Novel Prognosis and Therapeutic response model of Immune-related lncRNA pairs in Clear Cell Renal Cell Carcinoma

Gang Wang (wanggang1990@zju.edu.cn)
Zhejiang Provincial First Hospital: Zhejiang University School of Medicine First Affiliated Hospital
Panhong Liu
The second Affiliated Hospital zhejiang University school of Medicine
Jiangfeng Li
Zhejiang Provincial First Hospital: Zhejiang University School of Medicine First Affiliated Hospital
Ke Jin
Zhejiang Provincial First Hospital: Zhejiang University School of Medicine First Affiliated Hospital
Xiangyi Zheng
Zhejiang Provincial First Hospital: Zhejiang University School of Medicine First Affiliated Hospital
Liping Xie
Zhejiang Provincial First Hospital: Zhejiang University School of Medicine First Affiliated Hospital

Research Article

Keywords: Immune, ccRCC, lncRNA, Prognosis, Bioinformatics

Posted Date: May 11th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1616413/v1

Abstract

Background

Clear cell renal cell carcinoma (ccRCC) is the most common type of renal carcinoma. It is particularly important to accurately judge the prognosis of patients. Since most tumor prediction models depend on the specific expression level of related genes. Therefore, a better model needs to be constructed.

Objective

To provide an immune-related lncRNA (irlncRNAs) tumor prognosis model, which independent of the specific gene expression levels.

Methods

First, we downloaded and sorted out the data of ccRCC in TCGA database and screened irlncRNAs by co-expression analysis, and then obtained the differently expressed irlncRNA (DEirlncRNA) pairs by univariate analysis. In addition, we modified Lasso penalized regression. Subsequently, the ROC curve was drawn, compare the area under the curve, calculate the Akaike information standard value of the 5-year receiver operating characteristic curve, and determine the cut-off point to establish the best model to distinguish the high- or low-disease-risk group of ccRCC. Subsequently, we reassessed the model from the perspectives of survival, clinic-pathological characteristics, tumor-infiltrating immune cells, chemotherapeutics efficacy, and immunosuppressed biomarkers.

Results

A total of 17 DEirlncRNAs pairs were identified, all of them were included in the Cox regression model. With the cut-off point, we can better distinguish patients according to different factors, such as survival status, invasive clinic-pathological features, tumor immune infiltration, whether chemotherapy is sensitive or not, and expression of immunosuppressive biomarkers.

Conclusion

We constructed irlncRNA model by pairing, which can better get rid of the dependence on the expression level of the target genes, to obtain a better clinical prediction.

Introduction

Clear cell renal cell carcinoma is one of the most common pathological types of renal cell carcinoma(RCC). With the increase of physical examination rate and detection methods, the incidence rate is increasing and its mortality rate is still high. [1]. At present, the first-line treatments in clinical are partial or radical nephrectomy for patients with stage I or II in ccRCC, and the combination of targeted therapy and/or immunotherapy for those with stage III or IV [2]. As we all know, gene mutation is one of the initial factors of tumor. In ccRCC, more than 70% of patients found VHL gene mutation in gene testing [3]. Loss of VHL function can induce hypoxia inducible factors such as HIF-1α Or HIF-2α Increased expression level[4], and through its nuclear transcription factor function, it leads to the downstream genes VEGF, PDGF and TGF-β etc abnormal transcription and expression[5]. Therefore, many TKIs and monoclonal antibodies inhibiting VEGFR are used to treat ccRCC patients as first-line drugs, such as sunitinib and sorafenib etc [6]. Since a large amount of immune cell infiltration was found in ccRCC tissues, the immune sensitive of ccRCC was further verified by immunological therapy [7]. It is noteworthy that both the INF-α and IL-2 or immune checkpoint inhibitors (ICI) such as PD-1/PD-L1 blockers can significantly improve the overall survival rate (OS) of patients with ccRCC [8]. Recently, the theory that PD-1/PD-L1 monoclonal antibody combined with vascular targeted therapy has attracted the attention of many researchers and it has been demonstrated to be effective in prolonging OS in patients with ccRCC [9]. Even though, a great deal of research shown that expression of PD-L1 on the menbrance of cancer cells is not an indicator of the clinical outcomes of immunotherapy for ccRCC patients [10], which poses the uncertainty of when or which immunotherapy drugs should be used. Therefore, it is particularly important to find effective and accurate biological markers to help formulate an individualized treatment schedules.

In recent decades, the emergence of long noncoding RNAs (lncRNAs), a kind of RNA strand with nucleotide sequences longer than 200 bases, is receiving more and more attention [11]. The production method of lncRNAs is similar to that of coding genes, including a variety of regulatory pathways, such as histone modification and alternative splicing, etc [12]. Because there is no effective open reading frame, it does not encode any protein [13]. However, this does not affect its biological functions such as mRNA or protein expression regulation, including DNA transcription regulation, post transcriptional modification, protein translation and even participation in epigenetic modification [14]. Therefore, lncRNA is considered to play an indispensable role in the physiology and pathology of organisms, especially in cancer [15]. Recently, increasing evidences have indicated that lncRNAs are involved in the entire progression of ccRCC via a variety of molecular mechanisms such as alterations of the genomic, transcriptomic, and tumor immune microenvironment (TIME) [16]. According to reports, the biological markers of a risk model constructed based on the expression of lncRNAs have been demonstrated to predict the OS of patients with cancer, including ccRCC [17].

As immunotherapy, that is, the common treatment of patients with tumor in clinical brings constantly benefits for those, the signatures to predict the outcomes of the treatment are increasingly focused on [18]. For example, it has been researched and reported as these signatures that the infiltration score of

immune cells, the expression of immune checkpoints and immune-related genes, etc [19]. Lately, immune-related lncRNAs (irlncRNAs) were also considered to establish the signature to predict the prognosis of patients with cancer, for example hepatocellular cancer [20], gastric cancer [21], pancreatic cancer [22] and even ccRCC [23]. But, these previous characteristics depend on the risk score based on the expression of relevant lncRNAs. Nowadays, Hong W constructed a novel signature indepented with the expression of irlncRNAs in HCC, which was used to predict the prognosis of patients and its correlation with tumor-infiltrating immune cells [24]. However, the application prospect in ccRCC remains to be further studied.

In this study, we screened firstly immune-related lncRNAs (irlncRNAs) and identified the consequential irlncRNA pairs based on the ccRCC data from TCGA database. Then these irlncRNA signatures were utilized to construct a risk model by a novel algorithm, which was applied to explore the value of a predictor that assesses the os of patients, the infiltration condition of immune cells and the clinical effectiveness of immunotherapy drugs in ccRCC.

Result

Screening of differential expression of irlncRNA

We will conduct this study through the following steps. First, we obtained the transcriptome expression profile data of KIRC (as known as ccRCC) from TCGA database, including 72 normal and 539 tumor samples. Next, we annotated the date according to gene transfer format (GTF) files from Ensembl to convert the gene ID to gene symbols. Soon afterwards, we carryed on a co-expression analysis among lncRNAs and known immune-related genes. Finally, we obtained 433 irlncRNAs (correlation coefficient=0.7, p<0.001,shown in Supplementary materials S1-irlncRNAs), and 90 were considered as DEirlncRNAs (FDR=0.001，logFC=2, Figure 1A), 74 upregulat and 16 downregulat were included (Figure 1B, Supplementary materials S2-DEirlncRNAs).

Identification of DEirlncRNA pairs and a Risk Assessment Model

To obtain the DEirlncRNA pairs, we screened the matrix among 90 DEirlncRNA through an iteration loop and a 0-or-1 method. Finally, we got 2667 DEirlncRNA pairs (Supplementary materials S3-DEirlncRNAs pairs). Then a single factor analysis was performed followed by modified lasso regression analysis, 369 DEirlncRNA pairs were identified, among them 17 are included in Cox proportional hazard model (Figure 1C). After that, the ROC curve of 17 pairs was drown, shown that the area under the curve (AUCs) was 0.792 (Figure 2A). To validate our results, we plotted the 1-, 3- and 5-year ROC curves, respectively, which showed that all AUC values were over 0.792 (Figure 2B), then the 5-year ROC curves were compared with other clinical characteristics (Figure 2C). We recognized the maximum inflection point as the cut-off point on the 5-year ROC curve using the Akaike information criterion (AIC) values (Figure 2D). We collected data of 526 acceptable cases of patients with KIRC from TCGA and calculated the risk scores for all of them. We used the identified cut-off point to redistinguish high- and low-risk groups in the cohort for validation.

Application of risk models in clinical evaluation

On the basis of the above cut-off point, 273 cases were divided into the low-risk group and 253 cases were the high-risk group. Figure 3A and Figure 3B show the RiskScores and survival of each case. These results suggest that patients in the low-risk group have a better clinical prognosis. Kaplan Meier analysis further confirmed the above results ($p < 0.0001$) (Figure 3C). Then, we analyzed the relationship between the risk of KIRC and clinicopathological features by chisquare test. The strip chart (Figure 4A) and consequent scatter diagrams obtained by the Wilcoxon signed-rank test showed that Age (Figure 4B), tumor Grade (Figure 4C), clinical Stage (Figure 4D), T stage (Figure 4E), N stage (Figure 4F) and M stage (Figure 4G) were significantly related to the risk except the Gender (Figure 4H). Then, through the univariate Cox regression analysis, we know that Age ($p<0.001$, HR=1.029, 95% CI [1.016–1.043]), clinical grade ($p<0.001$, HR=2.286, 95% CI [1.862–2.807]) ,clinical stage ($p<0.001$, HR=1.897, 95% CI [1.660–2.167]) , and riskScore ($p<0.001$, HR=1.270, 95% CI [1.229–1.312]) have significant statistical differences (Figure 4I), whereas in the multivariate Cox regression analysis riskScore ($p<0.001$, HR=1.197, 95% CI [1.152–1.245]) was an independent prognostic predictor (Figure 4J). Table S1 shown the specific result of univariate and multivariate Cox regression analyses.

Risk assessment model of tumor infiltrating immune cells and immunosuppressive molecules

Since lncRNAs and immune-related genes were initially linked together, we studied whether this model was related to the tumor immune microenvironment. Our study described that the high-risk groups have a higher correlation with tumor-infiltrating immune cells such as Myeloid dendritic cells, NK cells, $CD4^+$ Th1 and T cell NK, whereas they were negatively associated with Hematopoietic stem cells, Macrophage and CD4+ memory resting, as revealed by the Wilcoxon signed-rank test (see Figure S1). Then we conducted a Spearman correlation analysis, and the resulting diagram shown by a lollipop shape (Figure 5A, Table S2). As we all know, ICIs plays an important role in the treatment of ccRCC, we investigated whether the risk model was related to ICI-related biomarkers and discovered that high risk scores were positively correlated with high expression of PDCD1 ($p < 0.001$, Figure 5B) and CTLA4 ($p < 0.001$, Figure 5C). However, the negatively correlated with low expression of EGFR ($p< 0.001$, Figure 5D), MTOR ($p< 0.001$, Figure 5E), FLT3 ($p< 0.01$, Figure 5F) and CD274 ($p< 0.05$, Figure 5G)

Correlation analysis between risk model and chemotherapy drugs

Except for checkpoint blockade therapy, we sought to determine the association between the risk and efficacy of conventional chemotherapy for KIRC in the TCGA project using the KIRC dataset. We discovered that there is an inverse ratio between the risk score and the half inhibitory concentration (IC50)

of chemotherapy agents. For instance, Cisplation (p=0.01), Paclitaxel (p<0.001), KU.55933 (p<0.001), Sunitinib (p<0.001) and Gefitinib (p=0.022), which showed that the model can be used as a potential predictor of chemosensitivity. However Sorafenib (p=0.74) showed no statistically significant.

Discussion

With the rapid development of genomics, increasing genes including coding genes and lncRNAs are regarded as novel biomarkers to assist the clinician to formulate the therapeutic schedule and evaluate the clinical outcomes of ccRCC patients after different treatments such as surgical operation, targeted therapy and/or immunological therapy [25]. A nearest research suggested that coding genes may be a promising prognostic biomarker of ccRCC, because there is a significant positive correlation between their expression and the low survival of ccRCC patients[26]. Meanwhile, other studies pointed out that lncRNAs may act as novel biomarkers for ccRCC diagnosis [27], prognosis [28], even in targeted therapy [29] and immunological therapy [30]. Previous studies usually explore the TIME and the curative effect of immunotherapy treatment via the signatures established by immune related genes (irGs) and irlncRNAs. Nowadays, a study proposed a novel statement for the first time that a novel signature based on the irlncRNAs pairs to predict the prognosis of patients with HCC [24], along with lung adenocarcinoma [31, 32] and colon adenocarcinoma [33]. Therefore, we are the first to explore the prospects of this novel model in ccRCC.

In this study, we extracted the expression data of ir-genes and lncRNAs from TCGA database and further obtained 90 DEirlncRNAs including 74 upregulation and 16 downregulation from 433 irLncRNAs by performing a differential co-expression analysis (Fig. 2). Then, 17 lncRNA-pairs identified by an iteration loop and a 0-or-1 method from 2667 pairs were applied to create the ROC curve, thereof, we got the most ideal pairs. Third, these results were further validated by the 1-, 3-, and 5-year ROC curves and 5-year clinical ROC curves with clinical characteristics, and then, the best cut-off point is determined according to the maximum AUC value (Fig. 3). Finally, we established a risk model according to the cut-off point and divided 526 patients with ccRCC into high (n = 253) and low-risk group (n = 273), and analysed the relationship between the risk score and the overall survival (OS) of patients and clinical pathological characteristics, respectively (Figs. 4 and 5). There results were revealed that the high-risk subgroups with ccRCC possessed the worse OS compared with the low-risk subgroups, as well as higher grade, T-, and M-stage, implying the prosperity of the novel model and the potency of the significant irlncRNA-pairs as the prognostic biomarker.

Formerly researches about lncRNAs as biomarkers were mainly divided into two ways. One is concentrated in a single abnormally expressed lncRNA, considering that it may potentially participate in the molecular mechanism of renal clear cell carcinogenesis and development. For example, Hong et al. considered that lncRNA HOTAIR may pass through mir-217/HIF-1α/Axl signaling pathway promotes the occurrence of ccRCC [34]. Hirata et al. considered that lncRNA MALAT1 promotes the invasiveness of ccRCC through miR-205/EZH2 axis [35]. Dong et al. pointed out that lncRNA GAS5 may act as a

competitive endogenous RNA (ceRNA) in competitive binding with miR-223 and indirectly regulate hZIP1, so as to regulate the progression of ccRCC [36]. Gang Wang et al. demonstrated that lncRNA OTUD6B-AS1 not only indicates poor prognosis but also inhibits ccRCC proliferation via the Wnt/β-catenin signaling pathway [37]. The other category pays more attention to multiple abnormally expressed lncRNAs, believing that their combination may better improve the predictive value of OS in ccRCC. For instance, Zeng et al. exploited a risk model accordin to the expression of 6 novel lncRNAs (, CTD-2263F21.1, LINC01510, CTA-384D8.35, RP11-395B7.2, RP11-352G9.1, RP11-426C22.4), which showed advantageous prognostic value for ccRCC [38]. Qu et al. constructed a classifier based on 4 lncRNAs expression (ENSG00000255774, ENSG00000248323, ENSG00000260911, ENSG00000231666), which has magnificent potential in predicting the OS of patients with stage I-III ccRCC [39]. However, as previously mentioned, the efficiency of these markers based on lncRNAs are mainly affected by their own expression levels. To circumvent this limitation, we performed a novel risk model dependent on the 17 irlncRNAs pairs obtained through iteration loop, 0-or-1 method and LASSO regression analysis, of which the critical value was acquired according to the cut-off point and implemented to divide patients into different risk groups. This novel model was not only independent of the expression level of irlncRNAs, but also did not distinguish risk groups just by the median value of the risk score. And we found that the model had an advantage of clinical applicability by analysing the relationship between risk subgroups and clinicopathological characteristics in ccRCC.

Some studies have shown the potential function of lncRNAs to regulate the TIME in ccRCC [40]. For instance, lncRNA MIR155HG is thought to be associated with immune checkpoint expression and immune cell infiltration in ccRCC [41]. What's more, an increasing number of irlncRNAs were manifested the promising and important prognosis prediction of ccRCC patients, including the potential clinical value of immunotherapy [42, 43]. For instance, Zhong et al. implied that 14 irlncRNAs are regarded as a prognostic signature to evaluate the OS of ccRCC patients because of their management with the level of immune cell infiltration [44]. Therefore, considering that the targeted gene in this study was the irlncRNAs pairs, we consequently investigated the features of TIME in high/risk subgroups with ccRCC produced based on the risk model. First, we estimated the immune cell infiltration in high/risk subgroups by performing 7 softwares and found that most types of immune cells prefer to infiltrate in subgroups with high risk (Fig. 6), which was consistent with the forepassed research results [45] that more numerous immune cells infiltrate in the tumor tissue of patients with more advanced ccRCC whether they are immune killer cells (such as $CD8^+$ T cell) or immunosuppressive cells (such as regulatory T cell). Meanwhile, we discovered that the classical immune checkpoint genes including PD-1 (PDCD1) and PD-L1 (CD274) were higher level in subgroups with high risk, which hinted that immune cells infiltrating in tumor tissue may lose their immunogenicity, resulting in the appearance of immunosuppression. Just as Braun DA thought, not all immune killer cells infiltrating in ccRCC can fulfill effective immunity lethality [46]. In addition, the model also suggested that subgroups with high risk were nonsensitivity to chemotherapeutics and targeted therapeutics, which reflected the characteristic of ccRCC patients to drugs, consistenting with the phenomenon in clinical.

In addition, our research still has a few limitations similar to the previous studies. First of all, the deviation of the consequence can not be ignored because all data producing them not only is obtained from public resources but also conducted by the public R package. Second, there are currently no ccRCC samples for the treatment of immunotherapy drugs in TCGA database, so the novel model can not reflect the sensitivity of ccRCC patients to immunotherapy drugs. Finally, the significant 14 irlncRNAs pairs based on the risk model, lack of clinical data validation due to all data obtained from public databases, which will be solved by recollecting ccRCC samples in clinical and further verifying the validity of these signatures.

In summary, according to the significative irlncRNA pairs, this study discovers a significant signature via a risk model constructed by a novel algorithm independent of the expression of irlncRNAs, which could predict the prognosis for patients with ccRCC and might assess the clinical effectiveness of immunotherapy drugs.

Materials And Methods

Obtained, Sorted and differential expression analysis of transcriptome data

First, we obtained the KIRC transcriptome data from TCGA database, which is consistent with fragment perkilobase million (FPKM). Next, for follow-up analysis, we gained the GTF files from the Ensemble database to distinguish between lncRNA and mRNA. After that, the known immune-related genes (ir-genes) were downloaded from ImmPort database (http://www.immport.org) to facilitate the screening of immune-related lncRNA through co-expression methods. Finally, we performed a co-expression analysis between all lncRNA and ir-genes. Which lncRNAas with correlation coefficient greater than 0.7 and P value less than 0.001 are called as irlncRNAs. We used R-package limma to analyze the differential expression of irlncRNAs to obtain DEirlncRNA. The thresholds were set to log fold change (FC) > 2 and the false discovery rate (FDR) < 0.001.

Construction of DEirlncRNAs pairs

The DEirlncRNAs were cyclically singly paired. In short, we divide the expression of lncRNA-A by lncRNA-B to get the coefficient C. If C is greater than 1, it is marked as 1, otherwise it is 0, so we can get a 0-or-1 matrix. Then, we further analyze the matrix. No relationship was considered between pairs and prognosis if the expression quantity of lncRNA pairs was 0 or 1 because pairs without a certain rank could not properly predict patient survival outcome. It was considered a valid match if the amount of lncRNA pairs with 0 or 1 expression represented more than 20% of the all pairs.

Acquisition of patients' clinical data

We downloaded the clinical data of KIRC patients from TCGA database. The valid data were obtained by deleting data with a follow-up time of 0 days, as well as duplicates.

Construct of a Risk Model for assessment of the RiskScore.

First, univariate analysis was performed, followed by 10 fold cross-validation of Lasso regression, with a p value of 0.05. Lasso regression was performed in 1000 cycles, and 1000 times random stimulations were set in each cycle. Next, record the frequency of each pair in the 1000-times-repeated lasso regression model, and the pair with a frequency of more than 100 times was selected for Cox proportional hazards regression analysis, and build the model. Then, we draw the ROC curve of each model and calculate its AUC value. If the curve reaches the highest point, which means the maximum AUC value, the calculation process was terminated, and the model was taken as the best candidate model. The ROC curves of 1-, 3- and 5- years were drawn. We calculated the risk score of the risk model for all clinical cases by the following formula: RiskScore= . In order to determine the maximum inflection, the AIC values of every point of the 5-year ROC curve were evaluated, which was defined as the cut-off point to distinguish between high or low RiskScores.

Validation of the Risk Model

To verify the cut-off point, the Kaplan-Meier analysis was performed to show the difference between patients in the high- or low-risk group, as well as visualization of the survival curve. R tool was used to visualize the specific risk-Score values of each sample in the model. The R packages applied in these procedures contain survminer, survival, survivalROC, pbapply, glmnet and pheatmap.

Next, we analyzed the relationship between the model and clinicopathological characteristic by Chi-square test to better verify the availability of our model. The bar chart was used for visualization and was marked as follows: <0.001=***, <0.01=**, and <0.05=*. The riskScore differences among different clinicopathological characteristic was computed by Wilcoxon signed-rank test. The results were shown by a box diagram. Furthermore, we performed univariate and multivariate analysis among the clinicopathological features and riskScore, which confirmed that the model can be used as an independent predictor of clinical prognosis and the result was showed by forest map. The R packages used in these analysis were survival, pHeat-map and ggupbr.

Studys on tumor infiltrating immune cells

As everyone knows, tumor immunization plays a significant role in the development of tumors. So, is there a relationship between our risk model and tumor immune cells? How's the relationship? In order to answer the above questions we take the currently accepted algorithms into account to calculate the immune infiltration statues among the samples from the TCGA project of the KIRC dataset, including TIMER, CIBERSORT, XCELL, QUANTISEQ, MCPcounter, EPIC, and CIBERSORT. The difference in the content of immune infiltrating cells between high and low risk groups was analyzed by Wilcoxon signed-rank test; The results are shown in a box chart. The correlation between risk score and immune infiltrating cells was analyzed by Spearman correlation analysis. The results of the correlation coefficient was visualized by LollipopT chart. P < 0.05 was considered statistically significant. The R package used in these analyses was ggplot2.

Guiding significance of the model for clinical treatment

To assess the clinical value of the model in the treatment of ccRCC, we calculated the IC50 of commonly administrated chemotherapeutic drugs in the TCGA project on the KIRC dataset. Antitumor drugs such as Cisplatin, Paclitaxel, KU.55933, Sunitinib, Gefitinib and Sorafenib are recommended for liver cancer treatment by AJCC guidelines. The difference of IC50 between high and low groups was compared by Wilcoxon signed-rank test, and the results are shown as box charts. The R packages used in these analyses were pRRophetic and ggplot2.

Declarations

Acknowledgement

The author would like to express his sincere thanks to the research team of TCGA database, who provided data for this model construction.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests exist.

Funding

This work was supported by National Natural Science Foundation of China [grant No.81972374] Postdoctoral Science Foundation of China [grant No.2019M662080].

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Figures

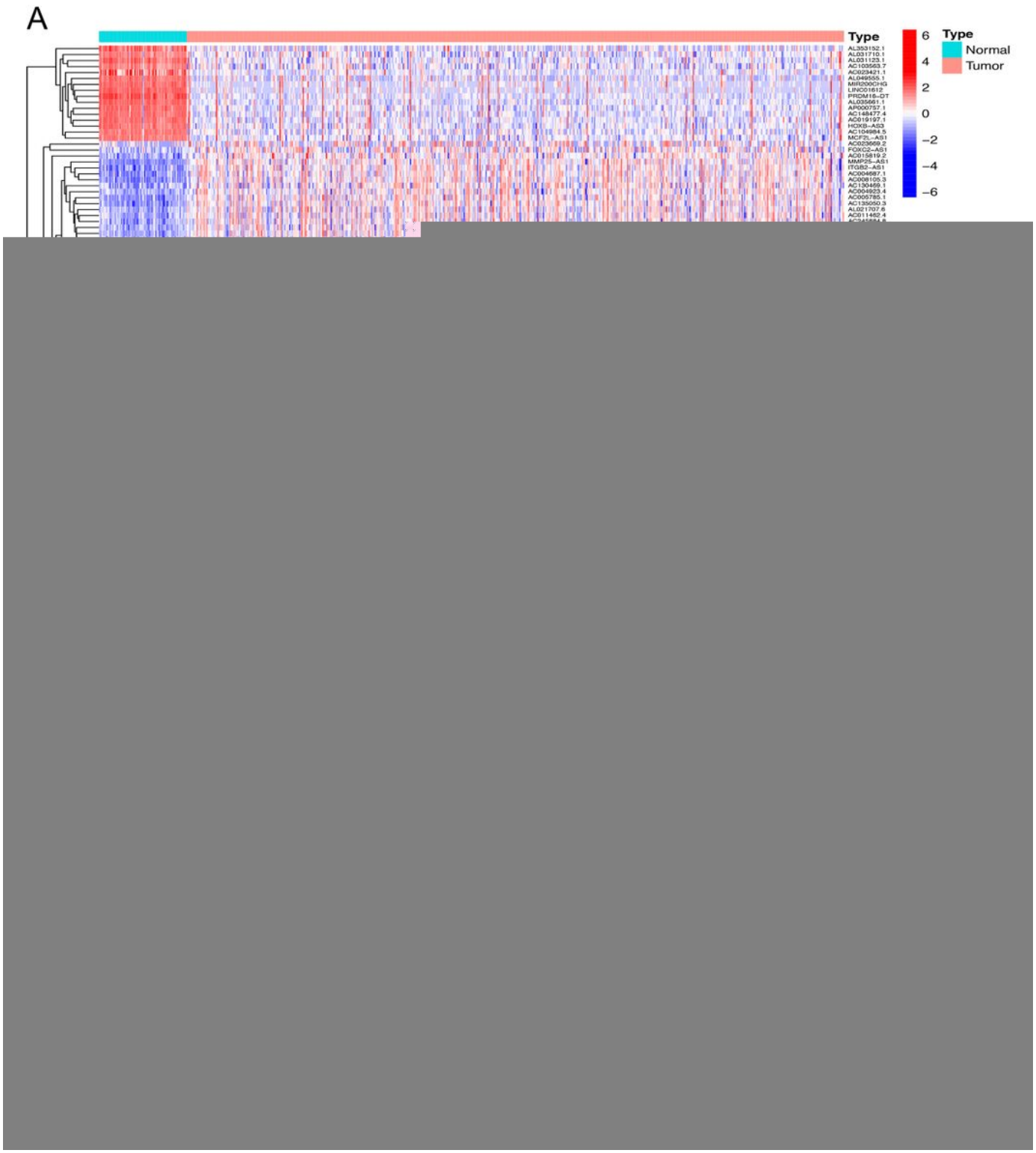

Figure 1

Construction of a risk assessment model using DEirlncRNA pairs. (A and B) Heatmap (A) and Volcano map (B) were drawn according to DEirlncRNA results. (C) The forest map showed 17 DEirlncRNA pairs determined by Cox regression analysis.

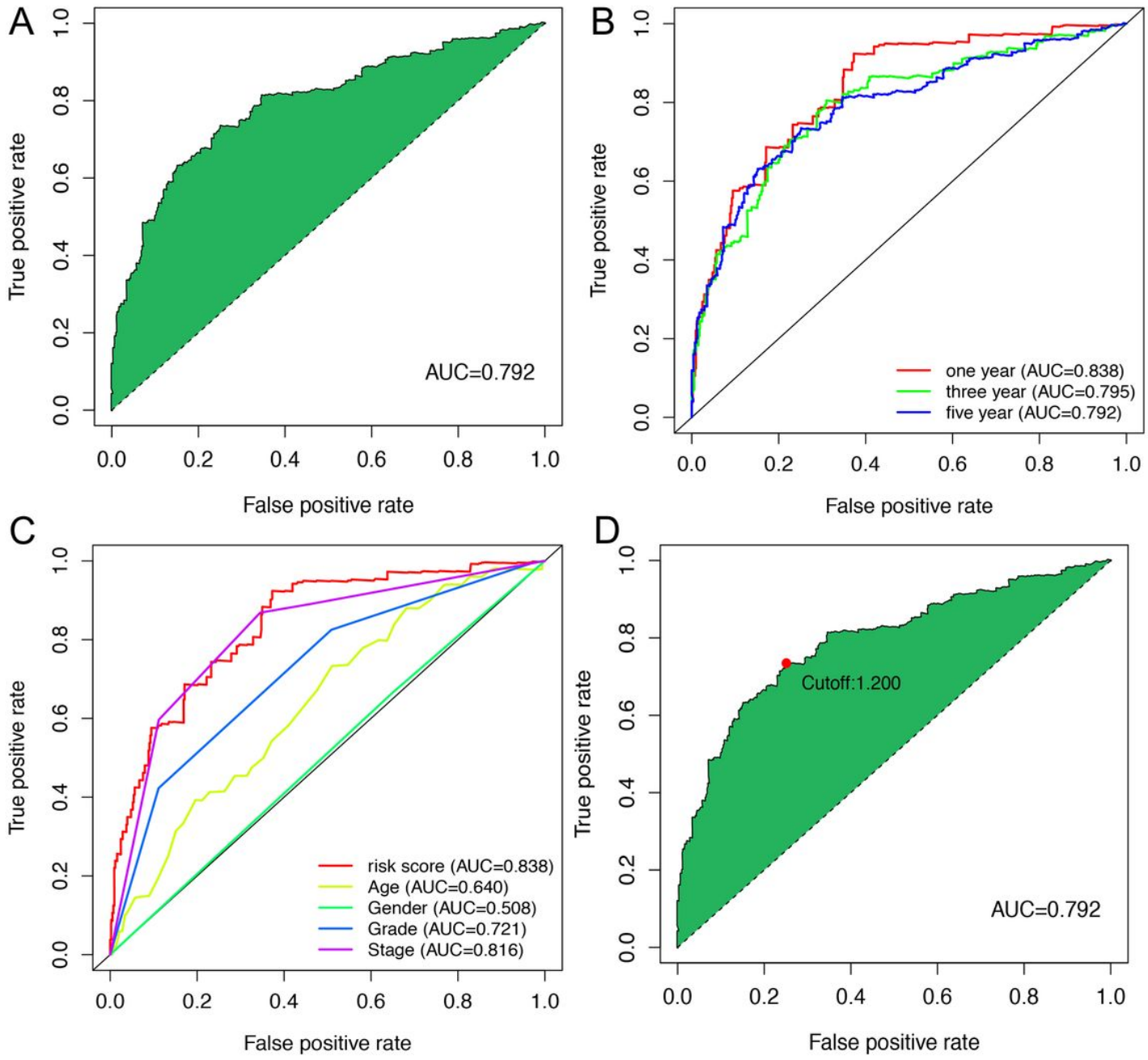

Figure 2

Construction of a risk assessment model using DEirlncRNA pairs. (A) The ROC of the optimal DEirlncRNA pair model. (B) The 1-, 3-, and 5-year ROC of the optimal model suggested that all AUC values were over 0.792. (C) A comparison of 5-year ROC curves with other common clinical characteristics showed the superiority of the riskScore. (D) RiskScore for 539 patients with KIRC; the maximum inflection point is the cut-off point obtained by the AIC

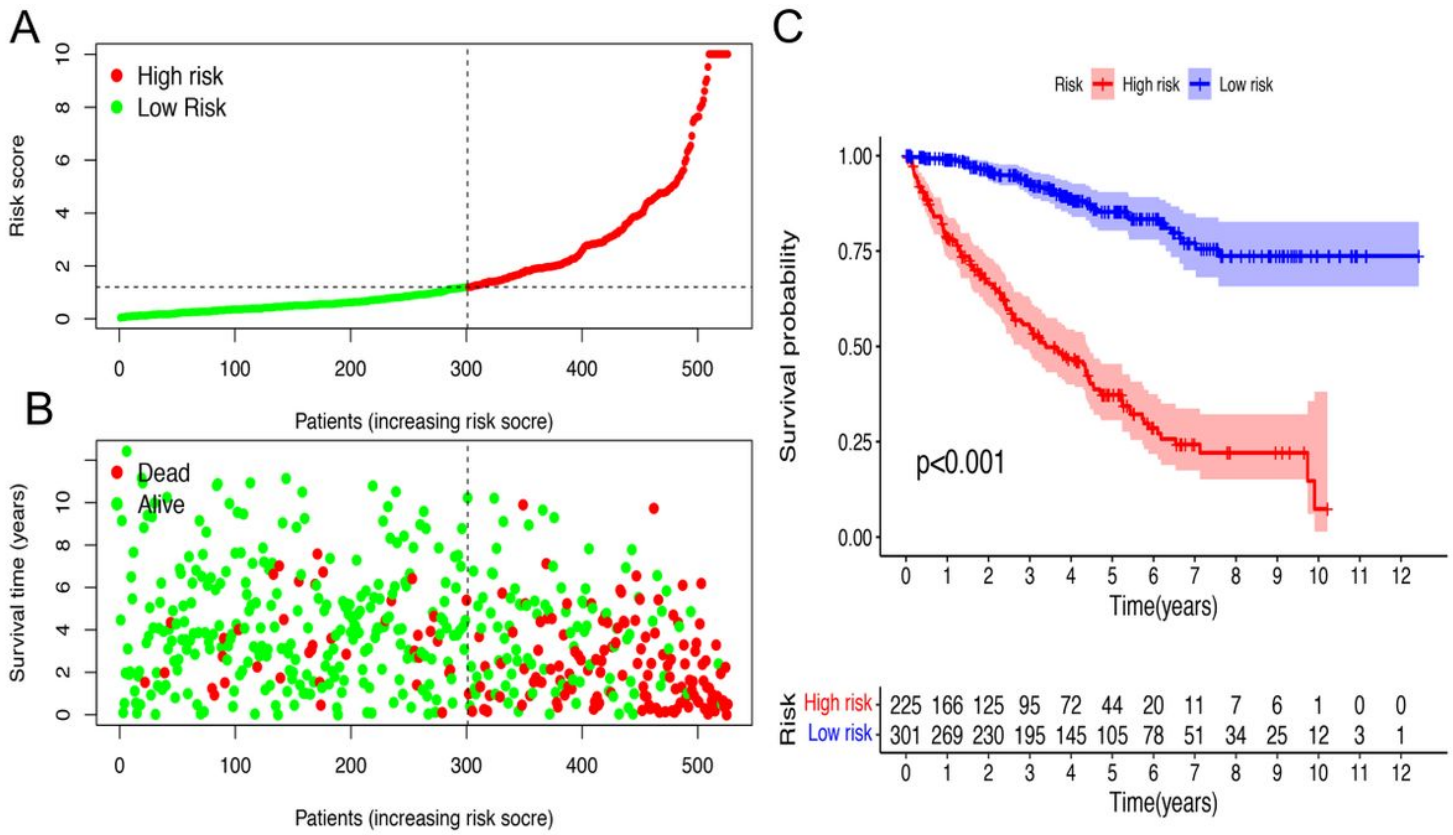

Figure 3

Risk assessment model for predicting prognosis. (A) Risk score; (B) Survival outcome (C) Patients in the low-risk group experienced a longer survival time tested by the Kaplan-Meier test.

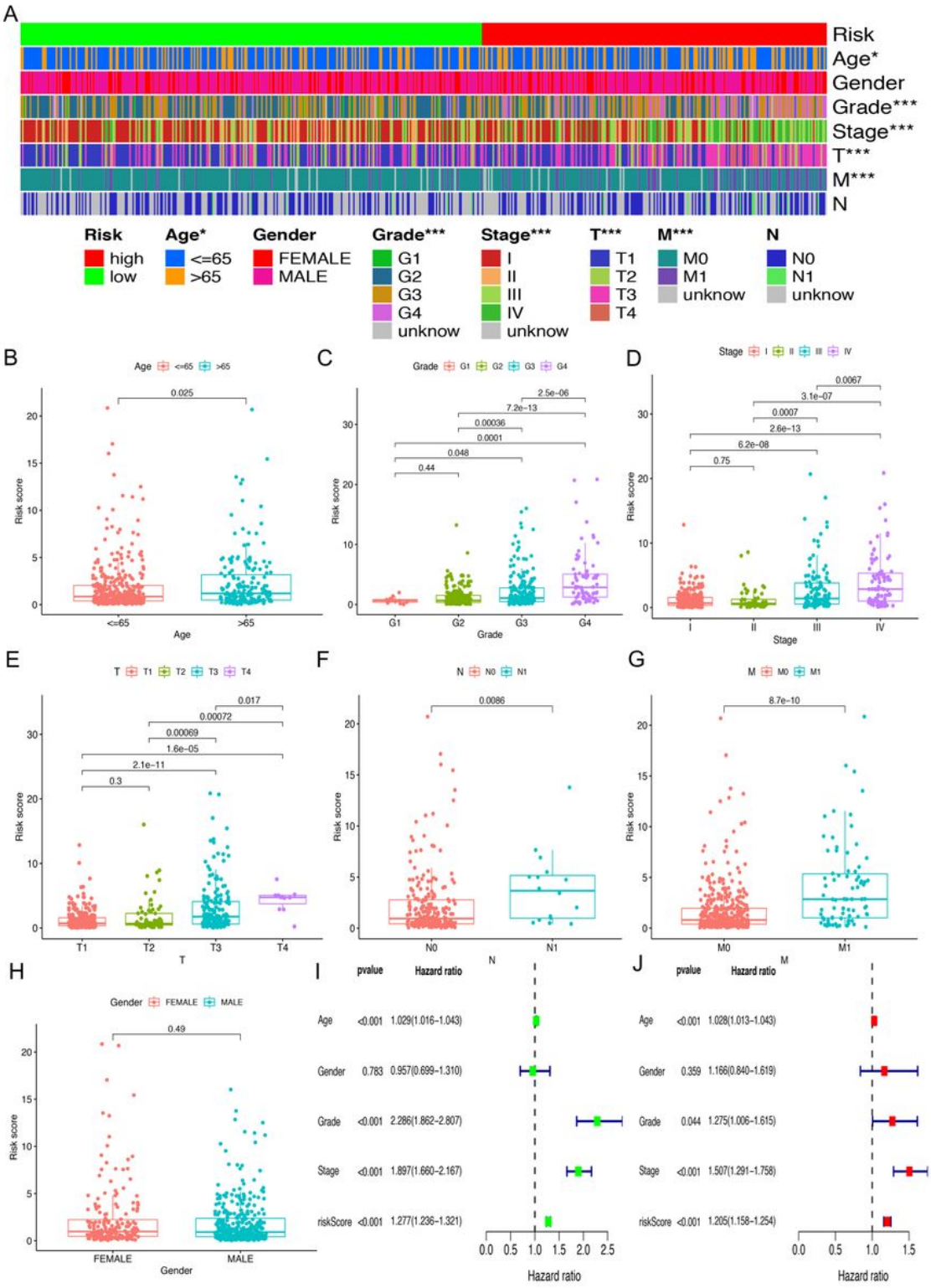

Figure 4

Application of risk assessment model for clinical evaluation. (A) Clinical correlation heat map; (B) Age; (C) Grade; (D) clinical stage; (E) T stage; (F) N stage; (G) M stage; (H) Gender; (I) The univariate Cox hazard ratio analysis results. (J) The multivariate Cox regression analysis results.

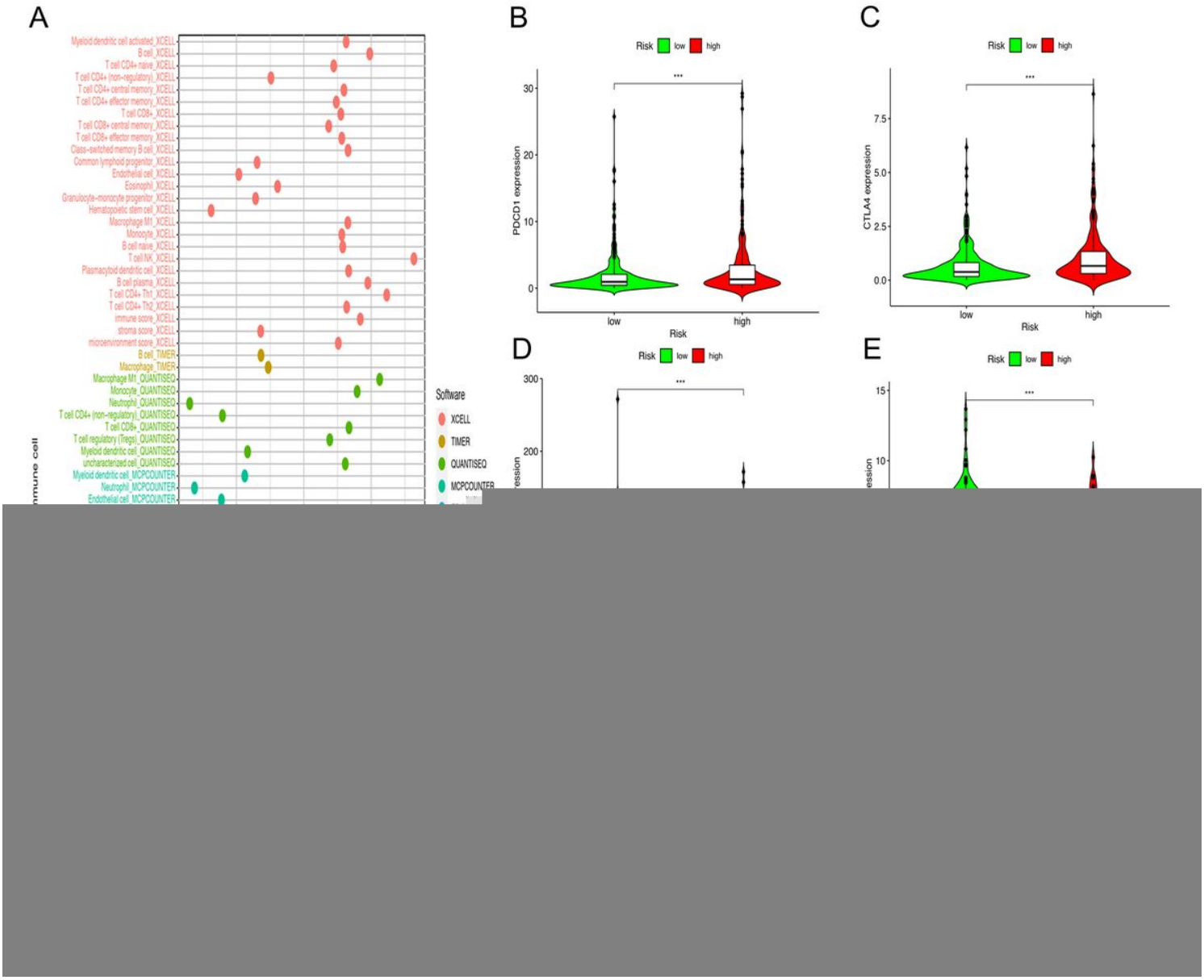

Figure 5

Evaluation of tumor immune cells and immunosuppressive molecules by risk assessment model. (A) Spearman correlation analysis showed that the correlation between patients in high or low risk group and immune cells. (B–C) High risk scores were positively correlated with upregulated (B) PDCD1, (C) CD274; (D–F) High risk scores were negatively correlated with low expression of (D) EGFR, (E) MTOR, (F) FLT3 and (G) CD274 levels in patients with KIRC.

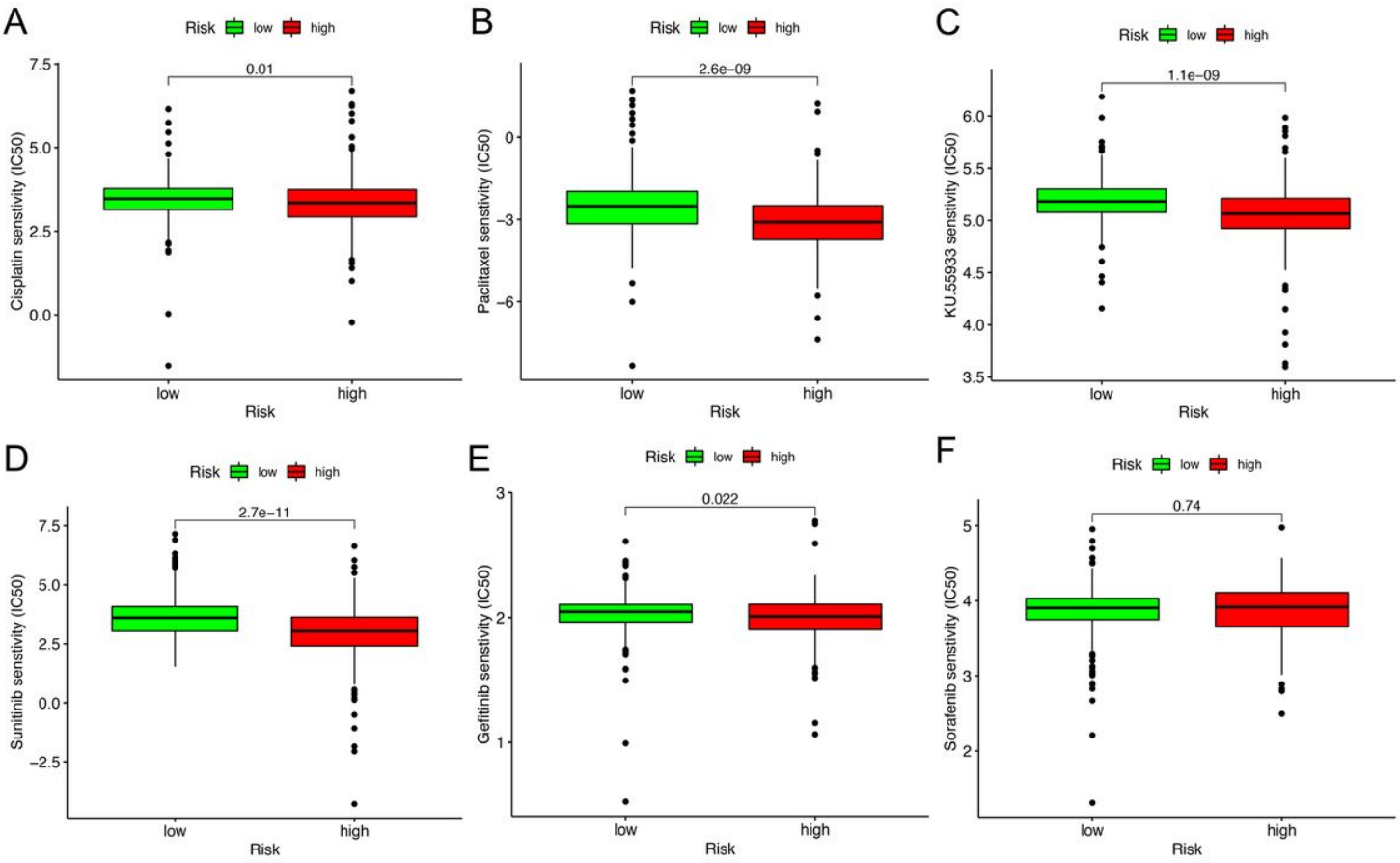

Figure 6

Calculation of the IC50 of KIRC related drugs by the risk assessment model. The model acted as a potential predictor for chemosensitivity as high risk scores were related to a lower IC 50 for chemotherapeutics such as (A) Cisplatin, (B) Paclitaxel, (C) KU.55933, (D) Sunitinib, and (E) Gefitinib. However (F) Sorafenib showed no statistically significant.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementalInformation.docx
- SupplementarymaterialsS1irlncRNAs.xls
- SupplementarymaterialsS2DEirlncRNAs.xls
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