Prognostic and predictive value of immune/stromal-related gene biomarkers in renal cell carcinoma

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Abstract. Immune/stromal-associated genes may be promising biomarkers for cancer diagnosis and the determination of clinical cancer treatment options. The aim of the present study was to identify prognostic stromal/immune-associated genes in renal cell carcinoma (RCC). RCC gene expression data (885 cases) were obtained from The Cancer Genome Atlas database. Immune/stromal scores were calculated by using the ESTIMATE package in R. Immune/stromal scores were significantly associated with Tumor-Node-Metastasis stage, clinical stage and overall survival rate ($P<0.05$). There were 419 differentially expressed genes (DEGs) based on immune scores and 738 DEGs based on stromal scores. Among these DEGs, 406 DEGs based on stromal scores and 252 DEGs based on immune scores were significantly associated with overall survival rate ($P<0.05$). The biological functions of these DEGs were primarily enriched in the 'immune response' and 'regulation of cell migration and proliferation'. These DEGs were observed in a protein-protein interaction network. A LASSO Cox regression model was used to build a prognostic 6 gene-based classifier, including the IL21R, ATP6V1C2, GBP1, P2RY10, GBP4 and TNNC2 genes [area under the curve (AUC) =0.776]. The predictive model which combined this classifier with clinical prognostic factors had a high accuracy in predicting patient survival in RCC (combined AUC =0.899). Taken together, these results demonstrated that there are significant associations between immune/stromal scores and clinicopathological staging. A set of tumor microenvironment-associated genes that have powerful prognostic value in patients with RCC were identified in the present study.

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Abbreviations: RCC, renal cell carcinoma; DEGs, differentially expressed genes; GO, Gene Ontology; BP, biological processes; MF, molecular functions; CC, cellular components; KEGG, Kyoto Encyclopedia of Genes and Genomes; PPI, protein-protein interaction

Key words: gene biomarker, tumor microenvironment, immune score, stromal score, renal cell carcinoma

Introduction

Renal cell carcinoma (RCC) is one of the most common urological tumors worldwide, and its incidence and mortality have been rising throughout the world (1). Traditional chemotherapeutic drugs cannot easily kill kidney cancer cells (2) and the clinical outcomes of patients with RCC can vary greatly (3). At present, the prognosis of patients with RCC is primarily determined by pathological staging (4). Although various studies have been investigated RCC, the clinical prognosis of patients with metastatic RCC remains poor (5). Therefore, there is a need to identify novel and more effective and accurate prognosis biomarkers for patients with RCC. At present, gene expression profiling has been increasingly used for the diagnosis of RCC (6). The genomic and molecular characteristics of tumors, such as RCC and bladder cancer, have been investigated in recent years (7). Molecular biomarkers should be considered in addition to standard clinicopathologic criteria to guide clinical treatment options.

Tumor-associated genes are important contributors to tumorigenesis and progression of cancer (8,9). In addition, the tumor microenvironment affects gene expression levels in tumor tissues, thus affecting clinical outcomes (10,11). In the tumor microenvironment, immune and stromal cells are the two main types of non-tumor components and are associated with the prognosis of tumors (12,13). Gene expression levels data from tumor databases can be analyzed using algorithms to predict cellular components in tumor tissues (14,15). For example, Yoshihara *et al*. designed an algorithm called ESTIMATE (16), which uses expression data to estimate stromal and immune cells in malignant tumor tissue. The algorithm can predict the infiltration of immune and stromal cells in the tumor microenvironment by analyzing specific gene expression profiles and calculating immune and stromal scores. At present, the value of immune/stromal scores in RCC has not been elucidated in detail. Novel useful biomarkers are required to estimate the prognosis and diagnosis of RCC.

In the present study, the RCC cohort of The Cancer Genome Atlas (TCGA) database and the ESTIMATE algorithm were both used to analyze the association of immune and stromal scores with clinicopathological features and a set of tumor microenvironment-related genes that could predict the prognosis of patients with RCC were identified. The results of the present study may provide effective genetic predictors for the diagnosis, prognosis and treatment of renal cell carcinoma.

Materials and methods

TCGA RCC data. Gene expression data for 885 patients with RCC were obtained from TCGA database (cancer.gov/tcga). In the data, 289 (32.7%) patients were female and 596 (67.3%) patients were male, with an age range of 34-90 years. The following are the selection criteria of TCGA IDs on the database: i) Disease type was adenocarcinomas; ii) primary site was the kidney; iii) program name was TCGA; iv) Workflow Type was HTSeq-FPKM; v) data category was transcriptome profiling and vi) data type was Gene Expression Quantification. RNA expression profiles of kidney adenomas and adenocarcinomas were analyzed using the Affymetrix Human Genome U133 Plus version 2.0 array. Clinical data such as sex, age, histological grading, Tumor-Node-Metastasis (TNM) stage, clinical stage, survival time and outcomes were also obtained from TCGA database.

ESTIMATE algorithm. The ESTIMATE algorithm can infer the infiltration of immune and stromal cells in tumor tissues by analyzing the transcriptional profile of cancer samples (16). Gene expression values were graded and ranked for each sample. The statistical significance value was calculated by integrating the difference between the empirical cumulative distribution functions of the signature gene and the remaining genes. The ESTIMATE algorithm output stromal and immune scores by performing single-sample Gene Set Enrichment Analysis.

Investigating the association between ESTIMATE scores and clinical features in renal cell carcinoma. Samples were grouped according to clinical/pathological stage, and the ESTIMATE scores of each group were then analyzed to evaluate the association between ESTIMATE scores and clinical/pathological stage. The mean score was used to divide these samples into low- and high-stromal/immune score groups, and the difference in overall survival rate between the two groups was evaluated using Kaplan-Meier plotter (17) and log-rank tests.

Identification of differentially expressed genes. Samples were grouped according to stromal/immune scores. The expression of individual genes was analyzed using the Limma package in R (18). All R language operations are performed in R Studio (19). A log2| fold-change (FC)| >2.0 and P<0.05 were considered to indicate a significant difference when screening for differentially expressed genes (DEGs). The Pheatmap package (CRAN.R-project.org/package=pheatmap) was used to generate heatmaps.

Enrichment analysis of differentially expressed genes. The Database for Annotation, Visualization and Integrated Discovery (DAVID v.6.8) database (david.ncifcrf.gov/) was used to identify Gene Ontology (GO) categories, including biological processes (BPs), cellular components (CCs) and molecular functions (MFs). Pathway enrichment analysis was also performed using the DAVID database with reference to the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (kegg.jp/).

Construction of protein-protein interaction (PPI) networks. PPI networks provide a valuable framework for an improved understanding of the interaction of proteins (20). The PPI networks were retrieved from the STRING database (21) and reconstructed using Cytoscape software v.3.7.1 (22). Nodes with a high degree of connectivity in the network were considered as regulatory hubs. The node size was determined to be proportional to the degree of interaction. The genes with a degree of connectivity ≥1 were reserved in the PPI network. Genes with a degree of connectivity >31.56 were considered hub genes.

Association between the expression of DEGs and overall survival rate. The log-rank method was used to evaluate the prognostic value of each gene. Genes that were significantly associated with overall survival rate were identified as prognostic genes. Subsequently, a LASSO Cox regression model was used to construct a prognostic classifier by selecting genes from these prognostic DEGs. The risk score based on the expression levels for each patient was calculated using the constructed Cox regression model. According to the optimum cut-off score (risk score=0.472), the patients were divided into high-risk and low-risk groups. The difference in survival rates between the two groups was then assessed using the Kaplan-Meier method.

Building a clinical predictive model based on the prognostic genes for RCC. A LASSO Cox regression model was used to select the most useful prognostic genes. A multiple-gene-based classifier was constructed to predict the survival rate of patients with RCC. Patients were randomly divided into training set and testing set (7:3). Cox regression analysis was performed by using the 'glmnet' package. The area under the curve (AUC) of the time-dependent receiver operating characteristic (ROC) curve was used to measure the predictive accuracy of the multi-gene based classifier. Univariate and multivariate survival analyses were performed by using the Cox regression model and a nomogram plot was generated according to the Cox regression coefficients.

Statistical analysis. The differences in ESTIMATE scores between different clinical/pathological staging groups were analyzed using the Kruskal-Wallis test. The differences in the levels of gene expression between the high-and low-score groups were compared using an unpaired t-test. Kaplan-Meier analysis and log-rank tests were performed in order to compare each prognostic gene signature. Univariate and multivariate survival analyses were performed with the Cox regression model. A LASSO Cox regression analysis was used to built the best classifier according to the regression

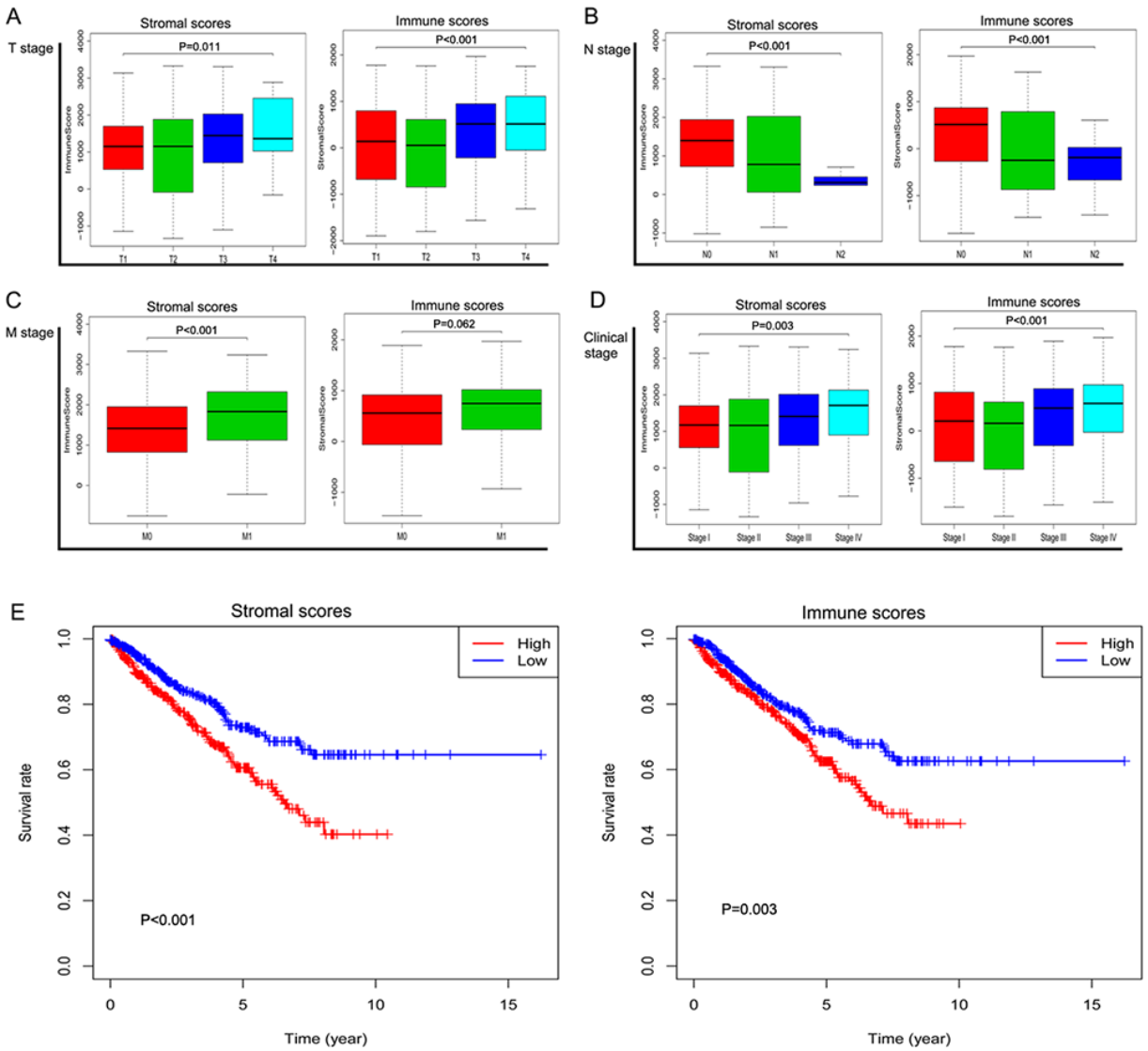

Figure 1. Immune/stromal scores are associated with clinicopathological features and overall survival rate. Association of stromal and immune scores with: (A) T stage, (B) N stage, (C) M stage and (D) clinical stage. (E) Median survival rate of the low-stromal score group was more favorable compared with the high-score group (P<0.001). Similarly, the median survival rate of the low-immune score group was more favorable compared with that of the high-score group (P=0.003). T, Tumor; N, Node; M, Metastasis.

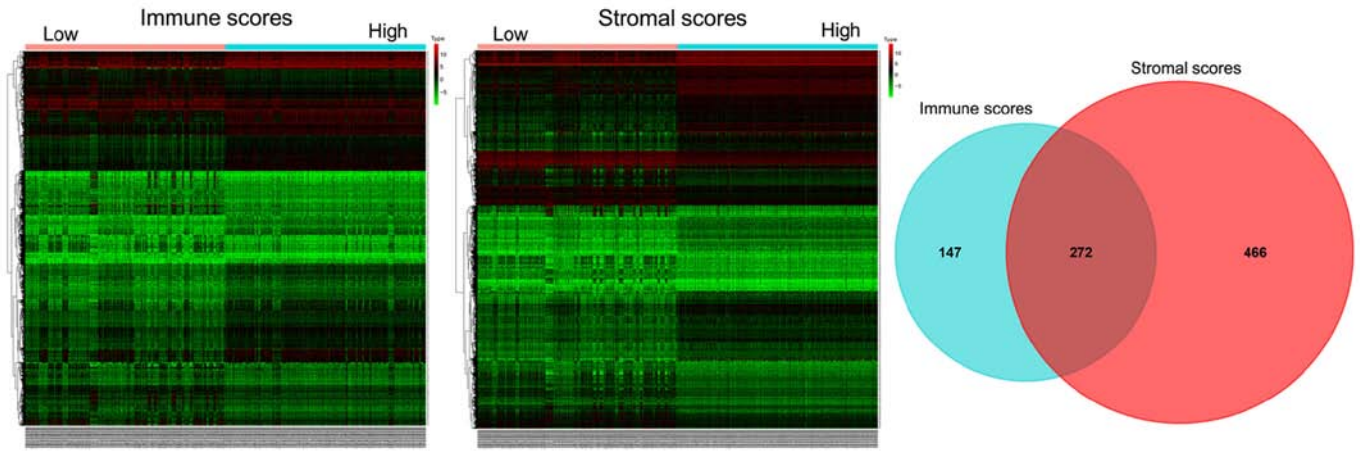

Figure 2. Comparison of gene expression profiles in renal cell carcinoma based on immune and stromal scores. (A) Heatmaps of the DEGs based on immune scores (high score vs. low score). log2| fold-change (FC)| >2.0 and P<0.05 were considered to indicate a significant difference when screening for DEGs. Highly expressed genes are shown in red and gene with low expression levels are shown in green. Similarly expressed genes are shown in black. (B) Heatmaps of the DEGs based on stromal scores (high-score vs. low-score; P<0.05; fold-change>2.0). (C) Venn diagram of the number of common DEGs in the stromal and immune score groups. DEGs, differentially expressed genes.

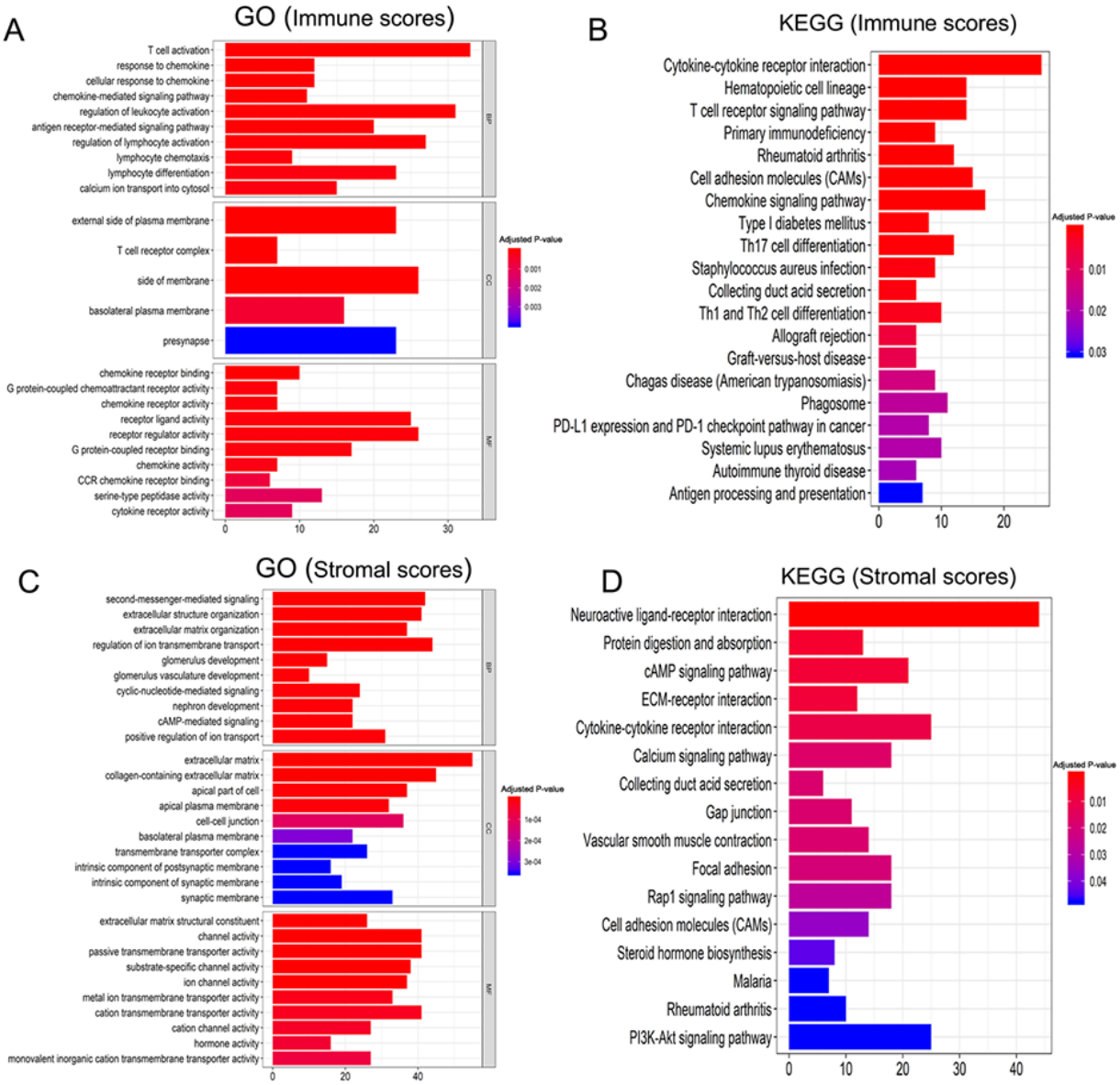

Figure 3. Functional enrichment analysis of differentially expressed genes. (A) Top 10 GO terms for the DEGs based on immune scores (BPs, CCs and MFs). (B) Top 30 KEGG pathways for the DEGs based on immune scores. (C) Top 10 GO terms for the DEGs based on stromal scores. (D) Top 30 KEGG pathways for the DEGs based on stromal scores. GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; BP, biological process; CC, cellular component; MF, molecular function; adjusted P-value.

coefficients and P-values. P<0.05 was considered to indicate a statistically significant difference.

Results

ESTIMATE scores were associated with clinicopathological features in RCC. The gene expression data and clinicopathological data of 885 patients with kidney adenocarcinomas were downloaded from TCGA database. Stromal scores were distributed between -1,897.14 and 1,967.19 and immune scores ranged from 1,673.78 to 3,459.07 (data not shown). As presented in Fig. 1A-D, apart from the M stage, the immune scores were significantly associated with T, N and clinical stage (P<0.001; P<0.001; P<0.001, respectively). Similarly, the stromal scores significantly increased with increasing T, N, M and clinical stage (P=0.011; P<0.001; P<0.001; P=0.003, respectively).

Immune/stromal scores were significantly associated with overall survival rate. Patients with RCC were divided into high- and low-groups based on their scores. As presented in Fig. 1E, the overall survival rate of the low-stromal score group was more favorable compared with the high-score group (P<0.001). Patients with high immune scores should theoretically have an improved survival rate, but the statistical results revealed that the survival rate of the high-immune score group was lower compared with the low immune score group (P=0.003).

Differentially expressed genes based on immune/stromal scores. The heatmaps in Fig. 2 present the genes with significant differential expression levels between the high- and low-immune/stromal score groups. A total of 141 genes were upregulated and 277 genes were downregulated in the high-immune score group compared with the low score group (FC

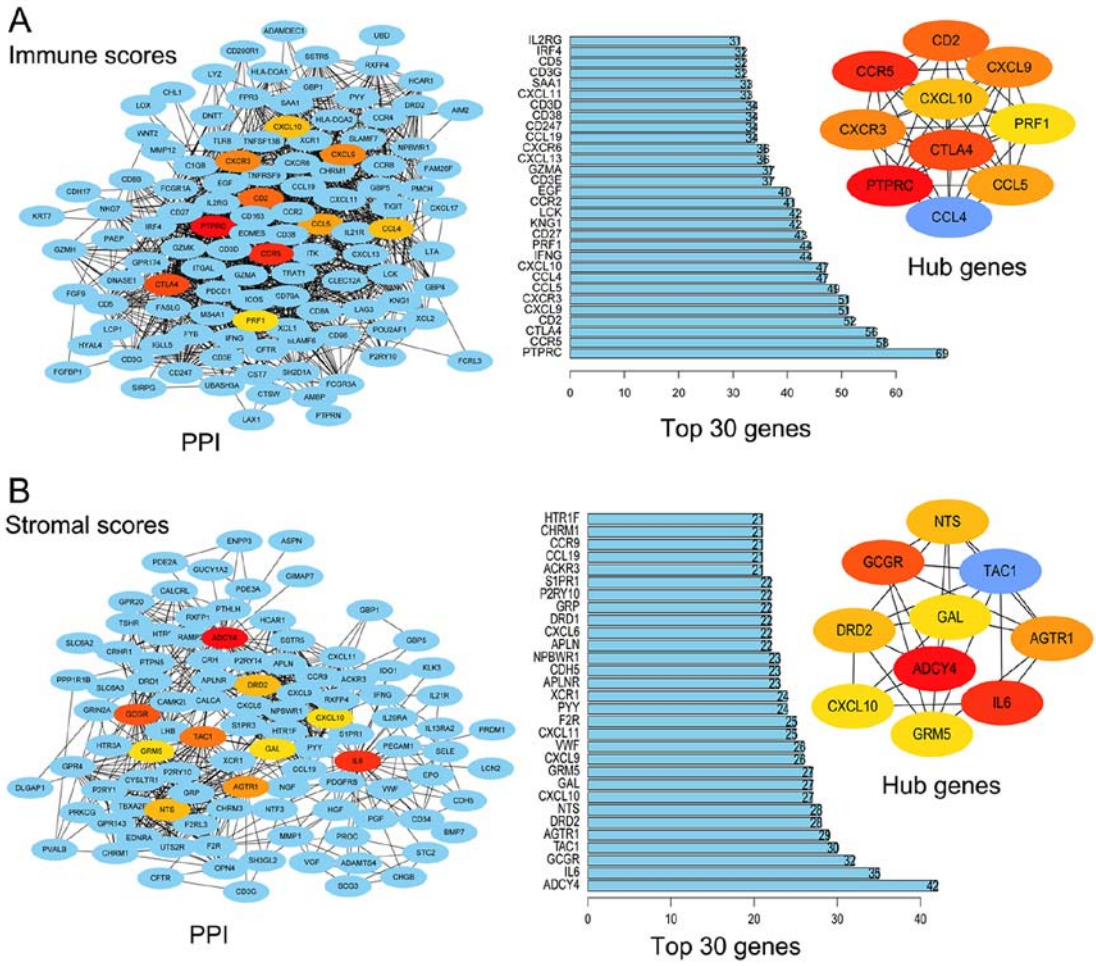

Figure 4. PPI network of differentially expressed genes and hub genes. (A) PPI network of DEGs based on immune scores, including the top 30 DEGs and the top 10 hub genes in the network. (B) PPI network of the DEGs based on stromal scores, including the top 30 DEGs and the top 10 hub genes in the network. PPI, protein-protein interaction, Hub, gene whose degree of interaction was >31.56.

>2.0; P<0.05). Similarly, 327 genes were upregulated and 410 genes were downregulated in the high stromal score group compared with the low score group (FC >2.0; P<0.05). In addition, the Venn diagram demonstrated that there were 272 common DEGs shared between the high-immune score group and low-stromal score groups.

Prognostic value of differentially expressed genes in overall survival rate prediction. As presented in Table SI, among the 738 DEGs based on stromal scores, 406 DEGs were significantly associated with overall survival rate (all P<0.05). Among the 419 DEGs based on immune scores, a total of 252 DEGs were significantly associated with overall survival rate (P<0.05). Among the 272 common DEGs, a total of 137 DEGs were significantly associated with overall survival rate (P<0.05).

Functional enrichment analysis of differentially expressed genes. GO analysis of the immune scores demonstrated a strong association with the 'immune response' (Fig. 3A). The BP terms were primarily enriched in 'T cell activation', 'response to chemokine', 'cellular response to chemokine', etc. The CC terms primarily included 'external side of plasma membrane', 'T cell receptor complex', 'side of membrane', etc. Top MF terms included 'chemokine receptor binding' 'G protein-coupled chemoattractant receptor activity', etc. The KEGG pathway analysis revealed that a number of these pathways were associated with the immune response (Fig. 3B), including the 'T cell receptor signaling pathway', 'Th1/Th2 cell differentiation', 'cytokine-cytokine receptor interactions' and the 'chemokine signaling pathway.'

Similarly, the BP terms based on stromal scores were primarily enriched in 'extracellular structure organization' and 'regulation of cell migration and proliferation' (Fig. 3C). The CC terms primarily included 'second-messenger-mediated signaling', 'extracellular structure organization', 'extracellular matrix organization', etc. Top MF terms included 'extracellular matrix', 'collagen-containing extracellular matrix', 'apical part of cell', etc. The KEGG pathway analysis identified pathways associated with 'PI3K-Akt signaling pathway', 'calcium signaling', 'protein digestion and absorption', 'Rap1 signaling pathway' and 'cAMP signaling' (Fig. 3D).

PPI networks and hub genes. The hub nodes in the regulation network were identified using Cytoscape software. Fig. 4A presents the PPI network of DEGs based on immune scores and displays the top 30 DEGs in the network. The top 10 noteworthy nodes were considered hub genes, including PTPRC, CCR5, CTLA4, CD2, CXCR3, CXCL9, CCL5,

Table I. List of hub genes that are significantly associated with overall survival rate of patients with renal cell carcinoma.

A, immune scores

Hub genes	P-value
CTLA4	<0.001
CCL5	<0.001
CXCL9	<0.001
CXCR3	<0.001
CD2	<0.001
PRF1	<0.001
CCL4	<0.001
CXCL10	<0.001
CCR5	0.003
PTPRC	0.026

B, stromal scores

Hub genes	P-value
IL6	<0.001
CXCL10	<0.001
NTS	0.014
GRM5	0.015
ADCY4	0.026

Hub gene, gene whose degree of interaction was >31.56.

CCL4, CXCL10 and PRF1. Similarly, Fig. 4B presents the PPI network of DEGs based on stromal scores and the top 30 DEGs in the network. The top 10 hub genes were ADCY4, IL6, GCGR, TAC1, AGTR1, DRD2, NTS, CXCL10, GRM5 and GAL. The results of the log-rank test demonstrated that all the top 10 hub genes based on immune scores and all of the top five hub genes based on stromal scores were significantly associated with overall survival rate (Table I).

Building a prognostic classifier using the LASSO Cox regression model. A LASSO Cox regression model was used to construct a prognostic classifier by selecting 6 genes from the 137 common prognostic DEGs, including IL21R, ATP6V1C2, GBP1, P2RY10, GBP4 and TNNC2 (Fig. 5A). The risk score based on the expression levels of the 6 genes for each patient was calculated using the constructed Cox regression model. According to the optimum cut-off score (risk score =0.472), the patients were divided into high-risk and low-risk groups (AUC=0.776; Fig. 5B). The 6 gene based classifier was a powerful prognostic factor in the training set [hazard ratio (HR), 2.8; confidence interval (CI), 2.3-3.4; P<0.001; Fig. 5C] and in the testing set (HR, 2.3; CI, 1.6-3.4; P<0.001; Fig. 5D). The survival rate of the low-risk score group was more favorable compared with that of the high-risk score group. These results suggested that this classifier effectively predicted the prognosis of patients with RCC.

Clinical predictive model based on prognostic genes in RCC. As presented in Fig. 6A, the 6 gene based classifier demonstrated a similar prognostic accuracy to other clinical prognostic factors. The AUC values of gene based classifier, age, clinical stage and TNM stage were 0.776, 0.566, 0.822 and 0.802, respectively. The AUC of the combined classifier and clinical prognostic factors was 0.899, thus, this classifier had value in improving the accuracy of prognosis prediction of patients with RCC. To provide clinicians with a quantitative tool to predict prognosis of patients with RCC, a nomogram was constructed that combined the 6 gene based classifier and clinicopathological prognostic factors (Fig. 6B).

Discussion

RCC is one of the most common primary renal malignancies (1). Personalized data from RCC specimens can provide doctors with more accurate information to select the appropriate treatment for patients (5). The interaction between tumor cells and the tumor microenvironment substantially affects the evolution of tumors, which subsequently affects tumor recurrence, drug resistance and overall survival rate of patients (23). There is an association between the components in the tumor microenvironment and tumor outcomes (24). Different tumor microenvironmental compositions lead to differences in sensitivity to different targeted drug therapies (25). Therefore, analyzing the components in the tumor microenvironment can provide guidance for selecting the best clinical treatment options for the patient. At present, gene expression profiling has been increasingly incorporated into clinical diagnostic criteria (6). Molecular biomarkers should be considered in addition to standard clinicopathologic criteria to guide clinical treatment options.

With the rapid development of high-throughput sequencing, tumor databases with large samples, such as TCGA, provide a wealth of information to help find ways to solve difficult clinical problems, such as improving diagnostic rates and choosing targeted drugs (26). The present study aimed to identify tumor microenvironment-associated genes that were associated with the overall survival rate of patients with RCC. In the tumor microenvironment, immune and stromal cells are two major types of non-tumor components that are valuable for the diagnosis and prognosis of tumors (27). The ESTIMATE algorithm is a useful tool to extract the genetic information of immune and stromal cells from a complex tumor microenvironment in order to identify the degree of infiltration of immune and stromal cells (28). Based on ESTIMATE scores, the present study aimed to evaluate the association between gene expression signatures of immune/stromal infiltration and RCC outcomes. In particular, a set of DEGs involved in the immune response and related to the extracellular matrix were identified. GO term analysis demonstrated that the majority of these DEGs were involved in the organization and regulation of the tumor microenvironment. It has been reported that the function of immune cells and extracellular matrix molecules are involved in the establishment of the tumor microenvironment of RCC (29).

The immune components in the tumor microenvironment are complex (30). Although immune cell infiltration has been associated with response to immune checkpoint blockade in

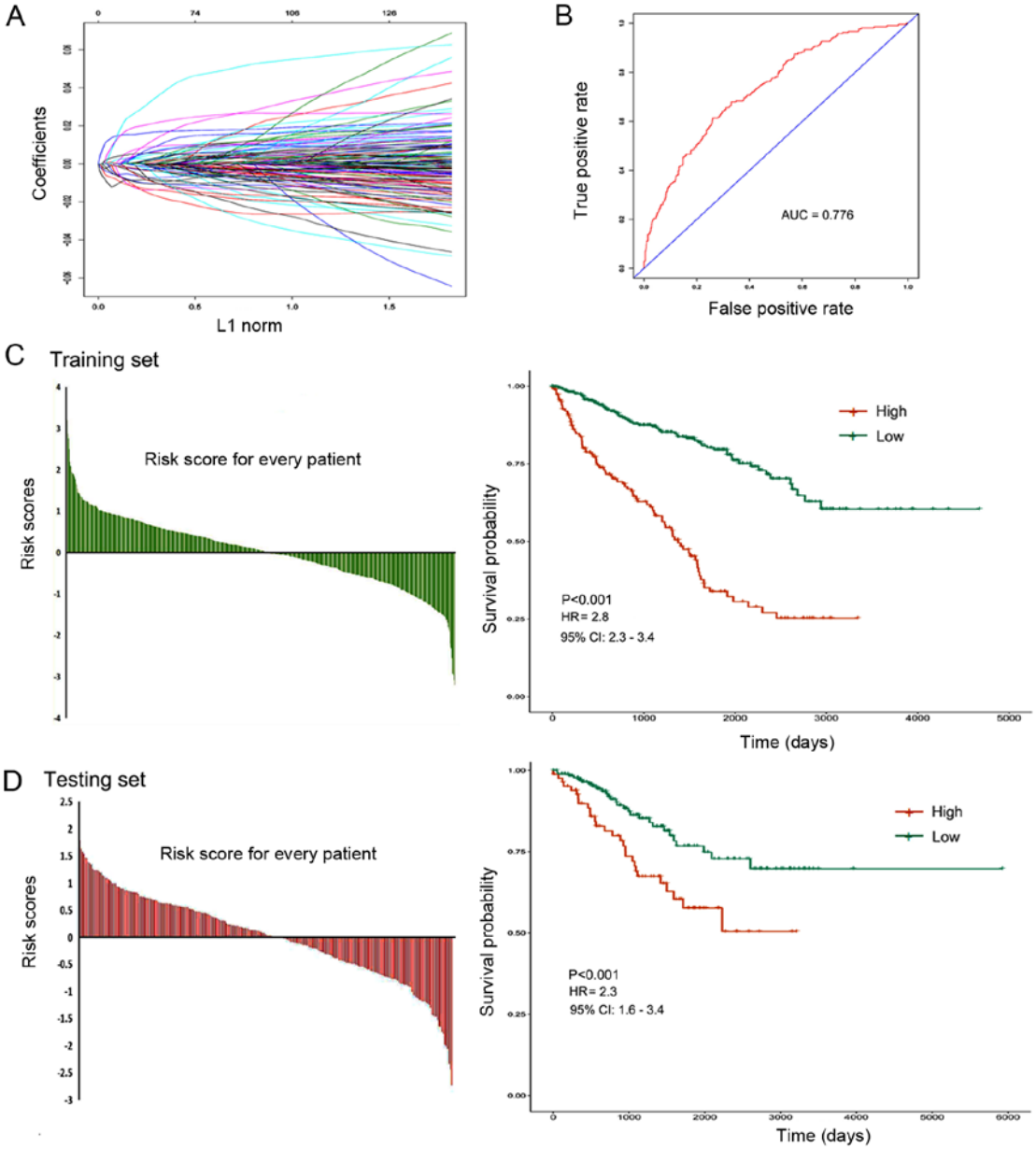

Figure 5. Construction of the 6 gene based prognostic classifier. (A) LASSO Cox regression model was used to build a prognostic classifier that contained 6 genes: IL21R, ATP6V1C2, GBP1, P2RY10, GBP4 and TNNC2. (B) The receiver operating characteristic AUC curve was used to measure the predictive accuracy of the 6-gene-based classifier (AUC=0.776). (C and D) Training and testing sets were divided into high-risk and low-risk groups according to the optimum cut-off score (risk score=0.472). The 6 gene based classifier was a valuable prognostic factor in the training set (HR 2.8; CI, 2.3-3.4; P<0.001) and in the testing set (HR, 2.3; CI, 1.6-3.4; P<0.001). L1, Manhattan Distance; ROC, receiver operating characteristic; AUC, area under the curve; HR, hazard ratio; CI, confidence interval.

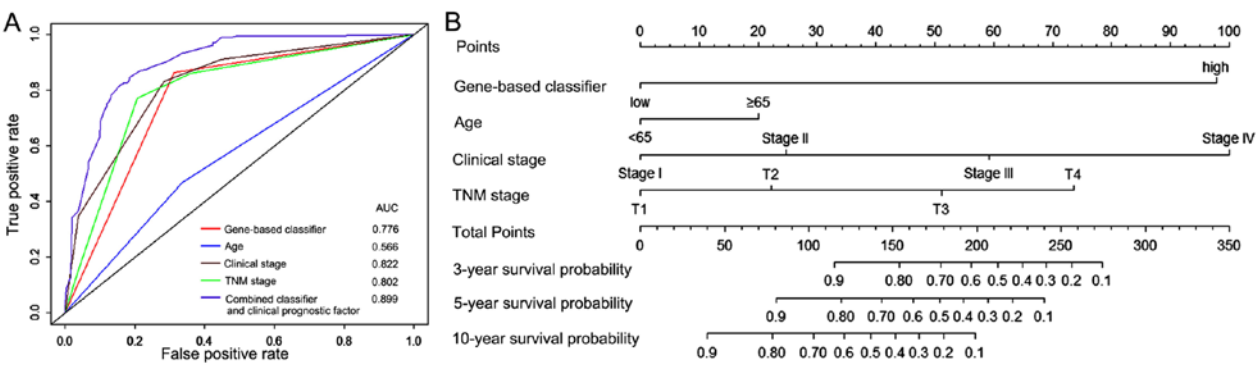

Figure 6. Prognostic value of the 6 gene based classifier in RCC. (A) Time-dependent receiver operating characteristic curves comparing the prognostic accuracy of the 6 gene based classifier with clinicopathological risk factors. (B) Nomogram to predict the survival of patients with RCC. RCC, renal cell carcinoma; TNM, Tumor-Node-Metastasis; AUC, area under the curve.

metastatic kidney cancer (31), it is difficult to predict prognosis of patients with cancer using one or several indicators. In the present study, by analyzing the clinical data, gene expression profiles and immune scores of 885 patients with RCC, it was demonstrated that the average immune scores significantly increased with increasing T, N stage and clinical stage, but were not associated with M stage. In addition, notable differences in overall survival rate based on immune gene expression signatures were observed. The presence of immune cells serves a role in killing tumor cells and inhibiting tumor growth, therefore, cases with high immune scores should theoretically have an improved survival. However, the present study demonstrated that patients with high-immune scores had less favorable survival rate when compared with those with low-immune scores. This result warrants further in-depth investigation, and such a study may identify a novel direction for immunotherapy in kidney cancer.

Non-immune cell components in the tumor microenvironment also affect the therapeutic response (32). In kidney cancer, the expression levels of transforming growth factor β signaling in fibroblasts surrounding the tumor are associated with immunologically-excluded phenotypes and a lack of response to immune checkpoint inhibition (33). Fibroblasts and other matrix components may also contribute to direct rejection or inactivation of chemotherapy (34). Based on the ESTIMATE algorithm, the present study reported that stromal scores are associated with TNM stage and clinical stage. The average stromal scores were significantly increased with increasing T stage, N, M stage and clinical stage. In addition, the overall survival rate of the low stromal score group was more favorable compared with that of the high score group. Stromal cells provide a suitable environment for tumor growth and progression (35). Analyzing the expression profiles of genes and identifying the underlying molecular mechanisms of gene expression levels affecting the tumor matrix, promoting tumorigenesis and development may provide novel insight into the clinical diagnosis and treatment of kidney cancer.

The aim of precision medicine is to implement personalized treatment based on the molecular characteristics of the patient (36). The present study aimed to identify a set of tumor microenvironment-associated genes that could predict the outcomes of patients with RCC. By comparing the gene expression levels low-score vs. high-score groups, a series of DEGs were identified. Kaplan-Meier analysis demonstrated that the 406 DEGs based on stromal scores and the 252 DEGs based on immune scores were significantly associated with overall survival rate in patients with RCC. The DEGs based on immune scores were primarily enriched in 'immune response' and the DEGs based on stromal scores were mainly enriched in 'extracellular structure organization' and the 'regulation of cell migration and proliferation'. Notably, the present study identified 10 hub genes based on immune scores and 5 hub genes based on stromal scores that were significantly correlated with overall survival rate of patients with RCC. These novel prognostic genes could be used as potential biomarkers for the diagnosis and selection of clinical treatment options for patients with RCC.

In order to investigate the prognostic value of these DEGS, a LASSO Cox regression model was used to construct a 6 gene based classifier, which was demonstrated to be an effective prognostic factor. The results of the statistical analysis demonstrated that the survival rate in patients with low-risk scores was higher compared with patients with high-risk scores. These results indicate that the 6 gene based classifier had high accuracy in predicting the prognosis of patients with RCC. Furthermore, combining this classifier with other clinical variables could lead to more accurate predictions of patient survival. A nomogram combining this classifier and clinical risk factors may aid clinicians in the prediction of prognosis of patients with RCC.

In summary, the immune/stromal gene signatures of RCC are associated with clinicopathological factors and overall survival rate. The present study identified a set of tumor microenvironment-associated genes that were associated with the prognosis of patients with RCC. The classifier based on these tumor microenvironment-associated genes had prognostic value in predicting the prognosis of patients with RCC. Further study of these genes may provide a comprehensive understanding of the association between the tumor microenvironment and the prognosis of RCC. Although validation in prospective trials is necessary, the results of the present study suggest that transcriptional analysis may be valuable to predict the prognosis of patients with RCC.

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Availability of data and materials

The results published in the present study are based upon data generated by the TCGA Research Network (http://cancergenome.nih.gov/).

Authors' contributions

SSC contributed to conception, data analysis, and final approval of the version to be published. SW contributed to the data analysis. XGZ and XLC made substantial contributions to data analysis and revising the manuscript critically for important intellectual content. XJS and SSC were involved in drafting the manuscript and contributed to the data analysis. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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