Original Article Identification and verification of a prognostic ferroptosis-related IncRNAs signature for patients with lung adenocarcinoma

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Abstract: Lung cancer has been identified as one of the deadliest malignant tumors worldwide. Mounting evidence suggests that ferroptosis is a well-known non-apoptotic cell death process that participates in pathological mechanisms and is a new cancer treatment strategy. Aberrantly expressed long non-coding RNAs (IncRNAs) that drive lung cancer progression have attracted increasing attention. Herein, we explored the prognostic significance of ferroptosis-related IncRNAs in lung cancer patients. LUAD gene expression patterns and clinicopathological data were downloaded from The Cancer Genome Atlas (TCGA) database. Based on LASSO-Cox regression, A 14 ferroptosisrelated differentially expressed IncRNAs (FRDELs) signature was constructed. Subsequently, a nomogram model for predicting the prognosis of LUAD patients was constructed based on clinicopathological data and the 14 - FRDELs signature. The signature was shown to be correlated with tumor mutational burden (TMB) and immune cell infiltration within the tumor microenvironment. Furthermore, Gene Set Enrichment Analysis (GSEA) confirmed that the signature was correlated with LUAD-related biological functions such as the P53 signaling pathway, DNA replication, and cell cycle. The roles and mechanisms of PACERR in the signature were explored by si-IncRNA-mediated knockdown and transfection-mediated overexpression via in vitro experiments in A549 and H1299 cells. PACERR was significantly upregulated in A549 and H1299 cells, and higher expression promoted LUAD cell proliferation, migration, and invasion via in vitro experiments, while knockdown of PACERR presented the opposite effects. In conclusion, our study provided information regarding ferroptosis-related IncRNA expression and established a prognostic nomogram based on 14 FRDELs to predict overall survival in LUAD accurately. Additionally, our results in vitro revealed that PACERR played an oncogenic role in LUAD proliferation and metastasis, which provides mechanistic insights into the roles of ferroptosis-related IncRNA in LUAD progression and that it may be a potential biomarker for LUAD treatment.

Keywords: Ferroptosis, IncRNA, IncRNA PACERR, LUAD, survival, TCGA

Introduction

Lung cancer is among the most prevalent lifethreatening malignant tumor worldwide, with a significant morbidity and mortality rate [1]. There were 2.1 million new diagnoses of lung cancer recorded; leading to approximately 1.8 million lung cancer related fatalities thus putting human health and lives at risk [2]. In the case of lung cancer, adenocarcinoma (LUAD) is a frequent pathological subcategory that accounts for around 45 percent of all instances of the disease. Despite significant advances in the treatment and management of LUAD, such as molecular targeting, chemotherapy, and radiation therapy, the prognosis remains dismal, with only an 18% five-year overall survival rate [3]. Therefore, novel and effective screening approaches are urgently required to increase diagnostic accuracy and treatment efficiency, enhancing LUAD patients' prognoses.

Ferroptosis is a well-known non-apoptotic cell death process induced primarily by increased lipid peroxidation and iron catalytic activity in the cell and is characterized by the build-up of reactive oxygen species [4, 5]. It participates in pathological mechanisms such as human growth, immunity, and aging and is critical to normal tissues and cells [6]. The cancer occur-

rence and progression are associated with ferroptosis, and ferroptosis activation in cancer cells is a new cancer treatment strategy [7, 8].

Long non-coding RNAs (IncRNAs) are defined as RNAs that exceed 200 nucleotides in length. Even though IncRNA does not participate in protein translation, it is necessary for gene regulation. IncRNA can affect gene expression in various biological, physiological, and pathological environments by modulating the function of chromatin as well as the function and assembly of nucleosomes that are membrane-less, thus altering the translation and stability of cytoplasmic mRNA and influencing signaling pathways [9]. The aberrant IncRNA expression or function, in particular, could be correlated with a variety of biological events such as ferroptosis [10]. For instance, the IncRNA LINC00336 reduces lung cancer ferroptosis via competitive endogenous RNA activity [11]. The IncRNA P53RRA induces tumor suppression through nuclear isolation of p53 and promotes ferroptosis and apoptosis in cancer [12]. Therefore, identifying important prognostic ferroptosisrelated IncRNAs in LUAD is critical for developing accurate prognostic assessment and treatment methods.

In this study, we constructed a ferroptosis-related differentially expressed IncRNAs (FRDELs) signature to predict the prognosis of patients with LUAD accurately and analyzed the relationship between the signatures and tumor mutational burden (TMB) and immune cell infiltration. We further carried out GSEA to explore the signature mechanisms. Most importantly, we explored the effects of PACERR on the proliferation, migration, and invasion of LUAD cells.

This study provides reference data for improving current diagnosis, treatment, follow-up, and prevention strategies.

Materials and methods

Acquiring data on gene expression and clinicopathology

The Cancer Genome Atlas (TCGA) database (https://portal.gdc.cancer.gov/) was utilized to obtain clinicopathological data (age, gender, stage, and follow-up data) as well as gene expression profiles (fragments per kilobase of transcript per million reads) of LUAD patients (downloaded on 2 June 2021; platform: Illumina

HiSeq 2000 RNA Sequencing). Subsequently, 54 normal and 497 LUAD tissues were obtained. GENCODE Release 29 (GRCh38.p12) was used to extract the matrix of mRNA and IncRNA expression. Patients having insufficient data on survival were not included in the present study. Eventually, 468 patients with LUAD were included and divided at random into the training (n=312), and validation (n=156) sets utilizing the R package 'caret'.

Detection of ferroptosis-related differentially expressed IncRNAs (FRDELs)

A sum of 259 genes associated with ferroptosis was acquired from FerrDb (http://www.zhounan.org/ferrdb) [13], a newly available handcurated repository for ferroptosis indicators and modulators launched in January 2020. The TCGA database was retrieved independently for the mRNA and IncRNA expression profiles. Subsequently, ferroptosis-related IncRNAs were identified via a correlation analysis between the expression levels of IncRNAs and ferroptosis-related genes utilizing Pearson's correlation coefficient to determine the relationship between the two expression levels (correlation coefficient >0.40 and P<0.001). Furthermore, we employed the R package 'limma' to discover FRDELs in 54 normal and 497 LUAD tissues, with cut-off criteria of P<0.05 and |log2 (fold change)| >1.

Construction of a FRDELs signature

We used the R package 'survival' and 'glmnet' to perform univariate Cox regression, Least absolute shrinkage and selection operator (LASSO), and multivariate Cox regression analyses on the training set (n=312) to construct the FRDEL Signature. We computed the risk score of each patient sample according to the expression of IncRNAs and the corresponding coefficient using the following equation: Σi coefficient ($IncRNA_{1}$) × expression ($IncRNA_{1}$) + coefficient (IncRNA) × expression (IncRNA) + + coefficient ($IncRNA_{}$) × expression ($IncRNA_{}$). Finally, patients in the training, validation and whole sets were classified into two low- and high-risk groups according to their median risk score values.

Predictive ability of the FRDELs signature

Receiver operating characteristic (ROC) curve analysis was carried out in the training, valida-

tion, and whole sets utilizing the R package 'timeROC' to thoroughly examine the prediction performance of the FRDELs signature. The FRDELs signature and corresponding clinicopathological data were used to identify the independence through univariate and multivariate Cox regression analyses on the whole set.

Tumor mutational burden (TMB), gene set enrichment analysis (GSEA), and immune cell infiltration

After normalizing the transcriptome data, we utilized the CIBERSORT algorithm to measure the proportion of 22 different kinds of immune cells in each patient [14]. The differences in the proportion of invading immune cells between the whole set's low- and high-risk groups were compared.

Furthermore, the TMB data were downloaded from the TCGA database and analyzed using the R package 'maftools' in the low- and highrisk groups of the whole set, and the TMB of each patient (mutations per million bases) was calculated. Then, we subjected RNA-seq profiles to GSEA to screen for the gene - related signaling pathways with differential expression in the low- and high-risk groups. Statistical significance was set at false discovery rate (FDR) <0.25 and nominal (NOM) P<0.05.

Establishment and validation of a nomogram

Based on the multivariate Cox regression analysis of the whole set's risk score and clinicopathological data, a nomogram was developed to anticipate the one-, three-, and five-year survival probability of LUAD patients utilizing the R package rms'. Furthermore, we employed the R package 'survivalROC' to examine the prediction performance of the nomogram. Subsequently, DCA was conducted to evaluate the clinical applicability of the nomogram [15].

Cell culture and transfection

Three human LUAD cell lines (A549, H1792, and H1299) and normal human lung epithelial cells (MRC-5) were purchased from the Bluef Biotechnology Development (Shanghai, China). LUAD cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, BRL, USA) supplemented with 10% fetal bovine serum (Hyclone, Logan, USA) at 37°C and were main-

tained in a humidified cell incubator with 5% $\rm CO_2$ and 99% relative humidity. MRC-5 cells were cultured in the Minimum Eagle medium (MEM, Gibco, BRL, USA) with 10% fetal bovine serum.

A549 and H1299 cells were seeded in 6-well plates 24 h before transfection and were incubated overnight. The si-IncRNA PACERR, pcD-NA-IncRNA PACERR, and NC were purchased from YUNZHOU Biotech (Guangzhou, China). Subsequently, 4 µg of each of these three plasmids were added to 250 mL of DMEM, and transfection was performed using the Lipofectamine[™] 2000 transfection kit (Invitrogen Life Technologies) according to the manufacturer's instructions. siPACER: 5'-CAUAGGAGA-UACUGGUAAAUU-3' and pcDNA-IncRNA PACE-RR: 5'-CTCCACGGGTCACCAATATAAA-3'. NC: 5'-UUCUCCGAACGUGUCACGUUU-3'. After 24 h, transfection efficiency in A549 and H1299 cells was verified using real-time reverse transcription polymerase chain reaction (gRT-PCR), and the cells were used for subsequent experiments.

RNA extraction and qRT-PCR

To extract total RNA, we employed the TRIzol reagent (Invitrogen, K0732). The PrimeScriptTM RT reagent Kit with a gDNA Eraser (Invitrogen, 4368813) was utilized to perform reverse transcription in line with the instructions provided by the manufacturer. qRT-PCR Master Mix (Invitrogen, 11762500) was used for the quantitative analysis of the PACERR. β -actin was used as an internal reference. The primer sequences for qRT-PCR are shown in **Table 1**.

The qRT-PCR reaction system: cDNA (1 μ L), forward and reverse primers (0.5 μ L each), SYBR Premix Ex Taq (10 μ L), and ddH₂O (8 μ L). Reaction conditions: 95°C/2 min (initial denaturation) followed by 40 cycles of 95°C (15 s) and 60°C (60 s). GAPDH gene was used as an internal control. The results were presented as the relative expression value using the 2^{- $\Delta\Delta$ Ct} method. The experiment was repeated three times in each set.

Cell proliferation assay

The detection of the vitality of A549 and H1299 cells was accomplished using Cell Counting Kit-8 (CCK-8). After being seeded onto 96-well

| Table 1. | Primer | Sequences | for | qRT-PCR |
|----------|--------|-----------|-----|---------|
|----------|--------|-----------|-----|---------|

| Gene | Primer Sequence |
|---------|---------------------------------------|
| PACEER | Forward: 5'-AGAGAAACCATGGGCAGAAG-3' |
| | reverse: 5'-CAAGCCATGAAACAGCATCT-3' |
| β-actin | forward: 5'-AATCGTGCGTGACATTAAGGAG-3' |
| | reverse: 5'-TGACAGGATGCAGAAGGAGA-3' |

plates at a density of 5000 cells/well in a complete medium for 2 hours, the cells were subjected to subsequent analyses. A total of 10 μ L of CCK-8 solution were introduced into each well after incubation, and then the cells were left to incubate for another 2 hours. Every 24 h after transfection, the proliferation rate was determined, and optical density was quantified at 450 nm on a spectrophotometer. All experiments were performed in triplicates.

Colony formation assay

A549 and H1299 cells were plated in 6-well plates at a density of 1000 cells/well in a complete medium and cultured for 14 d. Subsequently, the cells were washed twice with 10 mL of PBS, immobilized with 2 mL of 4% methanol for 10 minutes, then stained with crystal violet, followed by washing with water and drying overnight. Finally, visible colonies in randomly selected fields were subjected to count, and we computed the rate of cell clone formation.

Transwell invasion assay

A549 and H1299 cells were digested utilizing trypsin and centrifugated for 5 minutes and at 1500 rpm, and the supernatant was discarded. Subsequently, 200 μ L of the serum-free medium was used to prepare cell suspensions (2.5 × 10⁴ cells), and the cells were placed in the upper transwell chamber coated with Matrigel matrix and the medium. Twenty-four hours after migration, the cells were fixed with 4% paraformaldehyde (PFA), followed by subsequent staining with 0.1% crystal violet. Five random fields of vision were chosen for observation and counting of cells.

Wound healing assay

A549 and H1299 cells in the logarithmic growth stage were digested with trypsin, seeded in 6-well plates, and cultured at 5% CO₂ and 37° C.

After the cells achieved confluency of approximately 90%, a horizontal line was drawn in the cell monolayer with a pipette tip. Following 48 hours of culture in a medium containing 2% FBS, the cells were washed using PBS to eliminate non-adherent cells from the plates. The wounds were observed under an inverted microscope (magnification, 40×), and images of the scratches were taken at 0 h and 48 h.

Flow cytometry analysis

A549 and H1299 cells were digested and centrifugated for 5 minutes at 1500 rpm. Subsequently, the cells were suspended in PBS pre-cooled at 4°C and centrifugated for 5 minutes at 1500 rpm. Incubation of the cells was carried out with 10 μ L of PI for 10 min at ambient temperature, followed by staining with annexin V-FITC. After 1 h, cell apoptosis was analyzed using flow cytometry. The excitation and emission wavelengths were 488 nm and 530 nm, respectively.

Western blot assay

Total proteins were extracted from A549 and H1299 cells with the mixed liquor of RIPA lysis buffer. The protein concentration was determined using a BCA protein assay kit (Abcam, ab102536). Then 30 µg/lane of the extracted protein was isolated by 12% SDS-PAGE at a 110-V constant current for 2 h, and then transferred to polyvinylidene difluoride membranes. After that, the membrane was immersed in a TBST inhibiting solution prepared by adding 5% skim milk powder for 1 hour and overnight cultivation with 5 mL of the following primary antibodies: Bcl-2 (CST-15071, 1:1000), Bax (CST-5023, 1:1000), caspase-3 (CST-9662, 1:1000) and β-actin (CST-3700, 1:1000), which were all from Cell Signaling Technology (Beverly, MA, USA).

Immunoreactive bands were visualized using the enhanced chemiluminescence (ECL) kit (Thermo Scientific[™], USA) and integrated density of the bands was quantified by Quantity One software (Bio-Rad).

Statistical analysis

All statistical analyses were performed using R software (version: 3.6.3). Pearson correlation analysis was performed to evaluate the associ-

ation of IncRNAs with ferroptosis-related genes. Wilcoxon rank-sum test was used to compare the differences between the two groups. Kruskal-Wallis test was utilized to analyze differences among three groups. Kaplan-Meier method was used to generate survival curves and the Log-rank test to compare the survival curves of two groups. Univariate Cox regression with LASSO and multivariate Cox regression analyses was utilized to estimate the 14 -FRDELs Signature. The independent prognostic significance of the 14 - FRDELs Signature was assessed through univariate and multivariate Cox regression. *P*-values less than 0.05 (P< 0.05) were considered statistically significant.

Results

Data processing

Bioinformatic analysis was performed as described in the flow chart (Figure 1A). First, the RNA-sequencing and clinicopathological data of patients with LUAD were obtained from the TCGA database. Second, the expression data of 259 ferroptosis-related genes were extracted. According to co-expression and differential expression analyses, ferroptosis-related IncRNA profiles were obtained from ferroptosis-related genes and used to construct the profiles. Third, the entire set of patients (n=468) was divided into the training group (n=312) and verification group (n=156), and the R package "limma" was used to identify FRDELs. Then, the FRDELs were screened by univariate Cox regression, LASSO, and multivariate Cox regression in the training set.

Risk scores were calculated based on the FRDELs signature constructed in stepwise regression analysis, consisting of FRDELs and corresponding coefficients. Fourth, the risk score was verified using survival analysis in the training, validation, and whole sets. In addition, the association of risk score with TMB, immune cell infiltration, and GSEA was analyzed in the whole set, and a nomogram was subsequently constructed. Eventually, the PACERR of the FRDELs signature was validated in A549 and H1299 cells.

Identification of FRDELs

We first screened 259 ferroptosis-related genes (mRNAs) and obtained the available

expression data of 247 genes in the TCGA-LUAD set. Subsequently, 2634 ferroptosisrelated lncRNAs were identified by Pearson correlation analysis (<u>Table S1</u>). Furthermore, 497 LUAD and 54 non-tumor tissues from the TCGA set were used for analyzing differential expression. A total of 909 FRDELs with adjusted P<0.05 and $|\log_2$ (fold change)| >1 were identified (**Figure 1B**; Table S2).

Development of the 14 - FRDELs signature

The whole set (n=468) of patients was randomly classified according to a ratio of 3:1 into the training (n=312) and validation (n=156) sets. In the training set, 76 FRDELs were detected via a univariate Cox regression (P< 0.05). LASSO-Cox regression analysis was further performed (**Figure 2A** and **2B**), and 14 FRDELs were identified (**Table 2**). AP001610.2, AC004832.5, AL355472.3, PACERR, AC0077-73.1, AC116552.1, AC108451.2, LINC00941 and LINC01638 were identified as risk factors with hazard ratio (HR) > 1. AC034102.8, AF131215.5, AC026355.2, MIR223HG and AC246787.2 were identified as protective factors with HR < 1.

Validation of the 14 - FRDELs signature

The risk score was further used to verify its prognostic significance in the training, validation, and whole sets. The patients who belonged to the training (Figure 2C and 2D), validation (Figure 2F and 2G), and whole (Figure 2I and 2J) sets were classified into two sets, namely the low- and high-risk groups based on the risk score for median value. The survival curves of the low- and high-risk groups differed substantially; nevertheless, the low-risk group exhibited a longer survival duration in the training (Figure

Ferroptosis-related IncRNAs signature in lung adenocarcinoma



Figure 1. The flow chart regarding the present study and identification of FRDELs. A. Flow chart for data collection and analysis. B. Volcano plot of downregulated and upregulated differentially expressed ferroptosis-related lncR-NAs. The 'limma' package was utilized to detect FRDELs in 54 normal and 497 LUAD tissues, with cut-off criteria of adjusted P<0.05 and $|\log_2(\text{fold change})| > 1$. Red dots represent ferroptosis-related lncRNAs with high expression, and blue dots represent ferroptosis-related lncRNAs with low expression. LUAD, lung adenocarcinoma; TCGA, The Cancer Genome Atlas; FRDELs, ferroptosis-related differentially expressed lncRNAs; LASSO, least absolute shrinkage, and selection operator; TMB, tumor mutational burden; GSEA, gene set enrichment analysis.

2E), validation (Figure 2H), and whole (Figure 2K) sets.

Furthermore, ROC curves were constructed according to the risk score to evaluate prognostic efficiency. As demonstrated in **Figure 3A**, the AUC of the training, validation and whole sets were 0.733, 0.717, and 0.751. These results indicated that the 14 - FRDELs signature had acceptable sensitivity and specificity in predicting the individual survival of patients with LUAD. To assess the independence of the signature, 447 patients with LUAD in the whole set with complete clinicopathological data (including age, gender, and stage) were included for univariate (**Figure 3B**) and multivariate (**Figure 3C**) Cox regression analyses. The result illustrated that the risk score might serve as a prognostic factor (P<0.05) independent of age, gender, and stage. Therefore, the signature was verified as an independent predictor of good prognosis, irrespective of clinicopathological characteristics.



Figure 2. Development and Verification of the 14 - FRDELs signature. (A) LASSO regression for constructing the FRDELs signature based on the optimal parameter (lambda). (B) The LASSO coefficient profiles regarding the 14 FRDELs. The risk score distribution in the training (C), validation (F), and whole (I) sets. The scatter dot plot shows the survival status of LUAD patients in the training (D), validation (G), and whole (J) sets. Kaplan-Meier survival analysis demonstrates better overall survival in the training (E), validation (H), and whole (K) sets in the low-risk group as opposed to the high-risk group. LUAD, lung adenocarcinoma; FRDELs, ferroptosis-related differentially expressed lncRNAs; LASSO, least absolute shrinkage, and selection operator.

Association of the 14 - FRDELs signature with TMB and immune cell infiltration

We used the R package 'maftools' to summarise and analyze the mutational data of TCGA datasets and compared the 20 topmost mutated genes in the high- (**Figure 4A**) and lowrisk (**Figure 4B**) groups. KRAS, TP53, TTN, MUC16, FLG, and ADAMTS12 were the most frequently mutated genes. When comparing the high- and low-risk groups, the number of patients with TP53 mutations was considerably

| | | - 0 | | 0 | -) |
|------------|--------------|-------------|-------------|-------------|-------------|
| ID | Coeff | HR | HR.95L | HR.95H | P-value |
| AP001610.2 | 0.795370936 | 2.215262564 | 0.957799521 | 5.123606893 | 0.063001836 |
| AC034102.8 | -1.617288949 | 0.19843594 | 0.069489858 | 0.566655673 | 0.002519917 |
| AC004832.5 | 1.039294008 | 2.827220313 | 1.437467398 | 5.560595469 | 0.002600028 |
| AL355472.3 | 0.779997231 | 2.181466225 | 1.143234469 | 4.162571214 | 0.017980743 |
| PACERR | 0.430000161 | 1.537257771 | 1.032019417 | 2.289842047 | 0.034431239 |
| AF131215.5 | -0.454120663 | 0.635006107 | 0.401099121 | 1.00531947 | 0.052704937 |
| AC026355.2 | -0.323628834 | 0.723518738 | 0.54310619 | 0.963861901 | 0.027002455 |
| AC007773.1 | 0.477651612 | 1.612283686 | 1.178811574 | 2.205151987 | 0.002793366 |
| MIR223HG | -0.545276812 | 0.579681298 | 0.364648681 | 0.921518232 | 0.021135932 |
| AC116552.1 | 0.82781487 | 2.288313013 | 1.167008883 | 4.487006499 | 0.015974387 |
| AC108451.2 | 0.262514833 | 1.300195754 | 0.982915813 | 1.719891954 | 0.065880729 |
| AC246787.2 | -0.691589978 | 0.500779208 | 0.27363052 | 0.916490655 | 0.02491257 |
| LINC00941 | 0.41179945 | 1.50953167 | 1.146624643 | 1.987298875 | 0.003333334 |
| LINC01638 | 1.257207435 | 3.515590251 | 1.72230935 | 7.176048142 | 0.000553748 |

Table 2. Construction of the 14 - FRDELs Signature based on LASSO-Cox regression analysis

Abbreviations: HR, hazard ratio; Coeff, coefficient; L, low; H, high.



Figure 3. Prediction accuracy of the 14 - FRDELs Signature. (A) ROC curve demonstrated the signature's feasibility to predict prognosis in the training, validation, and whole sets. Forest plot of univariate (B) and multivariate (C) Cox regression analyses involving 447 patients with LUAD with complete clinicopathological data (including age, gender, and stage). FRDELs, ferroptosis-related differentially expressed IncRNAs; ROC, receiver operating characteristic; LUAD, lung adenocarcinoma.

greater in the high-risk group (P=0.047). The chi-square test revealed that mutations occurred more frequently in the high-risk group

in contrast with those in the low-risk group (91.53% versus 75.89%, respectively, P= 0.002). In addition, the Wilcoxon rank-sum test



Figure 4. Relationship between the 14 - FRDELs Signature and TMB levels and immune cell infiltration. A, B. The top twenty most frequently mutated genes in low- and high-risk groups are shown in a waterfall plot. The chi-square test revealed that mutations occurred more frequently in the high-risk group than in the low-risk group (91.53% versus 75.89% P=0.002). The number of patients with TP53 mutations was substantially elevated in the high-risk group compared to the low-risk group (P=0.047). C. The Wilcoxon rank-sum test revealed that the TMB level of the high-risk group was more significant in contrast with the level in the low-risk group (P<0.001). D. The plot showed the difference among the infiltration of 22 distinct immune cells in the low- and high-risk groups. FRDELs, ferroptosis-related differentially expressed IncRNAs; TMB, tumor mutational burden; LUAD, lung adenocarcinoma.

| Enriched pathways | | Es | NES | NOM <i>P</i> -value | FDR q-value | FWER <i>P</i> -valve |
|---|-----|-------|-------|------------------------|----------------|-------------------------|
| High-risk group | | | | | | |
| KEGG_CELL_CYCLE | 125 | 0.72 | 2.37 | 0.000 | 0.001 | 0.001 |
| KEGG_MISMATCH_REPAIR | 23 | 0.90 | 2.25 | 0.000 | 0.003 | 0.007 |
| KEGG_HOMOLOGOUS_RECOMBINATION | 28 | 0.84 | 2.24 | 0.000 | 0.003 | 0.009 |
| KEGG_DNA_REPLICATION | 36 | 0.88 | 2.13 | 0.000 | 0.008 | 0.026 |
| KEGG_PYRIMIDINE_METABOLISM | 98 | 0.60 | 1.12 | 0.000 | 0.008 | 0.030 |
| KEGG_P53_SIGNALING_PATHWAY | 68 | 0.55 | 2.12 | 0.000 | 0.007 | 0.030 |
| KEGG_PROTEASOME | 46 | 0.78 | 2.02 | 0.002 | 0.015 | 0.067 |
| KEGG_NUCLEOTIDE_EXCISION_REPAIR | 44 | 0.65 | 2.01 | 0.004 | 0.015 | 0.072 |
| KEGG_SPLICEOSOME | 127 | 0.64 | 2.00 | 0.018 | 0.014 | 0.079 |
| KEGG_AMINOACYL_TRNA_BIOSYNTHESIS | 41 | 0.70 | 1.98 | 0.004 | 0.016 | 0.097 |
| Low-risk group | | | | | | |
| KEGG_HEMATOPOIETIC_CELL_LINEAGE | | -0.67 | -2.11 | 0.004 | 0.038 | 0.031 |
| KEGG_INTESTINAL_IMMUNE_NETWORK_FOR_IGA_PRODUCTION | | -0.77 | -2.03 | 0.002 | 0.044 | 0.063 |
| KEGG_ALDOSTERONE_REGULATED_SODIUM_REABSORPTION | | -0.58 | -2.00 | 0.000 | 0.042 | 0.084 |
| KEGG_ASTHMA | 28 | -0.80 | -1.98 | 0.006 | 0.037 | 0.099 |
| KEGG_FC_EPSILON_RI_SIGNALING_PATHWAY | 79 | -0.51 | -1.97 | 0.002 | 0.033 | 0.106 |
| KEGG_FATTY_ACID_METABOLISM | 42 | -0.60 | -1.92 | 0.004 | 0.047 | 0.163 |

| Table 3. KEGG | pathways analysis | of the low- and | high-risk g | roups using GSEA |
|---------------|-------------------|-----------------|-------------|------------------|
| | padinajo anaijoio | or the form and | | |

Abbreviations: ES, enrichment score; NES, normalized enrichment score; NOM *P*-value, nominal *P*-value; FDR q-value, false discovery rate q-value; FWER *P*-value, familywise error rate *P*-value.

suggested that the TMB level of the high-risk group was elevated in contrast with that of the low-risk group (**Figure 4C**).

KEGG_AUTOIMMUNE_THYROID_DISEASE

KEGG_GRAFT_VERSUS_HOST_DISEASE

KEGG_LEISHMANIA_INFECTION KEGG_ALLOGRAFT_REJECTION

Furthermore, we used 22 immune cell types to investigate the potential of the 14 - FRDELs signature to reflect the state of the immunological microenvironment in LUAD (**Figure 4D**). The number of activated resting mast cells, CD4 memory T cells, and resting NK cells was elevated in the high-risk group as opposed to the low-risk group (P<0.05). However, the number of monocytes, M0 and M1 macrophages, activated mast cells, and resting dendritic cells was substantially reduced in the high-risk group in contrast with the low-risk group (P<0.05). These findings demonstrated that the 14 - FRDELs signature was related to immune cell infiltration in the LUAD.

Pathway enrichment analysis of the 14 -FRDELs signature

GSEA was performed in the low- and high-risk groups to examine the prospective molecular

mechanisms of the 14 - FRDELs signature in LUAD, and 80 enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were identified. The top 10 pathways in both sets are summarised in **Table 3** and **Figure 5**.

70 -0.61 -1.84 0.022 0.064 0.256

50 -0.67 -1.90 0.006

37 -0.76 -1.86 0.012

35 -0.78 -1.84 0.012

0.045

0.062

0.059 0.227

0.177

0.267

Establishment of a new predictive nomogram

We developed a nomogram to anticipate the individualized survival probability of LUAD patients according to independent prediction parameters generated from the multivariate Cox regression analysis (Figure 6A). The AUC values predicted by our nomogram for the one-, three-, and five-year overall survival probability were 0.783, 0.761, and 0.774 (Figure 6B). Generally, an AUC value of 0.5 suggests no discrimination, an AUC value of 0.7-0.8 suggests acceptable sensitivity and sensitivity, an AUC value of 0.8-0.9 suggests excellent sensitivity and sensitivity, and an AUC value higher than 0.9 suggests outstanding sensitivity and specificity. The results of the ROC analysis indicated that the nomogram was an acceptable prediction model.



Figure 5. The top 10 KEGG signaling pathways in the low- and high-risk groups were assessed via GSEA with FDR <0.25 and NOM *P*-value <0.05. GSEA, gene set enrichment analysis; KEGG, Kyoto Encyclopedia of Genes and Genomes; NOM, nominal; FDR, false discovery rate.

Furthermore, DCA demonstrated that the nomogram model was optimal for predicting one-, three- and five-year overall survival (**Figure 6C**). These data indicate that using the constructed nomogram to predict prognosis is of a more significant benefit than using a single parameter for prediction. Nomograms integrating clinicopathological data and risk score could enhance clinical management, decisionmaking, and patient counselling.

PACERR enhanced the migration and invasiveness of LUAD cells

PACERR is a recently discovered IncRNA that regulates cyclooxygenase 2 (COX-2) gene expression and promotes inflammation in primary human mammary cells and macrophagocytes. The association between COX-2 overexpression and survival in patients with lung cancer has been under investigation for more than a decade and is reported to be a poor prognostic indicator [16-18]. Therefore, the function of PACERR in LUAD was investigated further. First, PACERR was substantially up-modulated in A549, H1792, and H1299 cells compared to human normal lung fibroblast MRC-5 cells (P<0.05, **Figure 7A**).

The results showed that cell viability was significantly enhanced after PACERR overexpression but decreased after PACERR inhibition in A549 and H1299 cells compared with the NC set (P<0.01, **Figure 7B** and **7C**). Simultaneously, comparable findings were discovered in the cell proliferation assay (**Figure 7D**), and the ability of A549 and H1299 cells to form colonies was enhanced following the overexpression of PACERR (P<0.01) but suppressed after PACERR inhibition (P<0.01).

We found that the cell migration ability as well as the invasiveness of A549 and H1299 cells were substantially enhanced after PACERR overexpression (P<0.01) but suppressed after PACERR inhibition (P<0.01, **Figure 7E** and **7F**). These results indicated that PACERR significantly enhanced the migration ability and invasiveness of A549 and H1299 cells *in vitro*.

PACERR decreased apoptosis of LUAD cells

We used flow cytometry to evaluate the effects of PACERR on the apoptosis of A549 and H1299 cells. As illustrated in Figure 8A, the apoptosis rate of A549 and H1299 cells was significantly decreased after PACERR overexpression as opposed to the NC set (P<0.01) but increased after PACERR inhibition (P<0.01, Figure 8A). Moreover, we found that PACERR overexpression substantially enhanced Bcl-2 expression and suppressed Bax and caspase-3 expressions in A549 and H1299 cells (P<0.01, Figure 8B). However, PACERR inhibition significantly enhanced Bax and caspase-3 expressions and suppressed the expression of Bcl-2 (P<0.01, Figure 8C). Caspase-3 and Bcl-2 are pro-apoptotic proteins, while Bax is an antiapoptotic protein [19]. Therefore, PACERR inhibited LUAD cell apoptosis, indicating that PACERR-targeted drugs may be a novel therapeutic strategy for LUAD.

Discussion

Despite optimistic advances in the testing, diagnosis, and therapy of LUAD in the past few years, this disease remains among the most lethal malignancies due to the complexity of its molecular and genetic processes [20]. Ferroptosis is a recently recognized regulatory cell death that is triggered by an excess buildup of iron-dependent reactive oxygen species and lipid peroxides. It is strongly correlated with the pathological process of LUAD and has been described before [1]. As emerging biomarkers, IncRNAs perform a fundamental role in the incidence and progression of distinct tumors such

Ferroptosis-related IncRNAs signature in lung adenocarcinoma



Figure 6. Development and Verification of a nomogram integrating the 14 - FRDELs signature and clinicopathological data. A. A nomogram was constructed by integrating the 14 - FRDELs signature and clinicopathological data to anticipate the one-, three-, and five-year overall survival of LUAD patients. B. Time-dependent ROC curves of the nomogram to anticipate the one-, three-, and five-year overall survival of LUAD patients. C. Time-dependent DCA for determining the net benefits of the nomogram, age, and stage to anticipate the one-, three-, and five-year overall survival of LUAD patients.

as LUAD [21]. In addition, many IncRNAs are involved in the occurrence, progression, and medication resistance of malignant tumors and have been identified as novel biomarkers and treatment targets for diagnosing and treating tumors [22, 23]. Nonetheless, it is yet uncertain if ferroptosis-related IncRNAs could be used to anticipate the prognosis of LUAD patients. Given this, we developed a predictive model for LUAD and predicted the survival probability of patients by screening for IncRNAs associated with ferroptosis. In the present study, we identified 14 FRDELs including AP001610.2, AC034102.8, AC004-832.5, AL355472.3, PACERR, AF131215.5, AC026355.2, AC007773.1, MIR223HG, AC11-6552.1, AC108451.2, AC246787.2, LINC00-941 and LINC01638. LINC00941 increases colorectal cancer metastasis through the TGF/SMAD2/3 axis [24]. In addition, LINC01638 expression is upregulated in LUAD tissues and cells and promotes proliferation but represses apoptosis via the PTEN/AKT signaling pathway [25]. In a LUAD prognosis model [26-28],



Figure 7. PACERR promoted the proliferation, migration, and invasion of A549 and H1299 cells. A. Assessment of PACERR upregulation in H1299, A549, and H1299 cells via qRT-PCR. B, C. Evaluation of the viability of A549 and H1299 cells via MTT assay after transfection for 24 h, 48 h, 72 h, and 96 h. D. Assessment of colony formation in A549 and H1299 cells via a colony formation assay. E. Assessment of the migration of A549 and H1299 cells via wound healing assay. Scale: 100 μ M; magnification: 100 ×. F. Assessment of the invasion of A549 and H1299 cells via transwell assay. Scale: 100 μ M; magnification: 100 ×. All the data are shown as mean ± SD; **P<0.01, **P<0.001.

AF131215.5, AC026355.2, and MIR223HG have been identified as indicators that can

accurately predict prognosis. In this study, PACERR was overexpressed in LUAD cell lines.

Ferroptosis-related IncRNAs signature in lung adenocarcinoma



Figure 8. PACERR decreased the apoptosis of A549 and H1299 cells. A. Evaluation of the apoptosis of A549 and H1299 cells using flow cytometry. The apoptosis of A549 and H1299 cells (P<0.001) was induced after PACERR knockdown but decreased after PACERR overexpression (P<0.001) as opposed to the NC set. B, C. Evaluation of the expression of proteins associated with apoptosis, Bcl-2, Bax, and caspase-3 in A549 and H1299 cells via western blot assay, using β -action as an endogenous control. PACERR overexpression substantially elevated Bcl-2 expression and suppressed caspase-3 and Bax expression in A549 and H1299 cells (P<0.001), whereas PACERR inhibition significantly enhanced Bax and caspase-3 expression and suppressed the expression of Bcl-2. All the data are shown as mean ± SD; **P<0.01, **P<0.001.

It was found that PACERR might be a poor predictor of prognosis in LUAD, as it enhances cell proliferation, invasion, and migration, and is anti-apoptotic, suggesting that PACEER has potential value for the diagnosis and treatment of LUAD. Previous studies demonstrated that PACERR might facilitate the metastasis of pancreatic ductal adenocarcinoma via the nonclassical M1-M2 polarization [29]. As far as we know, this is the first research report on the functions of PACEER in LUAD. Although little is known about other FRDELs, their importance should not be underestimated. Furthermore, we developed a prognostic signature for LUAD based on 14 FRDELs, which may independently serve as a prognostic indicator for LUAD. The survival probability of the highrisk group was shorter than that of the low-risk group in the training, validation, and whole sets. In addition, the AUC values for the training, validation, and whole sets were 0.733, 0.717, and 0.751, respectively. Several evaluation methods have been developed to assess the prognosis of patients with LUAD. In a study by Li et al., a prognostic model for LUAD was constructed based on immune-related IncRNAs. The AUC value in their study for predicting 5-year survival rates was 0.702, which was lower than that of our model [30]. Therefore, the 14 - FRDEL signature developed in our study may be an excellent prognostic model for LUAD.

One of the significant challenges of immunotherapy for patients with LUAD is a lack of understanding of tumors' heterogeneity, complexity, and immune evasion mechanism. Furthermore, specific biomarkers for evaluating the benefit of tumor immunotherapy treatments are insufficient. The discovery of novel immunotherapy targets and prognostic indicators is thus of great significance [31]. Several clinicopathological studies have suggested a correlation between genetic alternations and responsiveness to immunological treatment [32, 33]. In the present research, the high-risk group exhibited a higher frequency of mutation events and a significant increase in TMB levels. This finding suggests that patients with LUAD can benefit from tumor immunotherapy, such as programmed death receptor 1 (PD-1) inhibitor therapy, because the response rate to immune checkpoint inhibitors is considerably elevated in patients with higher TMB levels in contrast with those with lower TMB levels [34, 35]. Moreover, the efficacy of immunotherapy is dependent on the coordinated responses of both innate and adaptive immune cells [36]. The tumor infiltrating immune cells may be a valuable prognostic tool in the treatment of cancer [37]. This study employed the CIBERSORT algorithm to examine the relative proportion of 22 distinct kinds of immune cells in tumor tissues in the TCGA set. We found that the number of activated CD4 memory T cells, resting NK cells, and resting mast cells was elevated in the high-risk set, confirming the role of the 14 - FRDELs signature in the regulation of immune cell infiltration in tumors. Notably, these findings were consistent with previous studies that LINC00941 [38] and AF131215.5 [39] served as regulators in tumor immunity. These findings illustrated that the signature proposed in the present study might anticipate the efficacy of immunotherapy in LUAD patients.

Due to the differences in genetic variations among patients, targeted treatment is highly likely to be a precise and personalized therapeutic strategy. Molecular pathways involved in LUAD should be identified to discover new treat-

ment targets. Therefore, we used GSEA to screen for signaling pathways involved in the 14 - FRDELs signature related to a range of cell life activities. The P53 signaling pathway is strongly correlated with the apoptosis and progression of lung cancer cells and regulates the immune response [40]. DNA replication is a critical biochemical process in tumor development [41]. According to a recent study, ailanthuses can delay the progression of LUAD by inhibiting DNA replication by decreasing RPA1 (replication protein A1) expression [42]. These findings suggested that the 14 - FRDELs signature is involved in some cancer-related signaling pathways and associated with the occurrence and development of LUAD.

The tumor stage, which is defined using the tumor, node, and metastasis (TNM) approach, is usually used to evaluate prognosis. With the advent of precision medicine, mounting data has revealed that IncRNAs may have prognostic significance in cancer prognosis [43]. Therefore, compared to currently available prognostic methods, a prognostic model created by integrating IncRNAs with tumor stage has superior predictive accuracy. Nomograms are easy-tounderstand prognostic prediction models that are increasingly used in medical research and clinical practice [44]. This study constructed a nomogram integrating age, TNM stage, and the 14 - FRDELs signature based on independent prediction factors generated from multivariable Cox regression analysis. The ROC curve revealed that the nomogram predicted a better prognosis than that predicted by individual prognostic factors, such as the tumor stage. In addition, DCA demonstrated that using the nomogram to predict prognosis can bring greater net clinical benefits. These results indicate that the nomogram based on the 14 - FRDELs signature may have reliable clinical applications and improved accuracy to predict prognosis.

To the best of our knowledge, a nomogram based on the 14 - FRDELs signature still hasn't been previously reported, and they may be shown to be practical tools in determining the prognosis of patients with LUAD. Targeted drugs to develop novel ferroptosis-related therapeutic strategies based on the 14 - FRDELs signatures may improve the prognosis of patients with LUAD in the near future. However, this study has several limitations: 1. Because the clinicopathological data of LUAD obtained from TCGA were limited and incomplete, the nomogram did not contain treatment or tumor marker data. 2. More functional experiments on the 14 FRDELs are necessary to further elucidate the fundamental molecular mechanisms. 3. The nomogram was constructed based on a retrospective research design; thus, the model's performance must be validated in multi-center, large-scale prospective clinical research.

Conclusion

In conclusion, we constructed a 14 - FRDELs signature, comprising nine risk factors (APO-01610.2, AC004832.5, AL355472.3, PACERR, AC007773.1, AC116552.1, AC108451.2, LIN-C00941, and LINC01638) and five protective factors (AC034102.8, AF131215.5, AC026-355.2, MIR223HG, and AC246787.2), which might provide an accurate prediction for the prognosis of LUAD patients. The signature is closely related to some infiltrating immune cells and mutated genes in tumors. Moreover, we established a nomogram incorporating the signature and clinicopathological factors to anticipate the survival outcomes of LUAD patients.

Among the 14 FRDELs, PACERR promoted cell proliferation in LUAD cells. Therefore, our results contribute to a better understanding of FRDELs and offer insights into developing novel treatment approaches. However, more comprehensive molecular mechanisms of PACERR involved in LUAD progression should be elucidated.

Acknowledgements

The Second Affiliated Hospital of Nanchang University approved our study. This study was conducted following the Declaration of Helsinki.

Disclosure of conflict of interest

None.

Abbreviations

LUAD, Lung adenocarcinoma; FRDELs, Ferroptosis-related differentially expressed IncRNAs; DCA, Decision curve analysis; PD-1, Programmed death receptor 1; PACERR or PACER, PTGS2 antisense NFKB1 complex-mediated expression regulator RNA; LASSO, Least absolute shrinkage and selection operator; GSEA, Gene set enrichment analysis; ROC, Receiver operating characteristic; TMB, Tumor mutational burden; CIBERSORT, Cell-type Identification by Estimating Relative Subsets of RNA Transcripts; NOM, Nominal; FDR, False discovery rate; TCGA, The Cancer Genome Atlas; CCK-8, Cell Counting Kit-8; ECL, Enhanced chemiluminescence; KEGG, Kyoto Encyclopedia of Genes and Genomes; TNM, Tumor, node, and metastasis.

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