructing four-gene prognostic signature containing HLA-G. Above all, the prognostic assessment by our model may be presented in terms of tumor immunity and cell apoptosis status.

Multivariate Cox regression analysis suggested that these 8 feature genes in this study may stand for candidate biomarkers for predicting ccRCC patient's prognosis. GSEA was performed to further explore the potential mechanism of these 8 genes. Results showed that high-risk and low-risk groups obtained by risk score had significant differences in enrichment of 6 signaling pathways: ENDOCYTOSIS, PEROXISOME, WNT, TGF_BETA, MAPK, and MTOR. Previous studies reported that these signaling pathways are important parts of internal mechanism of tumor development. For instance, activation of WNT/catenin signaling pathway is a key step for colorectal carcinoma [22]. ENDOCYTOSIS signaling pathway can mediate adult acute myeloid leukemia development [23]. TGF-BETA signaling pathway affects the malignant

Fig. 7. Validation of the feature gene-based prognostic prediction model. **a** Forest plot of the relationship between risk score, clinical features, and survival by univariate Cox regression analysis. **b** Forest plot of the relationship between risk score, clinical features, and survival by multivariate Cox regression analysis. **c** A nomogram was established based on TCGA-KIRC dataset. **d** The calibration curves of nomogram-predicted 3-year and 5-year survival.

progression of hepatocellular carcinoma [24]. LncRNA MALAT1 is expected to be a diagnosis-related biomarker in pancreatic cancer via activating mTOR and MAPK signaling pathways [25]. PEROXISOME signaling pathway participates in the development and progression of pancreatic cancer [26]. The above pathways are closely associated with cancer development and progression, indicating their similar role in ccRCC.

However, certain limitations still exist in this study. First of all, this is a retrospective study and all data used were accessed from public databases. Second, further in vivo and in vitro experiments are needed to clarify the molecular mechanism of feature genes to lay a foundation for their clinical application. All in all, these 8 genes screened through WGCNA may be relevant biomarkers of ccRCC diagnosis and prognostic prediction with impressive application prospects. Meanwhile, this study provides a certain theoretic basis for related studies.

Statement of Ethics

An ethics statement was not required for this study type, and no human or animal subjects or materials were used.

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Conflict of Interest Statement

The authors declare that they have no potential conflicts of interest.

Funding Sources

This study was supported by the funds from Project of Dalian Traditional Chinese Medicine Scientific Research (20Z11001).

Author Contributions

All authors contributed to data analysis, and drafting and revising the article, gave final approval of the version to be published, and agreed to be accountable for all aspects of the work.

Data Availability Statement

The data used to support the findings of this study are included within the article. The data and materials in the current study are available from the corresponding author on reasonable request.

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