A Risk Assessment Model of Immune-Associated lncRNA to Predict the Prognosis and Immune Landscape of Renal Clear Cell Carcinoma

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Abstract

Renal cell carcinoma (RCC) is one of the most common malignancies of the urinary system, accounting for 3% of adult malignancies. Long non-coding RNA (lncRNA) is abnormally regulated in many cancers and can be used as a molecular marker for early diagnosis and prognosis of RCC. Here, original lncRNA datas were retrieved from TCGA, differential co-expression analysis was performed to classify immune-related lncRNA (irlncRNA) with differential expression, and the improved 0 or 1 matrix cyclic single pairing method was used to verify lncRNA pairs. Then, we performed a univariate analysis in combination with an improved Lasso penalty regression that included cross-validation, multiple repetitions, and random stimulus procedures to determine different expression irlncRNA (DEirlncRNA) pairs. AUC values under Receiver Operating Characteristic curve (ROC) were calculated to obtain the optimal model, and AIC values of each point on AUC were calculated to obtain the optimal cut-off point to distinguish the high and low risk groups of Clear-cell renal cell carcinoma (ccRCC) patients. Finally, we evaluated the new model in a variety of clinical settings including survival, clinicopathological features, tumor-infiltrating immune cells, chemotherapy, and checkpoint related biomarkers, all showing promising clinical application.

Introduction

Renal cell carcinoma (RCC) is one of the most common malignancies of the urinary system, accounting for 3% of adult malignancies[1]. The most common pathological type of RCC is Clear-cell renal cell carcinoma (ccRCC), which has a high incidence and poor prognosis[2]. Although ccRCC is a disease that can be detected early and successfully treated with surgical or ablative strategies, up to one-third of cases will develop or progress to metastasis[3]. Among the deadliest types of cancer, metastatic ccRCC has a 5-year survival rate of no more than 10%, with a median survival of only 13 months[4], mainly due to early diagnosis failure and chemoradiotherapy resistance. Currently, molecular biomarkers have been reported to guide the diagnosis, prognosis and treatment of patients with ccRCC, such as METTL14[5], NR1B2[6], EPB41L1[7] etc. And there are many drugs targeting ccRCC, including targeting PDGF, VEGF, MET and immune checkpoints, but the effectiveness of targeted therapy varies widely among patients[8]. Therefore, the selection of individual therapeutic agents is a huge challenge in clinical practice, and the identification of new biomarkers and predictive models for the treatment and prognosis of ccRCC is imperative.

Long non-coding RNA (lncRNA) is a kind of non-coding RNA whose length is more than 200 nucleotides[9]. In general, lncRNA do not encode proteins or peptides. In addition to the size of other types of lncRNA (microRNAs, small interfering RNAs, small nucleoli/nuclear RNAs), lncRNA have secondary and three-dimensional structures that enable them to function as both RNA and protein[10]. LncRNAs are poorly conserved and can regulate gene expression at different levels of histone modification, transcription, post-transcription and epigenetics[11, 12]. More and more evidence suggests that lncRNAs may play key roles in almost all biological processes, including stem cell maintenance, cell proliferation,

apoptosis, cell invasion and metastasis[13–15]. Many studies have found that lncRNAs are abnormally regulated in many cancers and are associated with cancer metastasis[14]. Many lncRNAs have been identified as prognostic markers of cancer by lncRNA array or RNA sequencing. For example, LUCAT1 is associated with breast cancer, ovarian cancer, thyroid cancer and renal cell carcinoma, and is highly expressed in liver cancer and other malignant tumors, which has been proved to induce a variety of malignant tumors[15]; LINC00659 promotes colorectal cancer cell proliferation, invasion, and migration via Mir-342-3p/ANXA2 axis[16]; LncRNA NBR2 inhibits hepatocellular carcinoma by regulating autophagy[17].

Tumor microenvironment plays an important role in tumor genesis and development. Dysregulation of immune system may be the main cause of cancer development, so immunotherapy has become a promising cancer treatment strategy[18]. The prognosis of patients with ccRCC is largely related to immunity, and precise regulation of immune gene expression is essential for the generation of robust immunity. However, most researches so far focused on genetic coding, particularly the function of cell surface receptors, cytokines and transcription factors. LncRNA can lead to tumor immune cell infiltration by directing the expression of genes related to immune cell activation, thus altering the immune microenvironment which leads to malignant phenotypes of cancer[19, 20]. Immune-related lncRNA signaling has shown prognostic and predictive value in early diagnosis, real-time evaluation and targeted therapy of cancer. Hong et al. [21]constructed an irlncRNA signal that did not require any specific expression level through pairing and iteration to evaluate the diagnostic efficacy, chemotherapy efficacy and tumor immune invasion in HCC patients. Shen et al. [22] identified 11 lncRNAs as prognostic markers of breast cancer, which are new and important prognostic factors independent of a variety of clinicopathological parameters. Wu et al. [23]identified the value of eight irlncRNA markers as predictors of bladder cancer prognosis and immunotherapy response. Compared with a single biomarker, two or more biomarkers have higher sensitivity and specificity in cancer diagnosis and prognosis prediction. In this study, we used a new modeling algorithm to construct an irlncRNA marker by pairing and iterating and to evaluate its diagnostic value, prognostic prediction, and tumor immune invasion in ccRCC patients.

Methods

We used the experimental method of Hong's article on the role of lncRNA in liver cancer for reference to study the immune infiltration and prognostic value of lncRNA in ccRCC.

Retrieval of Transcriptome Data, Preparation, and Differentially Expressed Analysis

Transcriptome data and corresponding clinical data of ccRCC were downloaded from TCGA database (https://tcga-data.nci.nih.gov/tcga/). GTF files are downloaded from Ensembl (http://asia.ensembl.org) to annotate the transcriptome data to distinguish mRNA and lncRNA for subsequent analysis. Then we download a copy of the immune related genes - gene (ir) list from ImmPort database (https://www.immport.org/home), and screened the irlncRNA through co-expression. Correlation analysis

was conducted between ir-gene obtained and all lncRNAs. LncRNAs with correlation coefficient of immune genes greater > 0.4 and P value < 0.001 were considered as irlncRNAs. The limma package in R software was used for differential expression analysis of irlncRNAs, and the threshold was set as log fold change (FC) > 1.5 along with false discovery rate (FDR) < 0.05 to obtain DEirlncRNA.

Pairing DEirlncRNAs

The DEirlncRNA obtained by the above process was used to construct the immune lncRNA pair. If the expression amount of the former is greater than that of the latter, it is denoted as 1; otherwise, it is denoted as 0 to obtain the matrix with row name lncRNA and column name sample 0 or 1. When the number of lncRNA pairs with expression level of 0 or 1 accounted for more than 20% of the total number of pairs, it was considered as an effective match. The use of lncRNA pairs can avoid batch correction, and only need to consider the comparison of lncRNAs within data, rather than the correction of lncRNAs between data, which is conducive to the clinical application of the model.

Establishment of a Risk Model to Evaluate the riskScore

Univariate analysis was performed first, followed by Lasso regression and 10-fold cross-validation, with a p value of 0.05. Lasso regression loops were performed 1000 times, with 1000 stimuli randomly assigned to each loop. Then, the frequency of each pair of 1000 repetitions in Lasso regression model was recorded, and pairs with a frequency of more than 100 repetitions were selected for Cox proportional risk regression analysis, and the model was constructed. Calculate the AUC value of the model and draw the curve. When the curve reaches the highest point, namely the maximum AUC value, the calculation process is terminated and the model is taken as the optimal candidate model. ROC curves of the model for 1, 3 and 5 years were drawn. The riskScore of all clinical cases was calculated using the constructed risk model. The AIC value of each point of the ROC curve was evaluated to determine the maximum inflection point, which was used as the cut-off point to judge the riskScores.

Validation of the Constucted Risk Model

To verify this cut-off point, we used Kaplan-Meier analys to analyze differences in survival between high-risk and low-risk patients. To verify the clinical value of the constructed model, the Chi-square test was used to analyze the association between the model and clinicopathological features. To test whether this model can be used as an independent clinical prognostic factor, univariate and multivariate Cox regression analyses were performed on the risk model and clinicopathological features.

Investigation of Tumor-Infiltrating Immune Cells

To analyze the relationship between the risk model and immune cell characteristics, we used TIMER, CIBERSORT, XCELL, QUANTISEQ, MCPcounter, EPIC, and CI-BERSORT methods to calculate the immune infiltration status between samples. The Wilcoxon signed-rank test was used to determine differences in the content of various immune infiltrating cells between the high-risk and low-risk groups. Spearman correlation analysis was conducted to analyze the relationship between risk score and immune infiltrating

cells. The correlation coefficients of the results are shown in the lollipop chart. Significance threshold was set as $P < 0.05$. The program is executed using the R ggplot2 package.

Exploration of the Significance of the Model in the Clinical Treatment

To evaluate the application of this model in the clinical treatment of ccRCC, we calculated the IC50 of commonly used chemotherapy drugs including Vinblastine, Sunitinib, Rapamycin, Mitomycin.C, Cisplatin and Temsirolimus in the TCGA database. IC50 between high-risk and low-risk groups were compared using the Wilcoxon signed-Rank test.

Results

Identification of DEirlncRNAs

First, transcriptome data of renal clear cell carcinoma were retrieved from the Cancer Genome Atlas (TCGA) database, including 72 normal samples and 539 tumor samples, and the obtained transcriptome data were divided into mRNA and lncRNA groups. Then, through online website IMMPORT list (https://www.immport.org/home) to download the immune related genes. lrlncRNAs were identified by co-expression of immune-related genes and lncRNAs. A total of 1318 irlncRNAs were identified, and 689 were differentiated as differentially expressed irlncRNAs (Figure 1A), of which 581 were up-regulated and 108 down-regulated (Figure 1B).

Establishment of DEirlncRNA Pairs and a Risk Assessment Model

The irlncRNA obtained has been displayed in pairs, and a table containing 148484 valid DEirlncRNA pairs with row name irlncRNA pair and column name sample name is obtained. If the previous lncRNA expression level is higher than the latter, it will be labeled as 1; otherwise, it will be labeled as 0. Then irlncRNA and survival data were combined to obtain 22 irlncRNA pairs associated with prognosis (Figure 2A), and then the irlncRNA prognostic model was constructed. Then, we calculated that the area under ROC curve (AUCs) of 22 pairs of DEirlncRNAs was 0.875, and the optimal cutoff was 2.444 (Figure 2B). According to the optimal cutoff, the patients were divided into low risk group and high risk group. The AUC values of the 1-year, 3-year and 5-year ROC curves of the model all exceeded 0.85 (Figure 2C), and the comparison between the 5-year ROC curve and other clinical data showed that the AUC of the risk model was higher than that of age, sex, grade and stage (Figure 2D).

Clinical Evaluation by Risk Assessment Model

According to the previously confirmed cut-off points, patients were divided into the high-risk group and the low-risk group. The risk assessment and survival rate of each case were shown in Figure 3A and 3B.

400 was divided into the cut-off points of the high-low risk group, and the clinical outcome of the high-risk group was significantly worse than that of the low-risk group. Kaplan-Meier analysis showed that patients in the low-risk group had longer survival (P<0.001) (Figure 3C). We then produced a clinically relevant heat map (Figure 4A), which showed that tumor grade, clinical stage, T stage, M stage, and N stage were significantly associated with risk (P< 0.01). Subsequently, scatter plots of age (Figure 4B), tumor grade (Figure 4C) and clinical stage (Figure 4D), T stage (Figure 4E), M stage (Figure 4F), and N stage (Figure 4G) were plotted. Then, univariate and multivariate Cox regression analyses were performed to verify age (P<0.001, HR = 1.030, 95%CI [1.017 – 1.044]), tumor grade (P<0.001; 0.001, HR = 2.302, 95%CI [1.858 – 2.851]), clinical stage (P<0.001, HR = 1.926, 95%CI [1.678 – 2.209]), T staging (P<0.001, HR = 1.987, 95%CI [1.674 – 2.357]), M staging (P<0.001, HR =4.388, 95% CI[3.178 – 6.060]) and riskScore (P<0.001, HR = 1.041, 95% CI[1.034 – 1.048]) showed statistical difference (Figure 5).

Estimation of Tumor-Infiltrating Immune Cells and Immunosuppressive Molecules with Risk Assessment Model

Since immune-related lncRNAs were screened out by co-expression of immune-related genes and lncRNAs, we investigated whether this model was related to the immune microenvironment of ccRCC. We found that the high-risk group was positively correlated with tumor-infiltrating immune cells such as natural killer cells(NK), macrophages, T cell regulators (Tregs), CD4+ Th1, and B cells, while negatively correlated with neutrophils, hematopoietic stem cells, vascular endothelial cells, and CD4+T cells (Figure 6A). Due to the clinical use of immune checkpoint inhibitors (ICI) in the treatment of ccRCC, we investigated whether risk models were associated with ICI related biomarkers and found that PDCD1 (P < 0.05, Figure 6B), CTLA4 (P < 0.05, Figure 6C), LAG3 (P < 0.05, Figure 6D), TIGIT (P < 0.001, Figure 6E) were high expression, While HAVCR2 (p < 0.001, Figure 6F) was low expression in the high-risk group. However, ICOS (P > 0.05, Figure 6G) and IDO1 (P > 0.05, Figure 6H) showed no significant difference between the high and low risk groups.

Analysis of Correlation between the Risk Model and Chemotherapeutics

In addition to immune checkpoint blocking therapy, we sought to determine the association between this risk assessment model and the efficacy of common chemotherapeutic agents in ccRCC in the TCGA project of the LIHC dataset. And it turns out, Vinblastine (Figure 7A), Sunitinib (Figure 7B), Rapamycin (Figure 7C), Mitomycin. C (Figure 7D), Cisplatin (Figure 7E) and Temsirolimus (Figure 7F) were differences in sensitivity between the high and low risk groups, and their 50% inhibitory concentration (IC50) in the high-risk group was lower than in the low-risk group, suggesting that patients in the high-risk

group were more sensitive to these drugs. This suggests that the model can be used as a potential predictor of chemotherapy sensitivity.

Discussion

Renal cell carcinoma accounts for 3% of adult malignancies and is the deadliest of all urinary cancers. The most common type is ccRCC[24]. It has shown strong resistance to conventional therapies such as chemotherapy and radiotherapy, and 2-year survival rate in metastatic patients < 20%[25]. Due to the important role of the immune system in cancer, the immune microenvironment has attracted people's attention. The prognosis of ccRCC is mostly related to immunity. Studies have shown that CD8+ T cells in ccRCC are significantly correlated with the prognosis of patients[26]. Therefore, in order to improve the prognosis of ccRCC and provide reliable information for guiding correct individualized treatment strategies, it is urgent to screen out reliable immune predictors and prognostic indicators.

LncRNA is closely related to the occurrence, development and prognosis of cancer. It does not encode proteins, but in the form of RNA regulates gene expression at various levels, including epigenetic, transcriptional, or post-transcriptional regulation. The abnormal expression of some lncrnas may be related to the invasion, metastasis and poor prognosis of renal clear cell carcinoma[27]. LncRNA regulation of the immune microenvironment of ccRCC has become a research hotspot. Some studies have shown that a large number of different types of immune cells infiltrate around and in the stroma of ccRCC. However, current studies mainly focus on finding single genes as prognostic markers for patients with malignant tumors. These markers are based on quantitative expression levels of transcripts and may be subject to occasional errors. Therefore, in this study, two lncRNAs were used to form lncRNA pairs to construct a reasonable model to evaluate the prognosis and immune cell infiltration of patients with ccRCC.

First, we retrieved the original lncRNA data from TCGA, conducted differential co-expression analysis to classify DEirlncRNA, and verified the lncRNA pairs using the improved 0 or 1 matrix cyclic single pairing method. Second, we performed a univariate analysis in combination with an improved Lasso penalty regression that included cross-validation, multiple repetitions, and random stimulus procedures to identify pairs of DEirlncRNAs. Then, AUC values under each ROC were calculated to obtain the optimal model, and AIC values of each point on AUC were also calculated to distinguish the optimal cut-off points for HCC patients in the high and low risk groups. Finally, we evaluated the new model in a variety of clinical Settings including survival, clinicopathological features, tumor-infiltrating immune cells, chemotherapy, and checkpoint-related biomarkers.

In general, high abundance lncRNAs have important biological functions. GAS5 is a well-known lncRNA, as a tumor suppressor, its expression is down-regulated in bladder cancer, kidney cancer and other cancers, which is negatively correlated with tumor size, grade and prognosis[28]. Dasgupta et al. [29]investigated the role of the interaction between lncRNA CDKN2B-AS1 and miR-141-3p in the progression and metastasis of renal cancer. CDKN2B-AS1 overexpression was positively correlated with

poor overall survival in RCC patients, and miR-141 expression could also effectively distinguish malignant tissues from non-malignant tissues. Zhu et al. [30]found that lncRNA HIF1A-AS2 was highly expressed in renal carcinoma tissues and renal clear cell carcinoma cells. In vivo, HIF1A-AS2 interferes with cell proliferation, invasion and migration, and accelerates cell apoptosis. However, current studies mainly focus on the evaluation of cancer prognosis by single lncRNA, which is contingent to a certain extent, while two or more biomarkers have higher sensitivity and specificity in cancer diagnosis and prognosis prediction. Deng et al. [31]extracted 9 kinds of hepatocellular carcinoma related lncRNAs from TCGA database and established a model to analyze the prognosis and clinical characteristics of hepatocellular carcinoma, which is conducive to individualized treatment of cancer patients. Sun et al. [32]developed a new immune-related lncRNA marker and constructed 5 lncRNA prognostic models, which have important clinical significance for the prognostic prediction of renal cancer.

Based on Lasso Penalized Modeling proposed by Sveen et al. [33], we constructed a risk assessment model for renal clear cell carcinoma and improved the model to improve the prognostic accuracy. After differentiating the high and low risk groups with this new escalation, we reassessed survival outcomes, performed univariate and multifactorial clinicopathological analyses, and analyzed the efficacy of chemotherapy agents for ccRCC treatment, tumor immune invasion, and biomarkers associated with checkpoint inhibitors. This model only needs to detect pairs of high or low expression, rather than the specific expression value of each lncRNA, which has strong clinical practicability and can be used to distinguish high and low risk clinical cases.

Stephane Chevrier et al. identified 17 tumor-associated macrophage phenotypes, 22 T-cell phenotypes, and a unique immune component associated with progression-free survival by in-depth immunoassay of 73 patients with ccRCC and 5 healthy controls.[34] We used 7 commonly used immune infiltrating cell assessment methods, including TIMER, CIBERSORT, XCELL, QUANTISEQ, MCPcounter, EPIC and CIBERSORT-ABS, to explore the relationship between risk model and tumor infiltrating immune cells. Our results showed that natural killer cells, macrophages, T cell regulatory (Tregs), CD4+ Th1 and B cells which are tumor-infiltrating immune cells were positively correlated with DEirlncRNAs. Tumor infiltrating immune cells can regulate cancer progression, showing potential prognostic value. CD4+ helper T cells can target antigenic tumors and inhibit tumor growth[35]. CD4+ T cell infiltration regulates the proliferation of renal cell carcinoma cells by regulating TGFβ1/YBX1/HIF2α signaling[36]. Macrophages are a very important immune group in tumor immunity, which can promote or prevent tumor development and metastasis[37], high expression of M2 macrophages in KIRP is often associated with poor clinical prognosis[38].

For renal cell carcinoma, based on Hsieh et al. [39]the US Food and Drug Administration approved immune checkpoint inhibitors as second-line treatment for advanced renal cancer in 2015, bringing the treatment of advanced renal cancer into the era of immunotherapy. Immune checkpoint mainly refers to the negative regulatory molecules in the body's immune system that maintain tolerance and regulate immune response[40], mainly include cytotoxic T cell associated protein-4 (CTLA-4), programmed death molecule-1

(PD-1), lymphocyte activation gene-3 (LAG-3), IDO, etc[41, 42]. Immune checkpoint inhibitors can inhibit tumor growth by specifically blocking immune examination sites and eliminating their immunosuppressive function. Therefore, it is very important to determine the expression level of immune checkpoint related genes in patients for the accurate selection of immune checkpoint inhibitors[43]. In the high-risk group of our risk assessment model, the expression of PDCD1, CTLA4, LAG3 and TIGIT immune checkpoint genes was high, while the expression of HAVCR2 decreased, suggesting that PDCD1, CTLA4, LAG3 and TIGIT immune checkpoint inhibitors may have better efficacy for patients in the high-risk group.

Advanced metastatic ccRCC is preferred for targeted therapy, with different targeted drug pathways. VEGFR1-3 and c-KIT inhibitors include Asitinib, Prazopanib, Sorafenib, Sunitinib and Capotinib; VEGFR2 antibodies include Bevacizumab and EGFR inhibitors include Erlotinib. Among them, Sorafenib could inhibit Raf nodes in RAS-ERK pathway, while Temsirolimus, Everolimus and Captinib could inhibit mTOR nodes downstream of PI3K pathway[44, 45]. Therefore, determining whether patients are resistant to chemotherapy drugs is the premise of individualized treatment. In our risk assessment model, the sensitivity of commonly used targeted chemotherapy drugs Vinblastine, Sunitinib, Rapamycin, Mitomycin.C, Cisplatin and Temsirolimus is different between high and low risk groups. And their 50% inhibitory concentration (IC50) in the high-risk group was lower than in the low-risk group, suggesting that patients in the high-risk group were more sensitive to these drugs. They can as a potential predictor of chemotherapy sensitivity.

Declarations

Author Contributions

TM conceived and designed the study. TM, CZ, and YD performed the literature search and extracted the data. LC, JL, and DL analyzed the data and summarized results. CZ, YD and LC performed the validation. TM drafted the manuscript. GY and MY revised and proofread the manuscript. All authors contributed to the article and approved the submitted version.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Figures

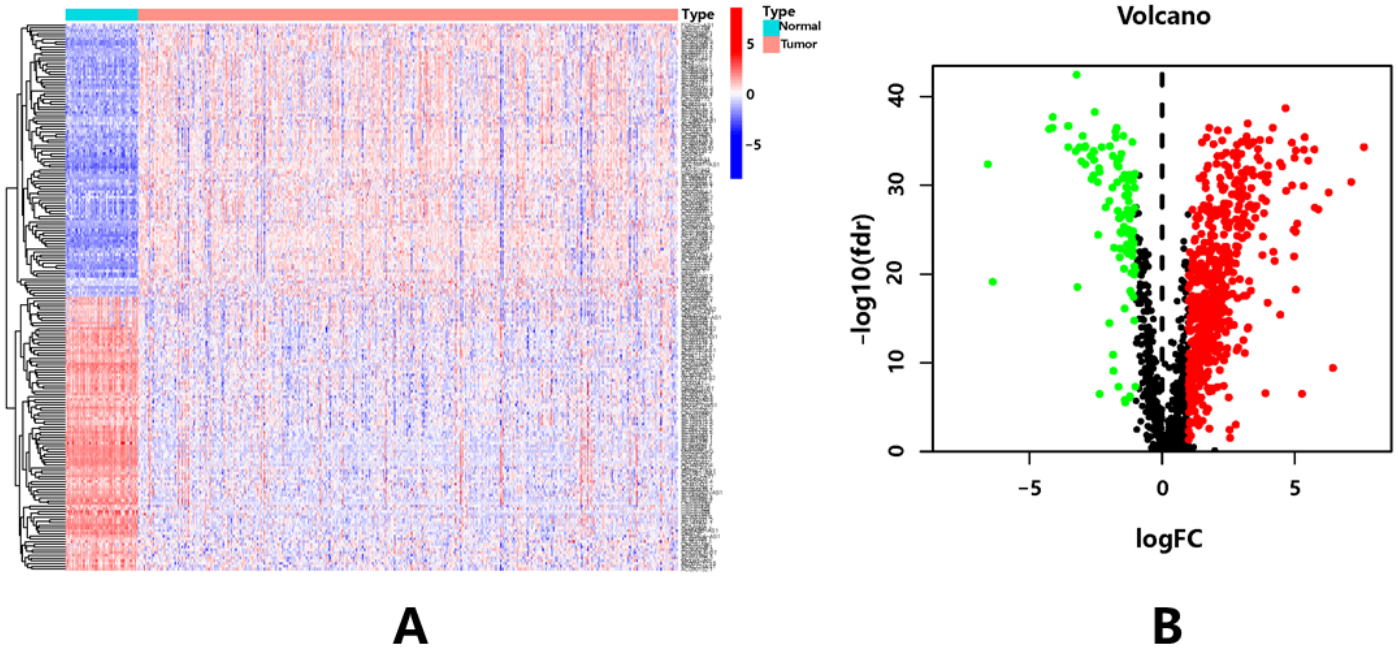

Figure 1

Differentially expressed immune-associated lncRNAs Differentially expressed immune-related lncRNAs (DEirlncRNAs) were identified using TCGA datasets and Ensembl annotations (A) as heat maps and (B) as volcano maps.

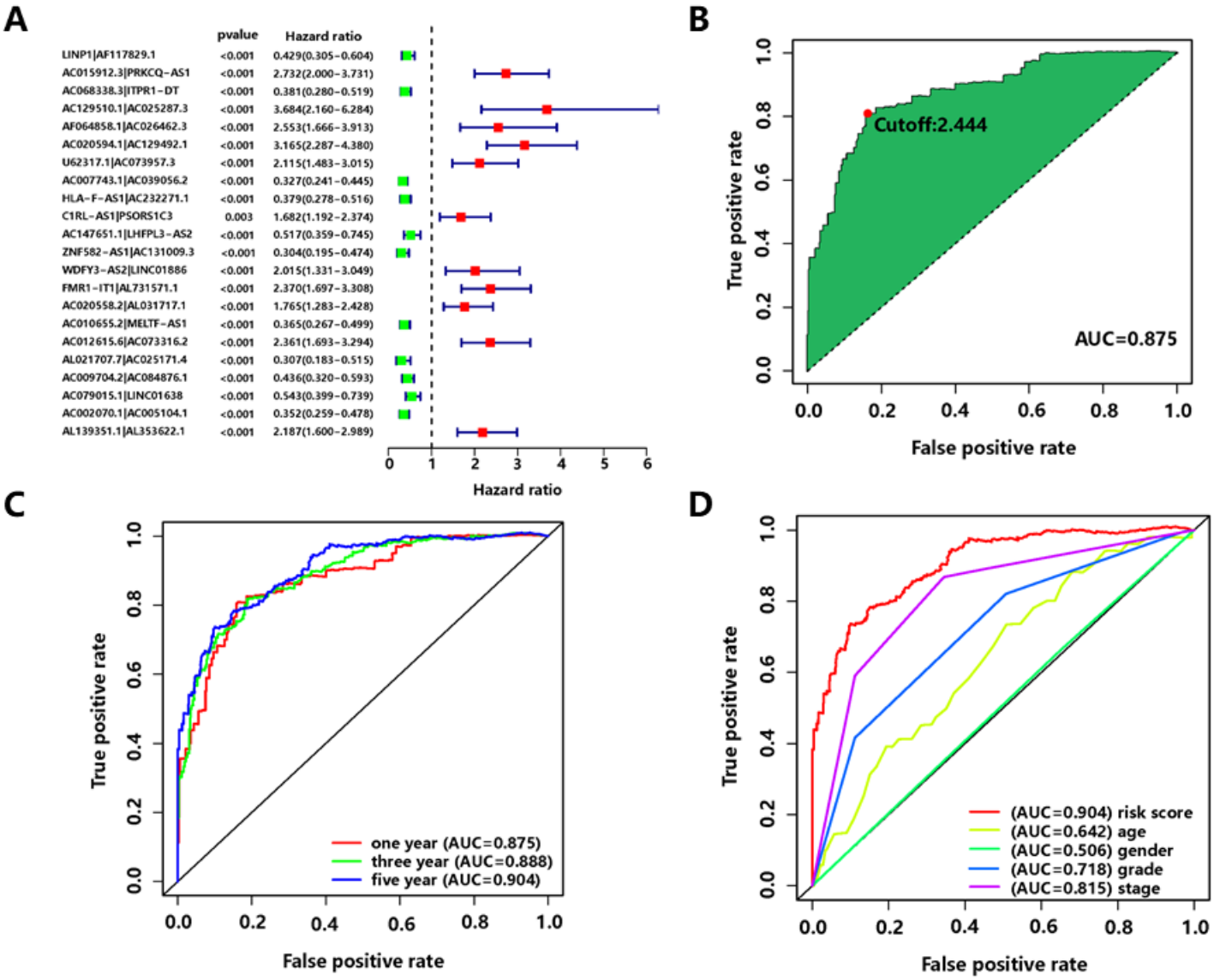

Figure 2

Risk assessment model was established using DEirlncRNA pair (A) The forest map shows 22 pairs of DEirlncRNAs progressively determined by Cox proportional risk regression; (B) The area under ROC curve (AUCs) of 22 to DEirlncRNAs is 0.875, and the optimal cutoff is 2.444; (C) ROC curves of 1, 3 and 5 years; (D) ROC curves at 5 years were compared with other clinical data.

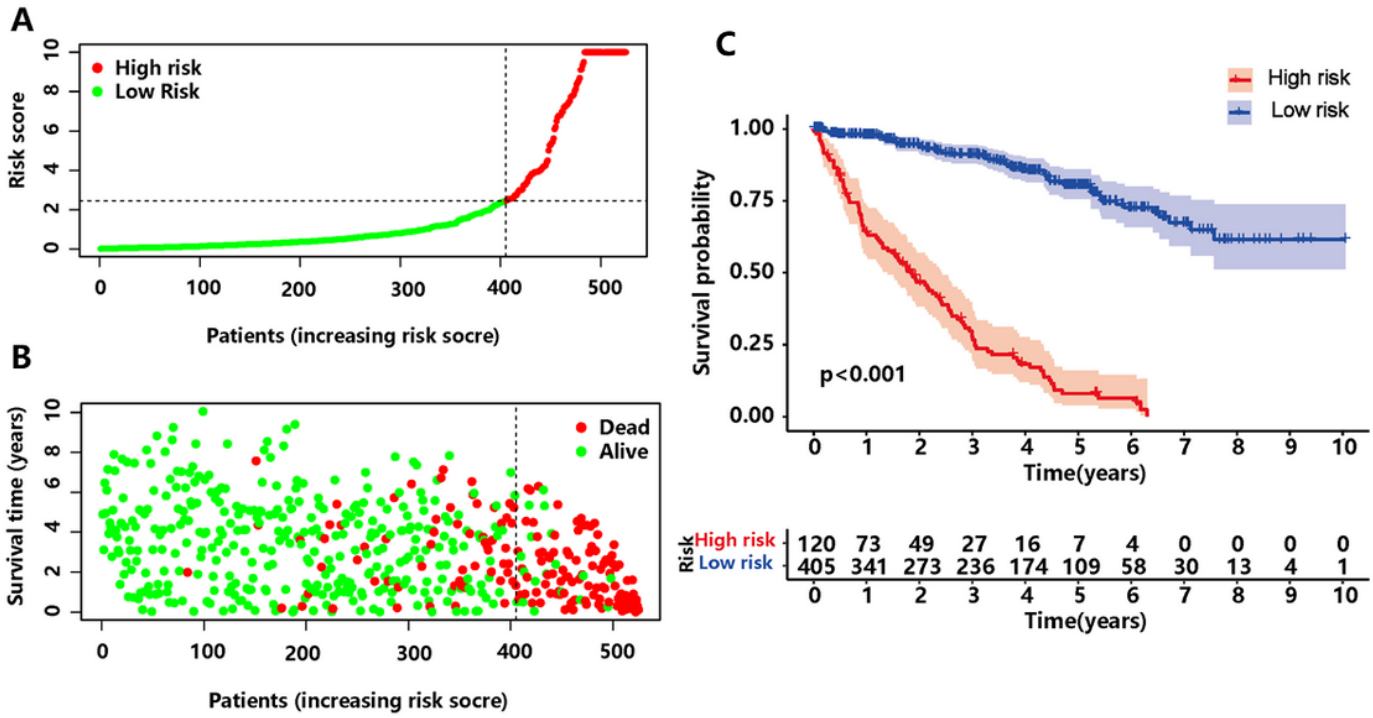

Figure 3

(A,B) Risk assessment and survival of clinical cases; (C) - Kaplan Meier analysis

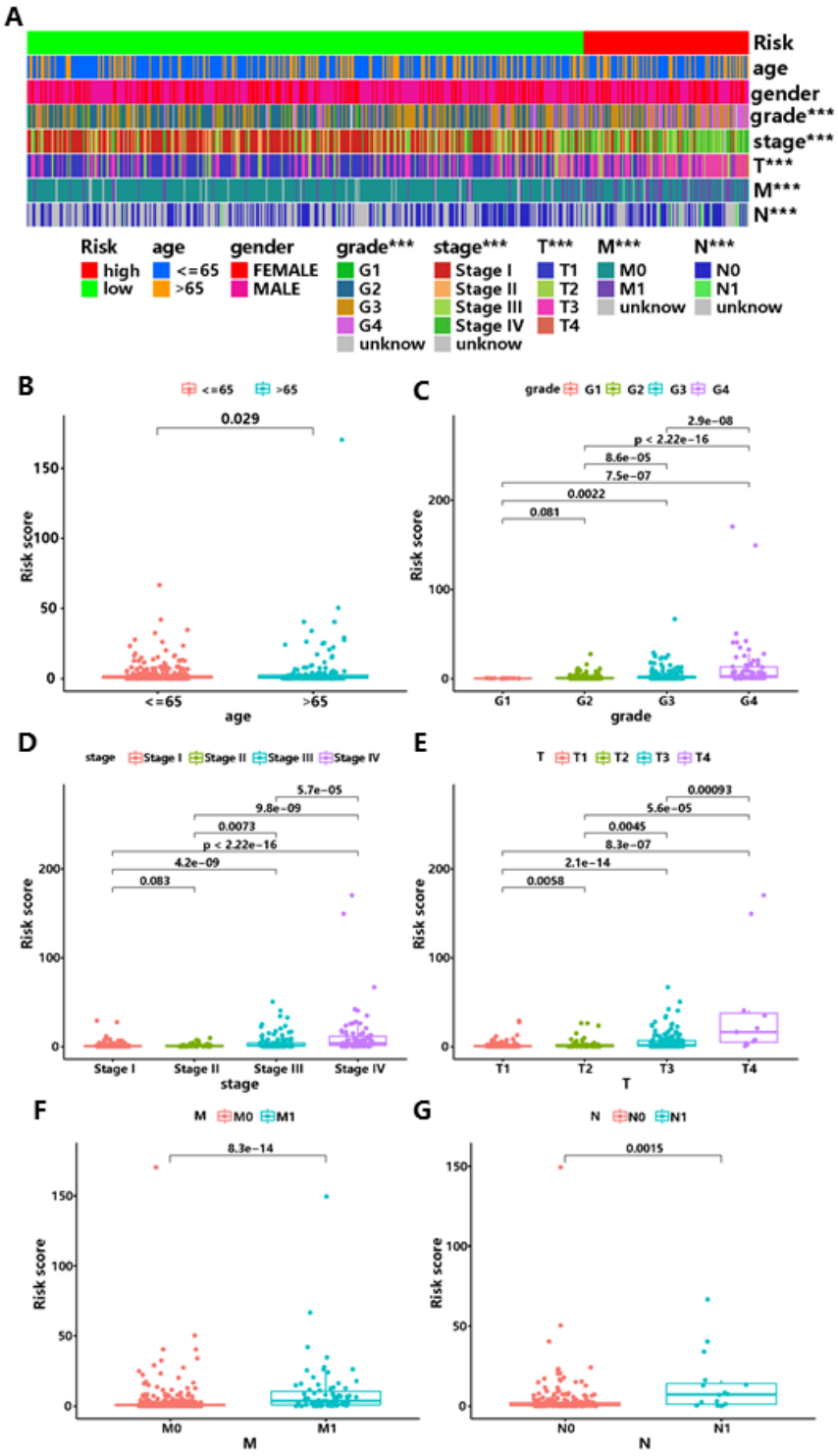

Figure 4

clinical evaluation of the risk assessment model (A) Clinically relevant heat map; Scatter plots of age (B), tumor grade (C), and clinical stage (D), T stage (E), M stage (F), and N stage (G).

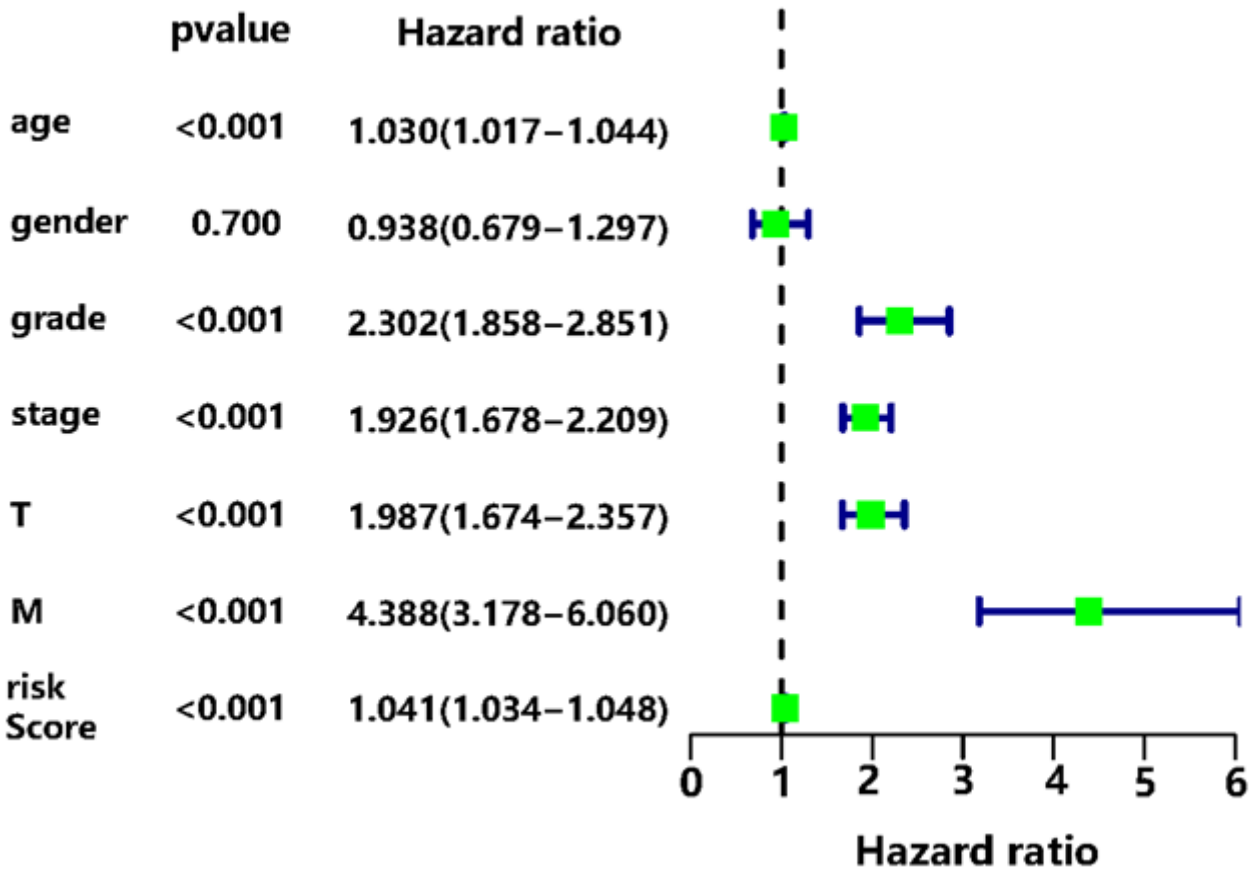

Figure 5

Cox regression analysis

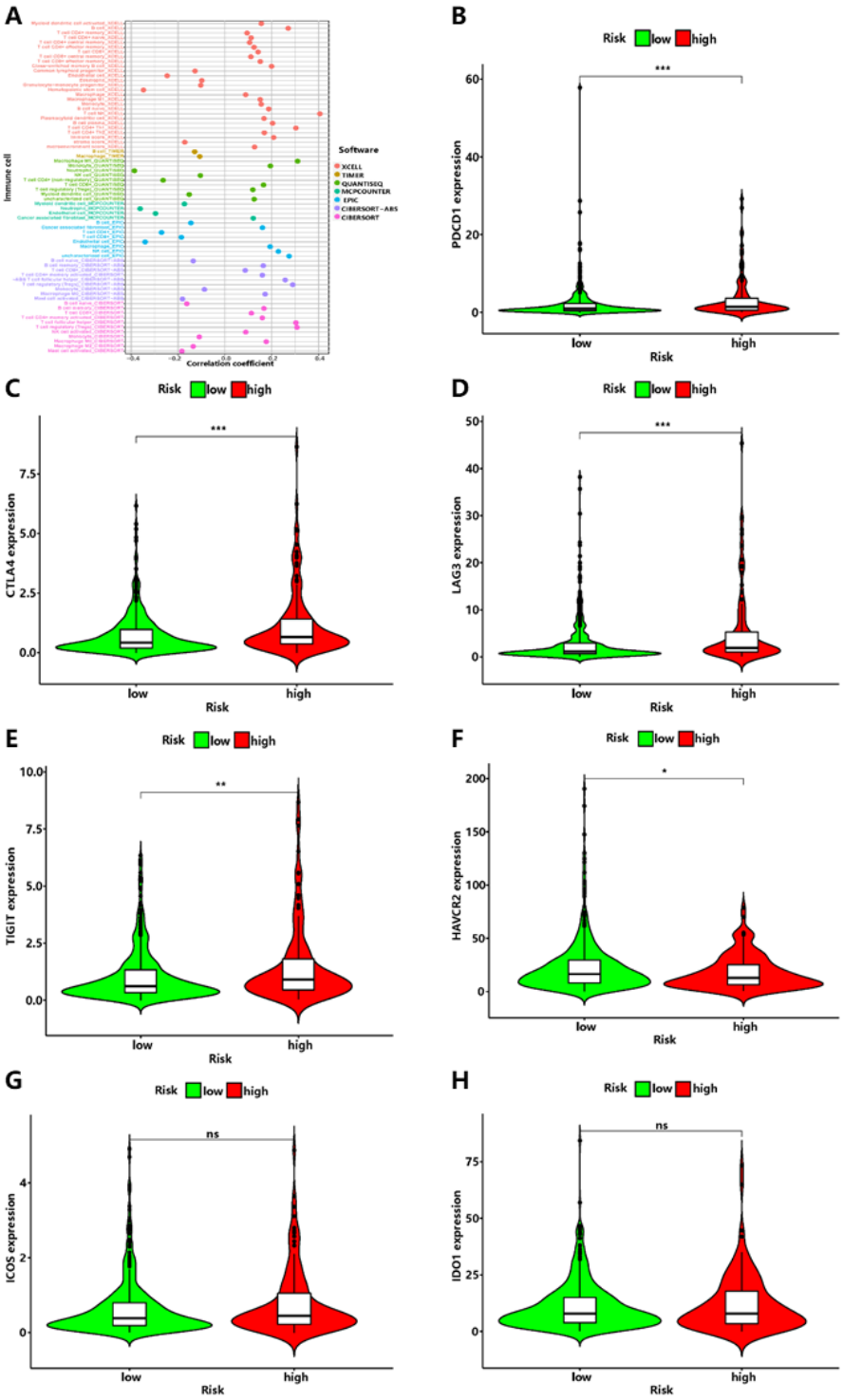

Figure 6

Immune validation of the risk assessment model. (A) Expression level of tumor-infiltrating immune cells in the high-risk group; (B-H) Expression differences of PDCD1, CTLA4, LAG3, TIGIT, HAVCR2, ICOS, IDO1 in high and low risk groups.

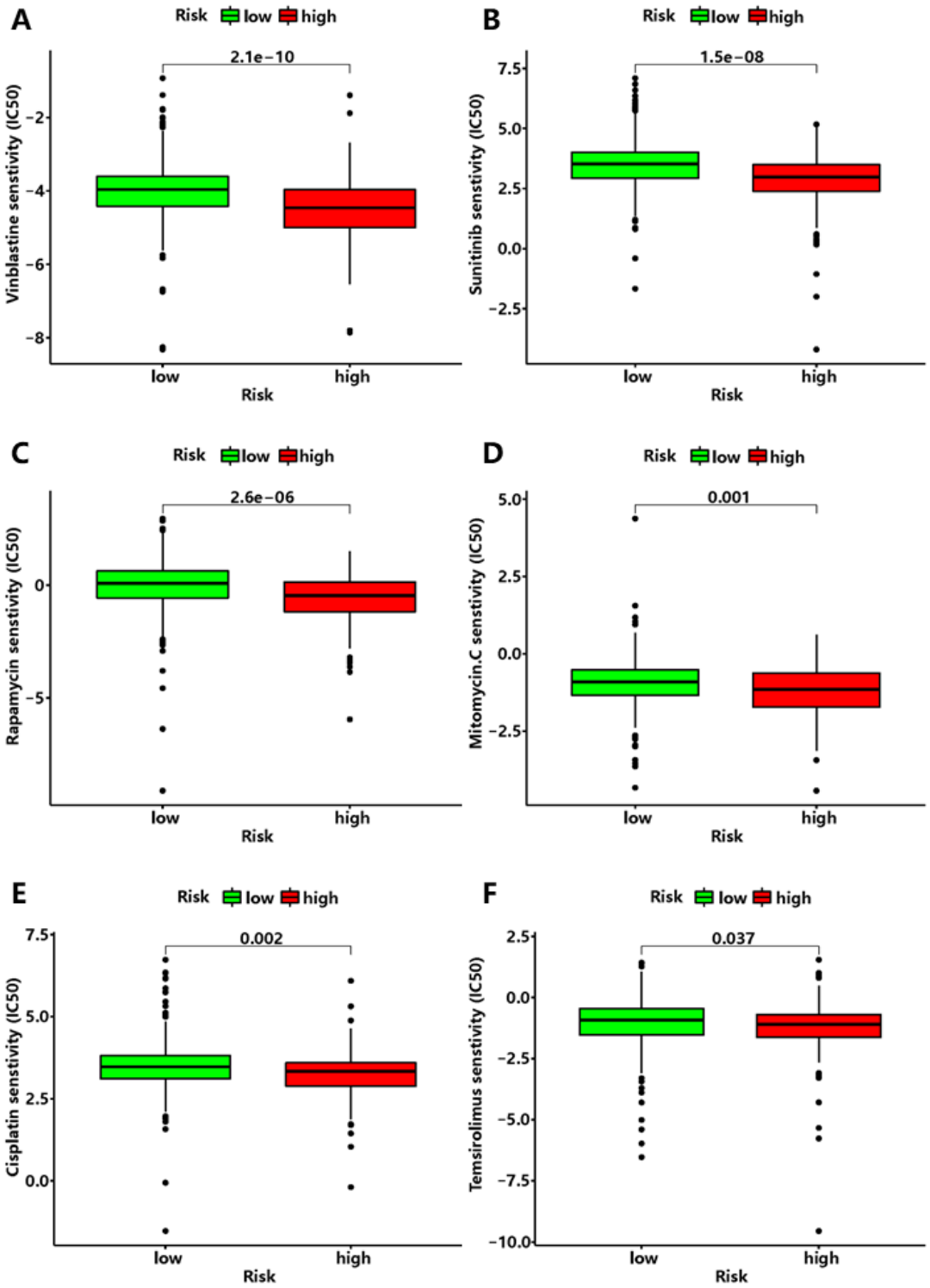

Figure 7

Difference levels of IC50 between commonly used chemotherapy drugs Vinblastine (A), Sunitinib (B), Rapamycin (C), Mitomycine. C (D), Cisplatin (E), and Temsirolimus (F) in the high and low risk groups.