

# A novel Signature Constructed using Ferroptosis-Related IncRNA Pairs May Predict the Prognosis of Bladder Cancer Patients

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## Research

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# Abstract

**Background:** Bladder cancer is one of the most common malignant tumors of the urinary system, and its incidence has been increasing in recent years. Ferroptosis is a recently discovered type of cell death, and some studies have suggested that it is closely associated with tumors. It can promote tumor apoptosis and also promote tumor development. Moreover, it has been reported that a correlation exists between long non-coding RNAs (lncRNAs) pairs and tumors. Herein, we developed an lncRNA pair signature associated with ferroptosis to predict the prognosis of bladder cancer.

**Methods:** We combined the bladder cancer transcriptome data from the Cancer Genome Atlas (TCGA) database to identify ferroptosis-related lncRNA (FRlncRNA) pairs. Using univariate and multivariate Cox analyses and LASSO regression analysis, we identified a FRlncRNA pair signature. We subsequently assessed the predictive prognostic value of this signature and validated the results.

**Results:** The signature included 18 lncRNA pairs and was highly accurate for clinical prediction in patients with bladder cancer. Univariate and multivariate Cox analyses and stratified analysis indicated that the model was an independent prognostic factor. Additionally, we detected a positive correlation between this signature and the tumor immune microenvironment.

**Conclusion:** The FRlncRNA pair signature has good prognostic and clinical predictive value in patients with bladder cancer.

## Background

Bladder cancer is the most common malignancy of the urinary system and its mortality rate ranks first among urinary system malignancies(1). Approximately 25% of bladder cancer cases are muscle-invasive bladder cancer (MIBC), and patients with MIBC usually have poor prognosis, with a five-year survival rate of less than 10%(2, 3). Although the prognosis of non-muscle-invasive bladder cancer (NMIBC) is better than that of MIBC, the recurrence rate of NMIBC is 50–70% after five years(4). At present, the main treatments for bladder cancer are surgery, chemotherapy, and immunotherapy(5, 6). Because of the poor survival rate of MIBC and the high recurrence rate of NMIBC, effective biomarkers are needed to predict bladder cancer prognosis.

Ferroptosis is a recently discovered mechanism of cell death and is characterized by excessive lipid peroxidation(7). The molecular mechanism between ferroptosis and tumors has not yet been clearly elucidated; however, tumor growth has been shown to be highly susceptible to ferroptosis(8). Long non-coding RNAs (lncRNAs) are > 200 nucleotides long(9, 10) and cannot encode proteins; however, they are involved in various biological functions(11). Recently, studies have shown lncRNAs may regulate ferroptosis and reduce tumor progression(12, 13). Wang et al.(14) showed that the lncRNA, LINC00336, plays a role in inhibiting iron ptosis in lung cancer. It has also been suggested that a nuclear lncRNA called LINC00618 plays a role in promoting iron ptosis in leukemia(15). However, the role of ferroptosis-related lncRNAs (FRlncRNAs) in bladder cancer remains unexplored to date.

Herein, we constructed a FRlncRNA pair signature characterized by the lack of specific expression of lncRNAs in the samples using the iterative and odd and even methods proposed by Sun et al.(16) Subsequently, we performed a series of assessments and validations of the prognostic value of the FRlncRNA pair signature in patients with bladder cancer.

## Results

### Identification of DEFRIncRNAs

The study workflow is illustrated in Fig. 1. The co-expression analysis of ferroptosis-related genes and lncRNAs is shown in Fig. 2A. Following differential analysis, we obtained 126 DEFRIncRNAs, which included 105 upregulated genes and 21 downregulated genes (Fig. 2B and C).

### Identification Of Prognostic Signature

Subsequently, univariate Cox, LASSO regression, and multivariate Cox regression analyses resulted in 18 DEFRIncRNA pairs being included in the prognostic signature (Fig. 3A–C). The risk value was calculated for each sample (Fig. 4A) and cut-off points were selected for high and low risks. The ROC curves at 1, 3, and 5 years were plotted, and all three curves had high area under the curve (AUC; Fig. 4B). The AUC values of the 5-year ROC curve were compared with other clinical characteristics, and the 5-year ROC AUC values were far higher than those of the other clinical features (Fig. 4C). Subsequently, we performed survival difference analysis for the high- and low-risk groups. We found survival in the low-risk group was significantly greater than that of the high-risk group (Fig. 5A–C).

### Independent Prognostic Validation Of DefrlnCrna Pairs Signature

We then performed univariate and multivariate Cox regression analyses of risk scores, age, sex, tumor grade, and tumor stage. Age, tumor stage, and risk score had independent prognostic values (Fig. 6A and B). Therefore, we conducted a stratified analysis where samples of patients with bladder cancer into two age groups: young (age  $\leq$  65 years) and elderly (age  $>$  65 years) and performed survival analysis of high- and low-risk patients within each age group. The survival rate of low-risk patients was higher than that of high-risk patients in both the young and elderly groups ( $p < 0.001$ , Fig. 6C and D). When patients were divided by tumor stage into early (stages I–II) and late (stages III–IV) and survival analysis was performed, low-risk patients had improved survival compared to high-risk patients ( $p < 0.001$ , Fig. 6E and F).

# Correlation Between DEFRlncrna Pairs Signature And Clinicopathological Features

The correlation between DEFRlncRNA pair signature and clinicopathological features was compared using the chi-square test (Fig. 7A) and the Wilcoxon signed-rank test (Fig. 7B–F). Both tests showed that the risk score was significantly correlated with age, grade, clinical stage, T stage, and N stage.

## Tumor immune infiltration in DEFRlncRNA pair signatures and analysis of ICI-related gene expression in different risk scores

Risk scores were then assessed by seven immune infiltration methods. Categorization in high-risk group was positively correlated with cancer-associated fibroblasts, macrophages, monocytes, neutrophils, NK cells, and CD8 + T cells, but negatively correlated with CD4 + naive T cells and B cell plasma (Fig. 8A).

We analyzed the association between several significant ICI-related genes and risk scores and found high risk correlated positively with the expression of *PDL1* (Fig. 8B,  $p < 0.05$ ), *CTLA4* (Fig. 8C,  $p < 0.05$ ), *LAG3* (Fig. 8D,  $p < 0.05$ ), and *HAVCR2* (Fig. 8E,  $p < 0.001$ ).

## Correlation analysis between DEFRlncRNA pair signatures and sensitivity to chemotherapeutic drugs

We analyzed the association between commonly used chemotherapeutic agents and the risk scores calculated using DEFRlncRNA pair signature. High-risk score was negatively correlated sensitivity to cisplatin (Fig. 9A,  $p < 0.001$ ), paclitaxel (Fig. 9B,  $p = 0.018$ ), and docetaxel (Fig. 9C,  $p < 0.001$ ). However, it was not significantly correlated with the  $IC_{50}$  of gemcitabine (Fig. 9D,  $p = 0.31$ ). We speculate that this signature may have the ability to predict chemosensitivity.

## Discussion

Ferroptosis is an iron-dependent mechanism by which various pathways decrease cellular antioxidant capacity, leading to the accumulation of reactive oxygen species and subsequent cell death(7, 17). Many studies have focused on inducing ferroptosis to treat cancer(18–20); however, it has also been suggested that ferroptosis may have both tumor-promoting and tumor-inhibiting actions (8, 21). Additionally, studies have suggested that lncRNAs play crucial roles in tumor development. Schmitt et al.(22) suggested that lncRNAs can promote the development of many tumor phenotypes through interactions with DNA and RNA. Elena et al.(23) showed that lncRNAs can affect the occurrence and development of urinary tumors and can be used as biomarkers for urinary tumors. Although many lncRNA signatures have been used as prognostic markers for bladder cancer patients(24–26), there is no signature of ferroptosis-related lncRNAs used to predict the prognosis of patients with bladder cancer. Therefore, we established a ferroptosis-related lncRNA signature and validated its ability to predict prognosis in patients with bladder cancer.

After performing numerous analyses on transcriptional and clinical data from TCGA we selected 18 lncRNA pairs to construct a signature for bladder cancer prognosis. Both KM survival analysis and ROC curve demonstrated that the signature successfully predicted the prognosis of patients with bladder cancer. Moreover, our calculated risk score based on this signature was positively associated with high-tumor grade and stage. Our analysis found that age and tumor stage also had independent prognostic values. To further validate the risk score, we performed a stratified analysis validation using age and tumor stage. The results also showed that the risk score has an independent prognostic value and can be used as an independent prognostic factor.

The 18 lncRNA pairs we obtained included some lncRNAs that have been associated with tumors, such as AC010186.3(27), LINC01605(28), LINC00641(29), and MAP3K14 – AS(30). Notably, LINC01605 and LINC00641 have been reported in bladder cancer. Qin et al.(31) suggested that LINC01605 promotes the proliferation and invasion of bladder cancer. In contrast, Li et al.(32) suggested that LINC00641 inhibits the progression of bladder cancer. In addition to well-known lncRNAs, we discovered novel lncRNAs, which we speculate could be new tumor biomarkers.

When we compared our risk score to immune infiltration, we found our risk score positively correlated with several cell populations, including CD8 + T cells. In addition, our calculated risk score correlated with the patient response to ICI, suggesting our model may predict responses to ICI. ICIs exert an anti-tumor effect mainly by reactivating immune cells and are most effective in inflammatory tumors with high CD8 + T cell infiltration and high PD-L1 expression(33). Our signature suggests that ICIs are effective for treating bladder cancer, in line with the National Comprehensive Cancer Network (NCCN) guidelines(34). When we analyzed ability of our signature to predict tumor sensitivity to commonly used chemotherapeutic agents, the results showed that chemosensitivity was lower in the high-risk group than in the low-risk group. Therefore, we believe that immunotherapy may achieve better results than chemotherapy in high-risk patients.

Our study also has some limitations. We only utilized TCGA database and did not validate our model with other external data sets. Further validation of our results would benefit from collecting additional samples and clinical trial data to verify our results. Our method offers some benefits compared to previous studies. The advantage of our lncRNA pair signature is that it does not need to adopt the specific expression level of each lncRNA in the sample. Additionally, it does not suffer from a batch effect, so the prognostic model constructed by this method is also more accurate than other signatures(16, 35).

## Methods

### Collection and processing of raw data

Transcriptome and clinical data from bladder cancer patients were acquired from the Cancer Genome Atlas (TCGA) database (<https://portal.gdc.cancer.gov/>). ene transfer format files from Ensembl (<http://asia.ensembl.org>) were analyzed using Strawberry Perl (version 5.32.01) to distinguish mRNAs

from lncRNAs. Then, 60 ferroptosis-related genes were obtained from the study by Liang et al.(36) Correlations between ferroptosis-related genes and lncRNAs were tested using the limma package in R (version 4.0.2) and significance was determined with correlation coefficient > 0.4 and  $p < 0.001$ . Resulting significant lncRNAs were defined as FRlncRNAs. Differential analysis of the FRlncRNAs was performed using the limma package of R, and significant differences were determined by a false discovery rate (FDR) < 0.05 and log fold change (FC) > 1.5. Differentially-expressed FRlncRNAs (DEFRLncRNAs) were used for subsequent analysis.

## Data analysis

Expression of DEFRLncRNA in each sample was compared in pairs and the value was defined by A. In each DEFRLncRNA pair, if the first lncRNA expression level was greater than that of the second lncRNA, A = 1; otherwise, A = 0. Univariate Cox analysis was subsequently used to determine prognostically-relevant DEFRLncRNA pairs. Then, prognostically-relevant DEFRLncRNA pairs were screened using LASSO regression analysis (iteration = 1000) and the DEFRLncRNA pair signature was constructed using multivariate Cox analysis. A risk score for each patient was calculated using the following equation:

$$\text{Risk score} = \sum_{i=1}^k C_i A_i,$$

where  $C_i$  represents the coefficient obtained after multivariate Cox analysis for the  $i^{\text{th}}$  lncRNA pair and  $A_i$  represents the expression value of the  $i^{\text{th}}$  lncRNA pair.

A time-dependent receiver-operating characteristic (ROC) curve was drawn for 5 years and the maximum inflection point was considered as the cut-off value. If the risk value was greater than the cut-off value, the DEFRLncRNA pair was divided into the high-risk group; otherwise, it was divided into the low-risk group.

ROC curves were constructed using the survivalROC package in R to assess the DEFRLncRNA pair signature precision. Survival differences between high- and low-risk groups were assessed using Kaplan–Meier (KM) survival analysis. Independent prognostic validation of the DEFRLncRNA pair signature was performed using univariate and multivariate Cox analyses. The association between this signature and clinicopathological features was assessed using the chi-square test and Wilcoxon signed-rank test. The risk scores were analyzed for immune infiltration using the Spearman correlation test by combining several accepted immune infiltration methods (XCELL, TIMER, QUANTISEQ, MCPOUNTER, EPIC, CIBERSORT – ABS, and CIBERSORT). The relationship between risk scores and genes involved in immune checkpoint inhibitors (ICIs) was assessed using the limma package of R. Risk groups were compared for their sensitivity to common chemotherapeutic drugs for bladder cancer by analyzing drug  $IC_{50}$  using the limma package and the pRRophetic package in R language. Statistical significance was determined at  $p < 0.05$ ; \* indicates  $p < 0.01$  and \* indicates  $p < 0.001$ .

## Conclusion

In conclusion, we constructed a novel ferroptosis-related lncRNA pair signature that can predict the prognosis of bladder cancer and guide immunotherapy and chemotherapy for bladder cancer patients.

## Abbreviations

lncRNAs

long non-coding RNAs; FRlncRNA:ferroptosis-related lncRNA;TCGA:the Cancer Genome Atlas; MIBC:muscle-invasive bladder cancer; NMIBC:non-muscle-invasive bladder cancer; FDR:false discovery rate; DEFRlncRNAs:Differentially-expressed FRlncRNAs; ROC:receiver-operating characteristic; KM:kaplan-Meier; ICIs:immune checkpoint inhibitors; AUC:area under the curve; NCCN:National Comprehensive Cancer Network.

## Declarations

### Ethics approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

### Availability of data and materials

All the data sets of this study are available in the TCGA (<https://portal.gdc.cancer.gov/>) and GEO (<http://www.ncbi.nlm.nih.gov/geo/>) databases.

### Competing interests

The authors declare that they do not have any conflict of interest.

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### Author Contributions

L.Z. and L.F.Z. were responsible for conceiving and designing this study, H.W. and Z.Y.Z. wrote the main manuscript, S.L.G., C.L. and Z.Z. were responsible for preparing figures, and H.W. was responsible for re-examination. All authors approved the submission of this study.

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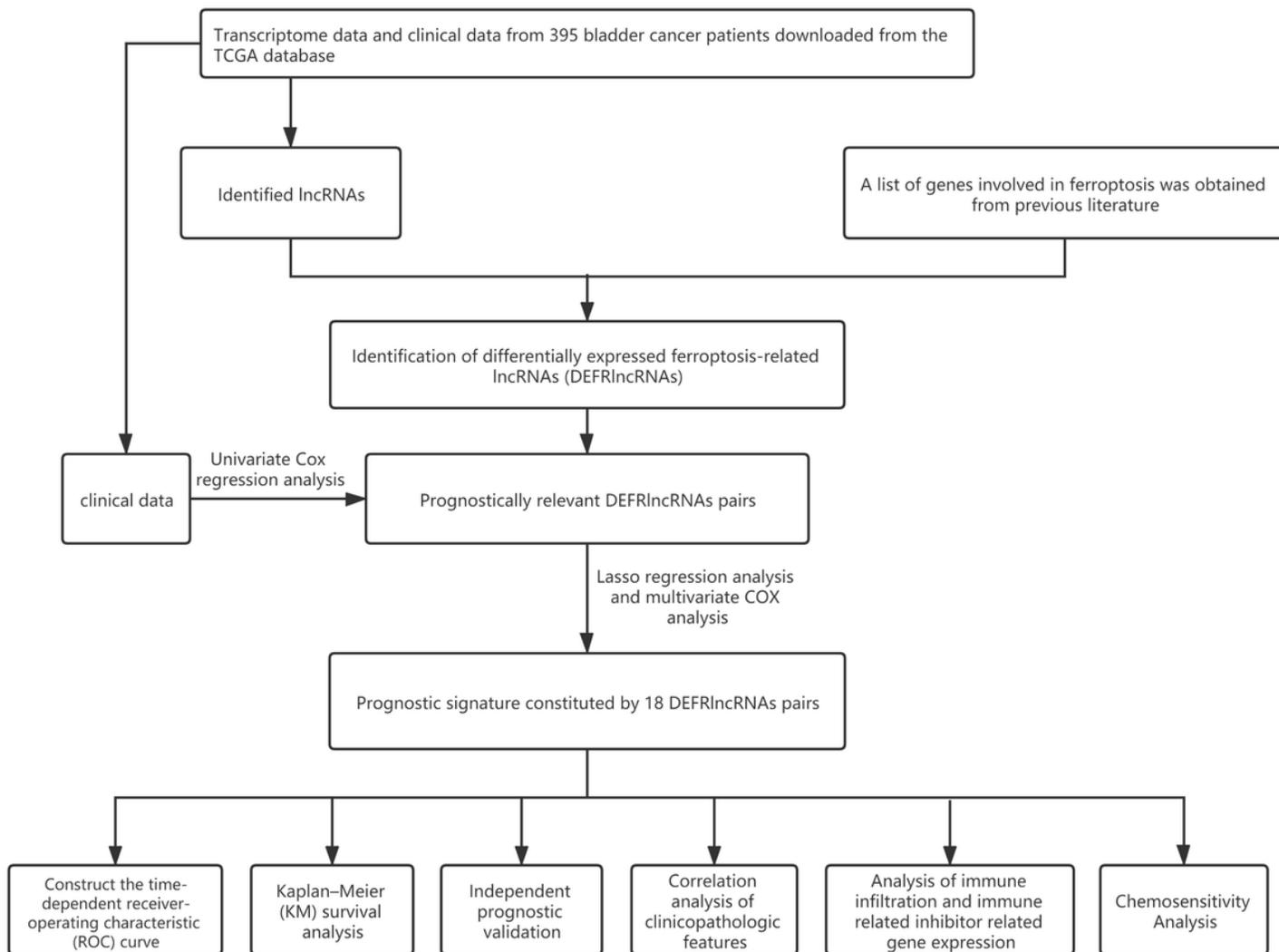
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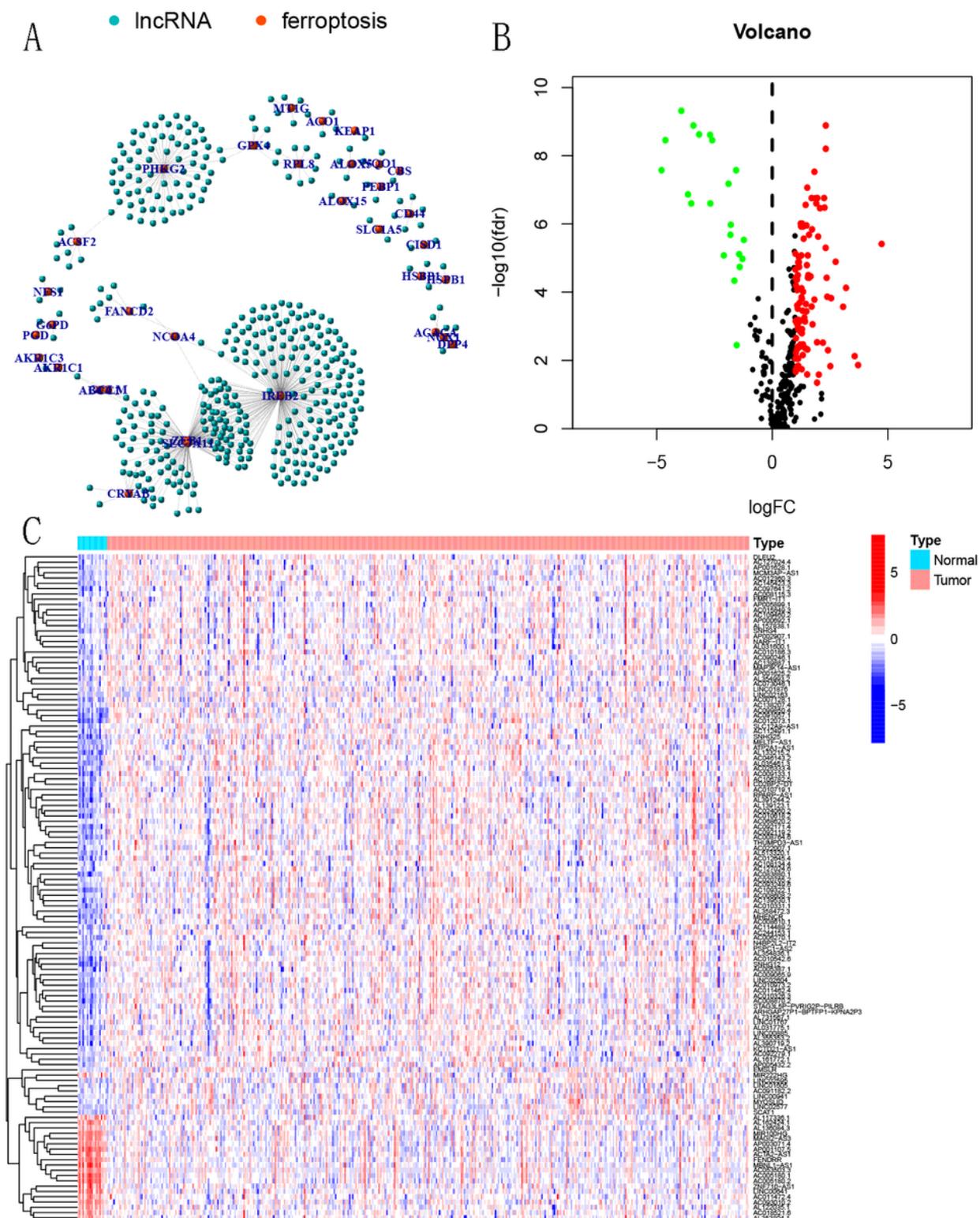
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## Figures



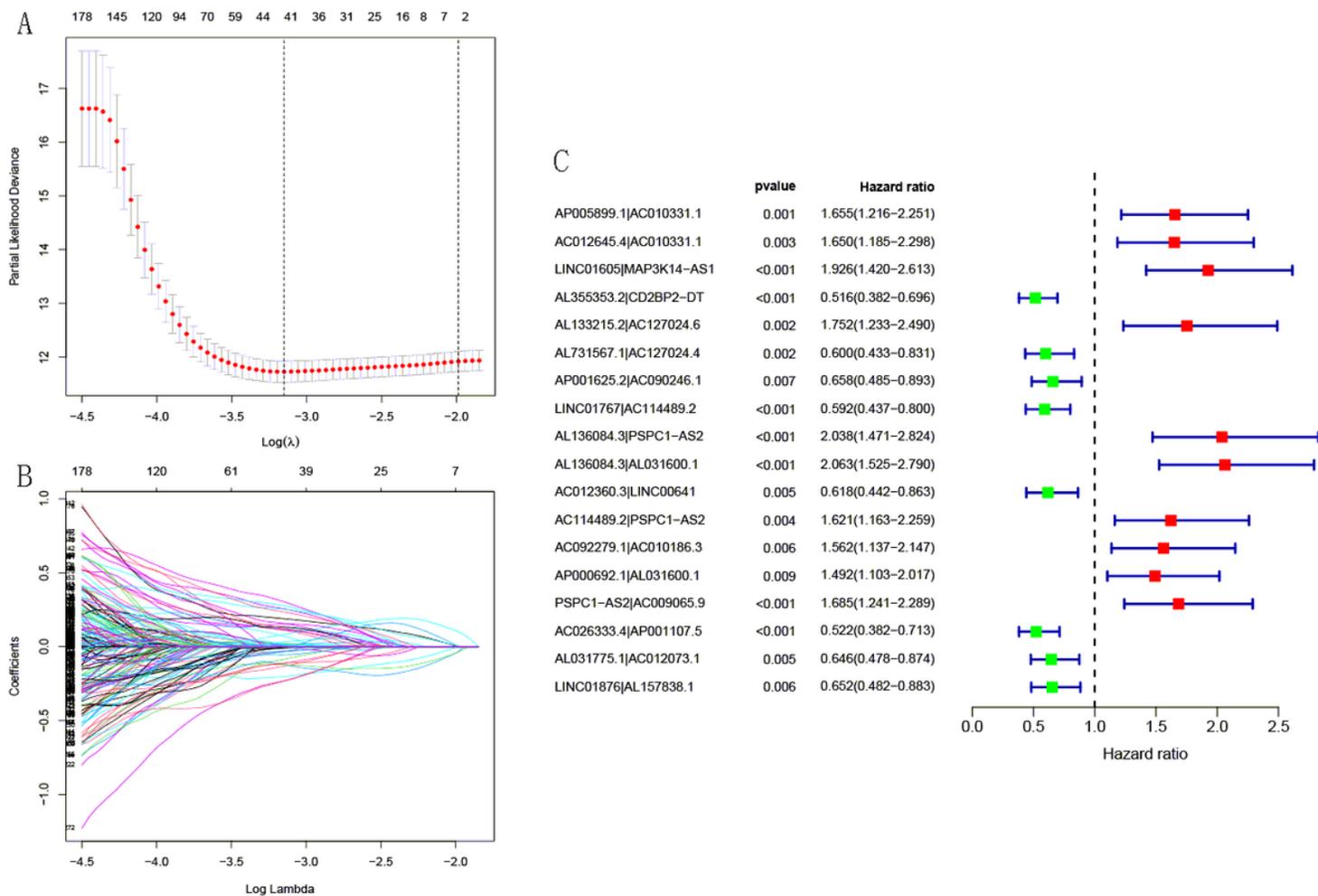
**Figure 1**

Flowchart of this study.



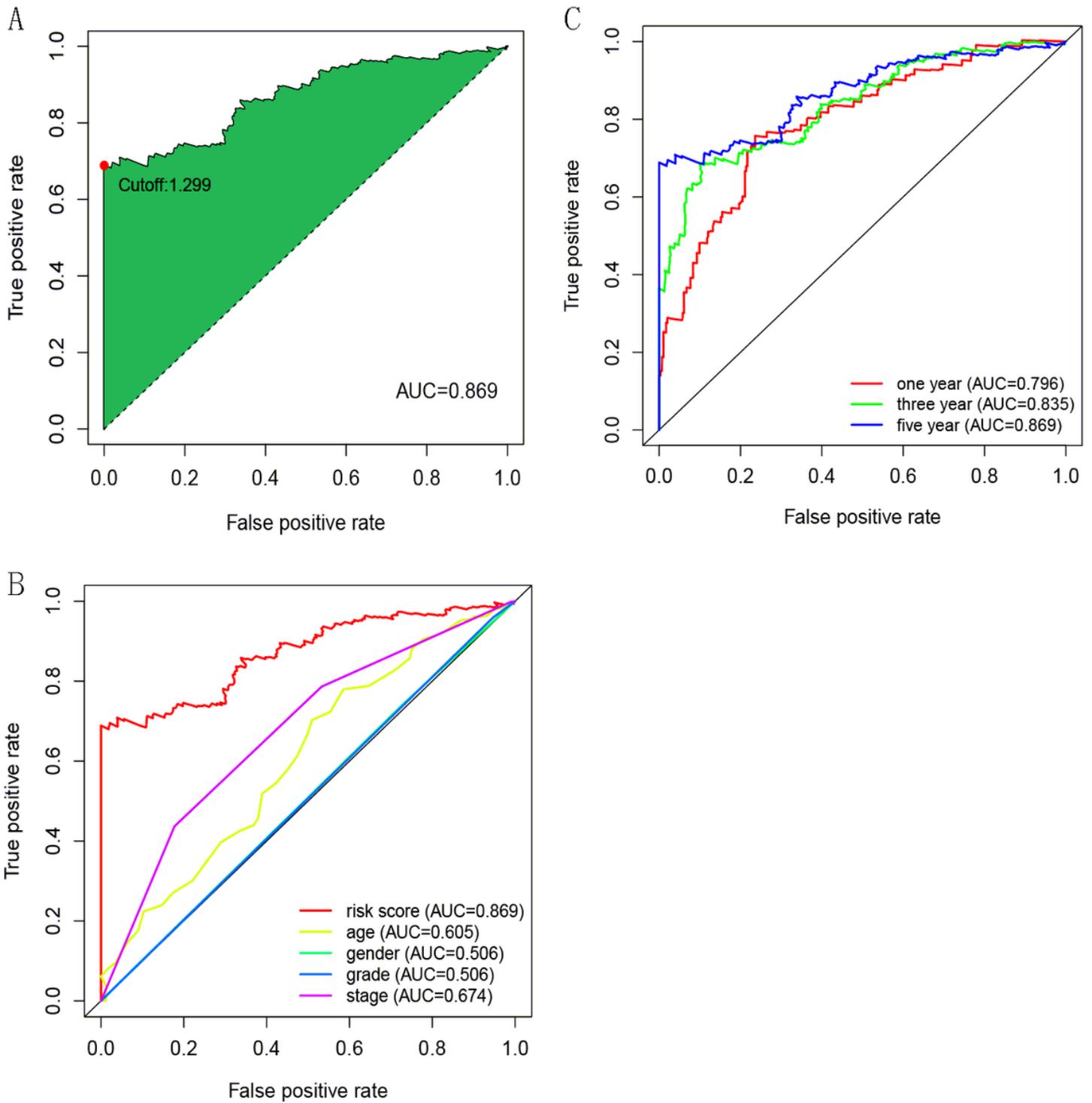
**Figure 2**

Identification of DEFRIncRNAs. (A): Co-expression networks of ferroptosis-related genes and IncRNAs. Volcano (B) and heatmap (C) and plot of DEFRIncRNAs.



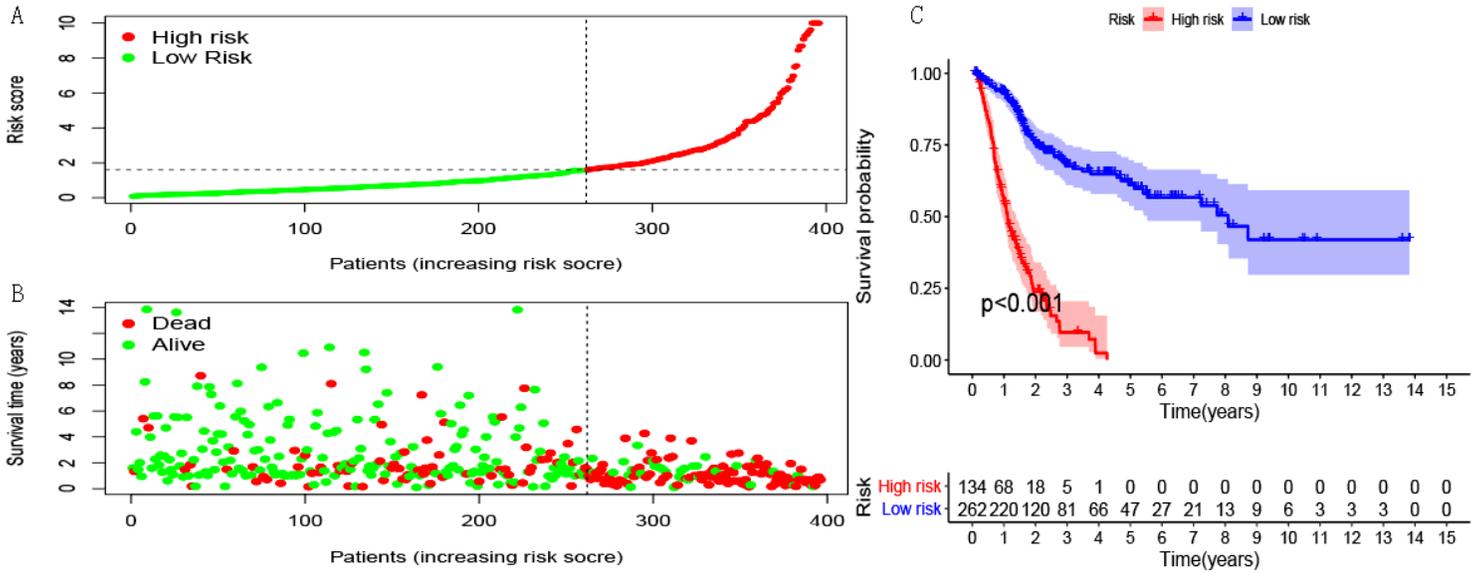
**Figure 3**

Identification of DEFRlncRNA pairs signature. (A-B): The Lasso regression model. (C): Forest plot of 18 DEFRlncRNA pairs formed after multivariate Cox regression analysis.



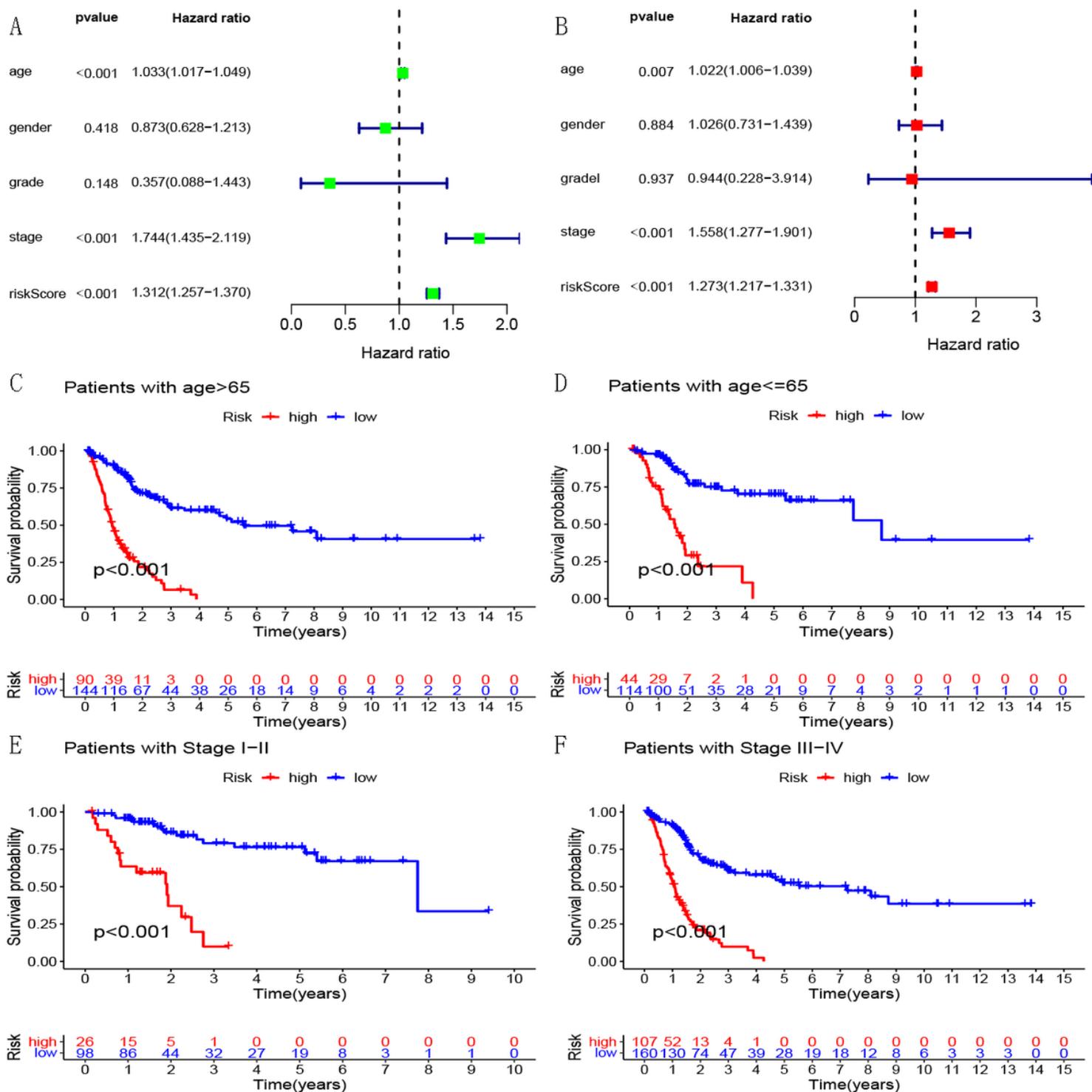
**Figure 4**

ROC curve of DEFRIncRNA pairs signature. (A): For the risk score of each bladder cancer patient, the cut-off value is the maximum inflection point. (B): The ROC curves at 1, 3, and 5 years. (C): Comparison of 5-year ROC curve with other clinical characteristics.



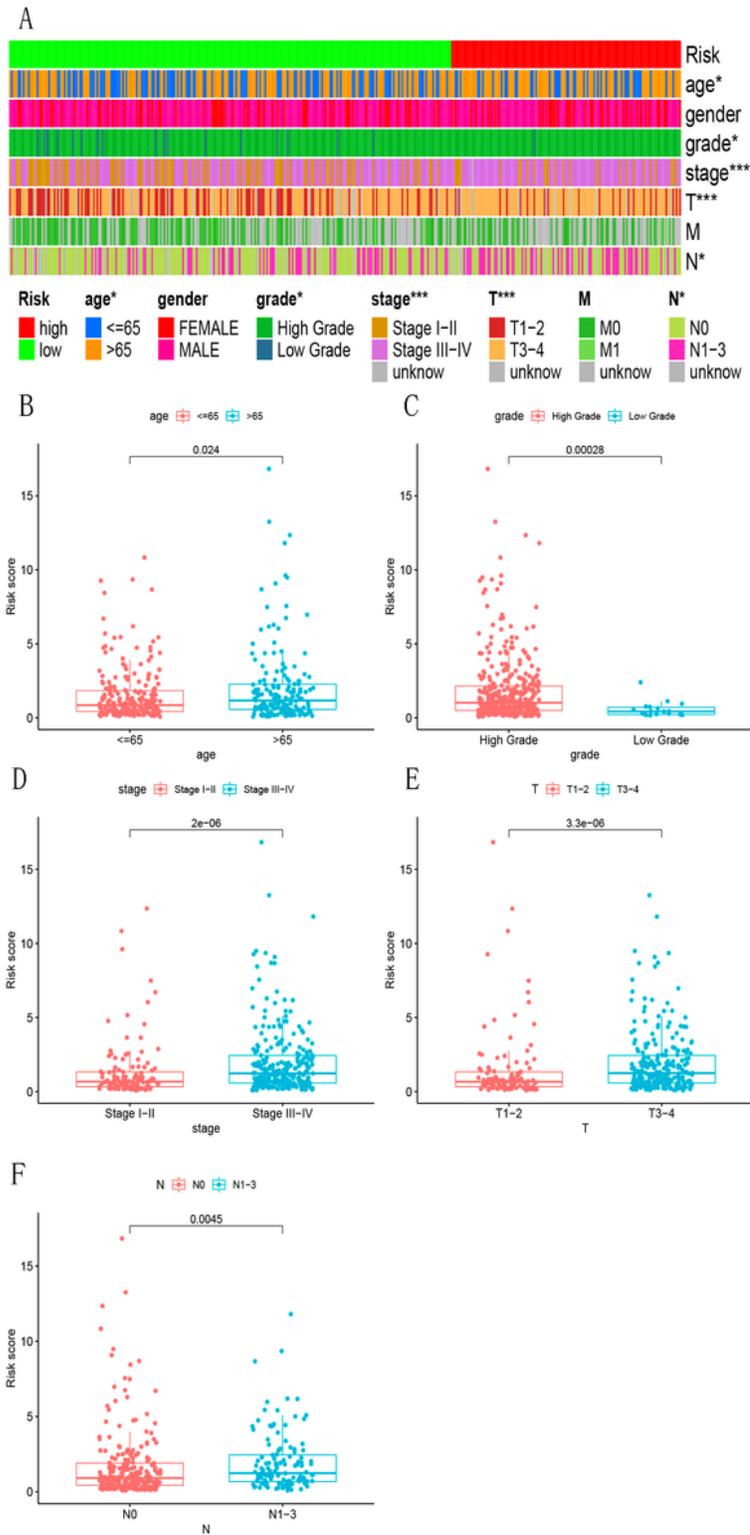
**Figure 5**

Survival analysis assessment of DEFRlncRNA pairs signature. Risk score (A) and survival status (B) of bladder cancer patients. (C): KM survival analysis between high-risk group and low-risk group.



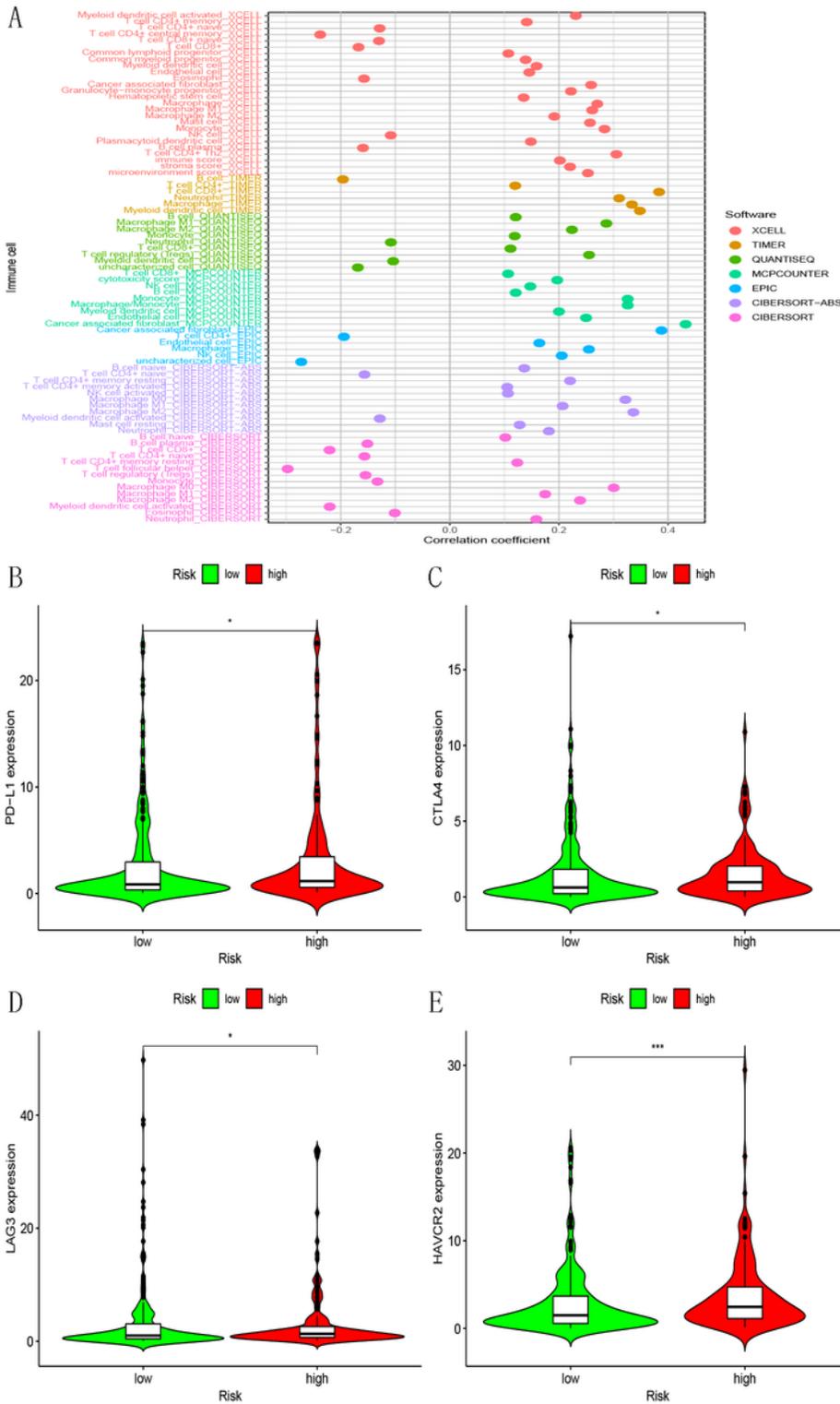
**Figure 6**

Independent prognostic validation of DEFR1ncRNA pairs signature. Forest plot obtained from (A) univariate COX regression analysis and (B) multivariate COX regression analysis. KM survival curves between (C) the young group and (D) the old group for the high-risk and low-risk groups. KM survival curves between (E) the early group and (F) the old group for the high-risk and low-risk groups.



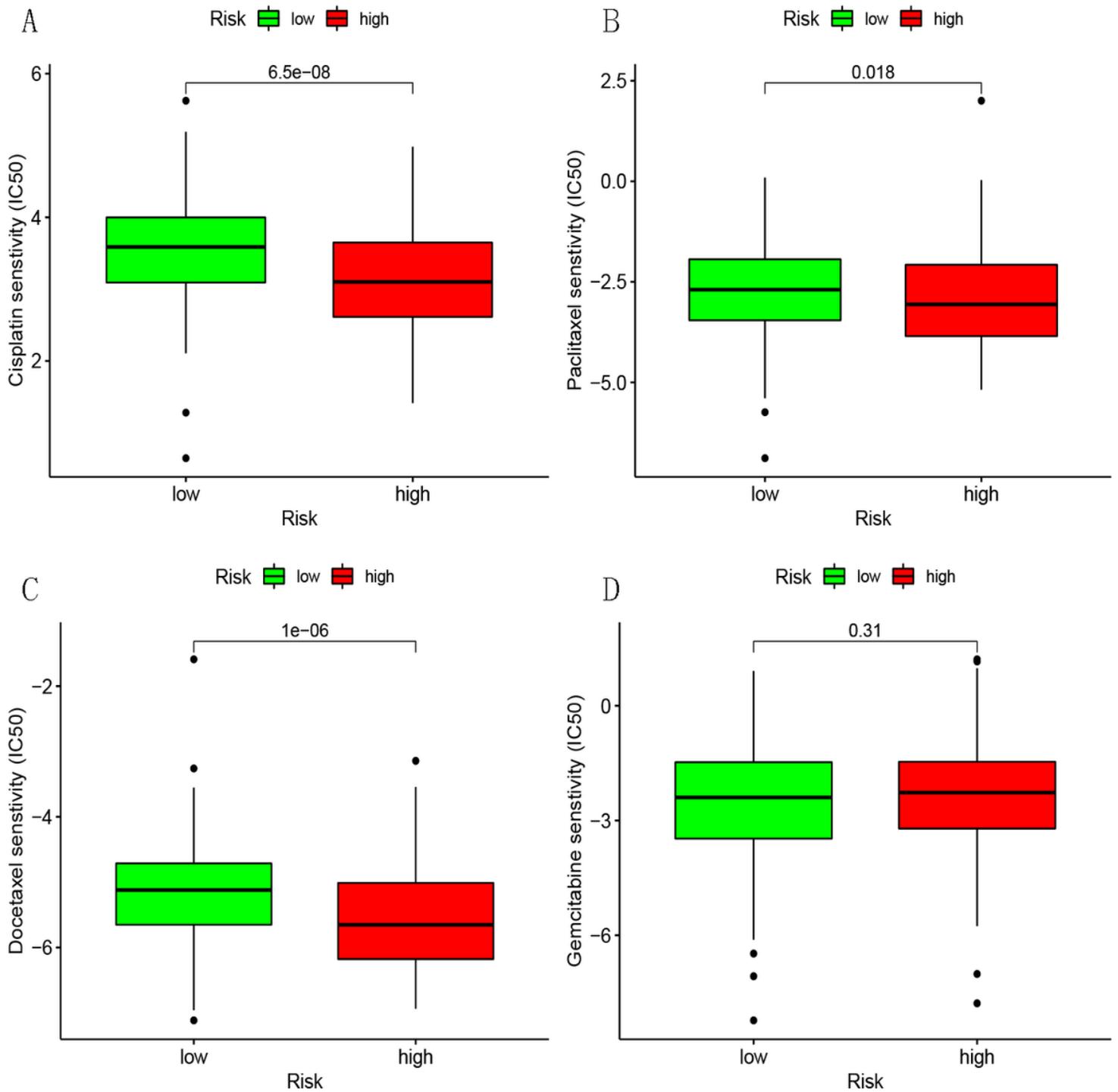
**Figure 7**

Clinical relevance evaluation of DEFRIncRNA pairs signature. (A-F) Heat Plot and Scatter Plot of Risk Score and Clinical Relevance. The results showed that (B) age, (C) grade, (D) clinical stage, (E) T stage and (F) N stage were significantly associated with risk score.



**Figure 8**

Immune infiltration analysis and ICIs correlation analysis of DEFRLncRNA pairs signature. (A): The results of immune infiltration analysis showed that the risk score was positively correlated with most of the immune infiltrating cells. The results of correlation analysis between risk score and ICIs gene showed that high risk score was significantly positively correlated with (B) PD-L1, (C) CTLA4, (D) LAG3 and (E) HAVCR2 expression levels.



**Figure 9**

Chemosensitivity of DEFRIncRNA pairs signature. The results showed that the high-risk score was negatively correlated with the IC50 of (A) cisplatin, (B) paclitaxel and (C) docetaxel, but was not statistically significant with the IC50 of (E) gemcitabine.