

A Ferroptosis-Related Long Non-Coding RNA Signature Predicts Prognosis and Immune Microenvironment for Lung Squamous Cell Carcinoma

Zhiyang Liu

University of South China

Zhongcheng Xie

University of South China

Chenxi Zhi

University of South China

Xiaoyan Lin

University of South China

Wentao Ma

University of South China

Xiaoqian Tan

University of South China

Pin Li

University of South China

Siyu Ouyang

University of South China

Jiang Yu

University of South China

Xi Chen

University of South China

Tianhong Peng

University of South China

Liang Li

University of South China

Wei Xie (✉ weixiehy@126.com)

University of South China

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Abstract

Background: Ferroptosis-related lncRNAs (FerLncRNAs) were developed to play a significant role in cancer treatment and prognosis. However, the relationship between FerLncRNAs and Lung squamous cell carcinoma (LUSC) remains unclear.

Method: Based on ferroptosis-related differentially expressed lncRNAs in LUSC, we established a prognostic 8-lncRNA signature.

Results: 8 Ferroptosis-related lncRNAs (LUCAT1, AL161431.1, AL122125.1, AC104248.1, AC016924.1, MIR3945HG, C10orf55 and AP006545.2) had prognostic value for LUSC by multivariate COX analysis ($P < 0.05$), and possessed significant association with patient outcomes. Kaplan–Meier curves showed an obvious difference in OS that the high-risk group patients exhibited poorer survival than the low-risk group patients. The clinical receiver operating characteristic (ROC) curve and decision curve analysis (DCA) revealed that the ferroptosis-related lncRNAs prognostic signature can (FerRLSig) emerged more outstanding performance than clinical features in predicting the prognosis of LUSC. GSEA revealed that the majority of the novel Ferroptosis-related lncRNAs signature-regulated immune responses and the immune system processes were enriched in the high-risk group. 34 immune checkpoints (ICs) were detected significantly different expression between high-risk and low-risk groups.

Conclusion: A novel FerRLSig based on 8 Ferroptosis-related lncRNAs provided important prognostic value for LUSC patients and developed new insights about ferroptosis-related immunotherapy targets in clinic.

1. Introduction

Despite great efforts made in recent years, the incidence of Lung squamous cell carcinoma (LUSC), a histological subtype of non-small cell lung carcinoma (NSCLCs) (Travis, 2002; Travis et al., 2015; Youlden, Cramb, & Baade, 2008), remains increasing and is a leading cause of cancer death worldwide (Stinchcombe, 2014). A poor 5-year overall survival (OS) rate of LUSC by current treatments further prompts that novel and efficient clinical managements are urgent to be established (Garon et al., 2019; Miller et al., 2012). Therefore, it is strongly required that definite diagnostic biomarkers, new therapeutic targets, and favorable prognostic signatures for patients with LUSC be found.

Iron not only is a vital microelement in normal cell physiological growth and development, but also plays a vital role in tumor cell of LUSCs (Babu & Muckenthaler, 2016; Feger et al., 2001; G. X. Wang et al., 2017). Previous studies have shown that ferroptosis, as a unique iron-dependent form of regulated cell death characterized by overwhelming lipid peroxidation due to accumulation of reactive oxygen species (ROS) (Doll et al., 2017; Yang et al., 2014; J. Zheng & Conrad, 2020), which differs from others such as apoptosis, necrosis and autophagy in respects of morphology, genetics and biochemistry (Dixon et al., 2012), is involved in the pathological process of lung cancer cell proliferation, migration and prognosis (Han et al., 2021; C. Y. Wu et al., 2021). Meanwhile, the adjustable sensitivity of lung cancer cells to ferroptosis by pharmacological strategies can affect the outcomes of lung carcinoma (Alvarez et al., 2017; Freire Boullosa et al., 2021). Overexpression of $\Delta Np63a$ protects LUSC from oxidative damage-induced ferroptosis to implement cancer cell with high viability (G. X. Wang et al., 2017). Thus, exploring regulations of ferroptosis is significant for the development of novel anti-LUSCs drugs and the improvement OS of patient with LUSC (J. Guo et al., 2018).

Long non-coding RNAs (lncRNAs) are transcripts composed by nucleotides ranging in length from 200bp to 10kbp (D. Wang et al., 2020). Although limited in protein-encode function, lncRNAs have been found to perform various functions in a wide variety of important biological and pathological processes, such as chronic inflammatory response, cell migration and invasion (Bai et al., 2020; Qian et al., 2018). Notably, previous studies have shown lncRNAs play as the essential regulators in ferroptosis and iron metabolism in lung cancers. For instance, LINC0036 exerts anti-ferroptosis effect in lung carcinoma by acting as an endogenous sponge of microRNA 6852 to regulate the expression of cystathionine- β -synthase (CBS), a surrogate marker of ferroptosis (M. Wang et al., 2019). Metallothionein 1D pseudogene (MT1DP) sensitizes NSCLCs toward ferroptosis via elevating lipid reactive oxygen species (ROS) (Gai et al., 2020). Silencing nuclear enriched transcript 1 (NEAT1) aggravates erastin-induced ferroptosis through decreasing levels of ACSL4, SLC7A11, and GPX4 in NSCLCs (H. Wu & Liu, 2021). Those have indicated ferroptosis-related lncRNAs (FerLncRNAs) are closely related to pathological outcome of NSCLCs, but whether to regulate LUSC and the specific mechanism maintains unclear. Therefore, identifying FerLncRNAs in LUSC is a key link in creating a prognostic signature based on FerLncRNAs, which may be contribute to a theoretical basis for novel strategies to treat patients with LUSC.

Immune checkpoint molecules, the receptors expressed on immune cell, inhibit immune response by triggering immune cells into a state of “exhaustion” (Wykes & Lewin, 2018; Zhou et al., 2017). Tumor cells activate immune checkpoint pathways to escape from host immune surveillance and create a microenvironment benefitting for neoplasia (McGranahan et al., 2016; Zhou et al., 2019). Administration of immune checkpoint inhibitors (ICIs) as an immunotherapy has been applied in clinical treatments partly to cancer patients (Grasselly et al., 2018; D. Liu, 2019). There have been studies finding lncRNAs are closely correlated with tumor immunity, but the role of FerLncRNAs in LUSC tumor immunity remains largely unexplored.

In this study, a prognosis signature of FerLncRNAs was constructed to evaluate actual scientific relevance and applicability to LUSC and identified the roles in tumor immunity, which contributes to clarifying the relationship between FerLncRNAs and LUSC, providing potential diagnostic biomarker and therapeutic targets to LUSC in clinic.

2. Materials And Methods

2.1. Data Source

The RNA sequencing (RNA-seq) data (49 normal and 502 tumors) and corresponding clinical information of patients with LUSC were from TCGA-LUSC. Table.1 showed the clinical features of the patients. What's more, some patients were excluded with incomplete clinical information. Eventually, a total of 102

patients were excluded from the study (Table 1). The ferroptosis-related genes were extracted from FerrDb. Finally, 246 ferroptosis-related genes were identified (Supplementary Table S1). Pearson correlation was used to assess the relationship between the ferroptosis-related lncRNAs and LUSC ($|R2| > 0.4$ at $P < 0.001$). The criteria was set for the ferroptosis-related lncRNAs to $FDR < 0.05$ and $|\log_2 FC| \geq 1$. First, we made expression and enrichment analysis of both upregulated and downregulated ferroptosis-related differentially expressed genes (DEGs). Then Gene ontology (GO) was used to evaluate the biological pathways associated with the DEGs. Through the use of ggplot2 package of R software, further function regulated by ferroptosis-related DEGs was analyzed based on Kyoto Encyclopedia of Genes and Genomes (KEGG) data.

2.2. Development and Validation of a Ferroptosis-Related lncRNAs Prognostic Signature

In order to establish the FerLncRNAs prognostic signature (FerRLSig), Lasso-penalized Cox regression and Univariate Cox regression analysis were performed. The computational formula was as follows: Risk Score = sum (each lncRNA's expression \times corresponding regression coefficient). Next, we calculated the scores of each patient in the data collected from TCGA. Patients were classified into low-risk and high-risk groups on the basis of the median risk score. By Kaplan-Meier analysis, the OS of high-risk and low-risk groups were compared. Time-dependent receiver operating characteristic curves (ROC) and risk survival status together proved the sensitivity of our model in predicting the prognosis of LUSC. And decision curve analysis (DCA) verified the specificity of FerRLSig.

2.3. Gene set enrichment analyses and the predictive nomogram

Gene set enrichment analysis (GSEA) was used to study the high-risk and low-risk groups, and visualized their pathways closely related to immunity and tumorigenesis. In order to explore the clinical significance of the FerRLSig, a nomogram integrating prognostic signals was constructed to predict the 1-, 3- and 5-year OS of HNSCC patients.

2.4. Immune correlation analysis

The difference of immune response, between high-risk and low-risk groups, under distinct algorithms (TIMER, CIBERSORT, CIBERSORT-ABS, QUANTISEQ, MCPOUNTER, XCELL, EPIC) was revealed by heat map. Furthermore, we used ssGSEA to quantify the tumor-infiltrating immune cell subgroups and immune function between the two groups. Potential immune checkpoints between high-risk and low-risk groups were listed.

2.5. Statistical analysis

All statistical analyses were completed by using Bioconductor packages in R software, version 4.1.0., Cytoscape and GSEA 4.1.0. Benjamin Hochberg method was used to identify different expressed FerLncRNAs. "Gsva" (R-package) was utilized in ssGSEA. Logistic regression analysis was used to evaluate the relationship between FerLncRNAs and clinicopathological manifestations. Statistical tests were bilateral with P value ≤ 0.05 indicated statistically significant differences.

3. Results

3.1 The expression and enrichment analysis of ferroptosis-related DEGs in LUSC

The 102 ferroptosis-related differentially expressed genes (FerDEGs) were first discovered in total from The Cancer Genome Atlas (TCGA) database which contains clinical information of patients with lung squamous cell carcinoma (LUSC), including 35 downregulated genes and 67 upregulated genes (Supplementary Table S2). The GO enrichment significantly revealed that FerDEGs were obviously enriched in many immune-related biological processes (BP) which generally appeared in the apical part of cell plasma membrane, such as response to oxidative stress, cellular response to chemical stress, cellular response to oxidative stress and reactive oxygen species metabolic process (Fig. 1A). The overexpressed FerDEGs was mainly involved in fluid shear stress and atherosclerosis, HIF-1 signaling pathway, Bladder cancer and AGE-RAGE signaling pathway in diabetic complications through the KEGG pathway analysis (Fig. 1B).

3.2 Identification of FerLncRNAs associated with prognostic signature in LUSC

1209 FerLncRNAs were identified by Pearson correlation analysis ($|R2| > 0.4$ and $P < 0.001$) (Supplementary Table S3) and 505 FerLncRNAs were found differentially expressed between normal and tumor tissue with filtering criteria of $FDR < 0.05$ and $|\log FC| > 1$ (Supplementary Table S4). 29 out of the 505 FerLncRNAs had prognostic value for LUSC by univariate Cox regression ($P < 0.05$), possessing significant association with patient outcomes. Among the 29 FerLncRNAs, 5 were poor prognostic factors ($HR > 1$, Fig. 1C) while 24 were favorable. Subsequently, 8 FerLncRNAs were identified in multivariate COX analysis to establish a FerRLSig, including LUCAT1, AL161431.1, AL122125.1, AC104248.1, AC016924.1, MIR3945HG, C10orf55, AP006545.2). The visualized co-expression network between 8 FerLncRNAs and ferroptosis-related genes constructed by Cytoscape was shown in (Fig. 1D). Profiting from this model, patients with LUSC were classified into low-risk and high-risk groups on the basis of the corresponding median risk score which was calculated by the favorable prognostic signature ($0.1052 \times \text{Expression}_{LUCAT1} + 0.004 \times \text{Expression}_{AL161431.1} - 0.319513084 \times \text{Expression}_{AL122125.1} + 0.146203641 \times \text{Expression}_{AC104248.1} + 0.592710446 \times \text{Expression}_{AC016924.1} + 0.434557078 \times \text{Expression}_{MIR3945HG} + 0.20.170496616 \times \text{Expression}_{AP006545.2}$).

3.3 Multivariate examination of FerLncRNAs prognostic Signature

Kaplan-Meier curves showed an obvious difference in OS that the high-risk group patients exhibited poorer survival than the low-risk group patients ($P < 0.001$, Fig. 2A), suggesting that the building-related signature of the 8-FerLncRNAs effectively predicts survival. Time-dependent receiver operating characteristic curves exhibited the AUC (Area Under the Curve) at 1, 3, 5-years were 0.658, 0.693, 0.687 respectively, showing predictive value of the novel FerRLSig (Fig. 2B). Risk survival status was shown in (Fig. 2C). The death risk augmented and survival duration diminished following the increased risk score, indicating risk score was inversely proportional to the survival rate of patients with LUSC. The heatmap also demonstrated that the 8-FerLncRNAs was positively correlated

with the prognostic signature (Fig. 2. D). Moreover, the clinical receiver operating characteristic (ROC) curve and decision curve analysis (DCA) revealed that the FerRLSig emerged more outstanding performance than clinical features in predicting the prognosis of LUSC (Fig. 2.E,F). In the univariate and multivariate Cox independent prognostic analysis, risk scores ($P < 0.001$) revealed the risk signature was prognostic factor for predicting the OS of LUSC patients (Fig. 3.A). To investigate whether the FerRLSig participated in the development of LUSC, the heatmap analyzed the association between 8 FerLncRNAs and clinicopathological manifestations, which showed there were significant differences between high-risk and low-risk groups (Fig. 3.B). The clinical nomogram based on prognostic signature combined with the clinical factors also confirmed a FerRLSig might be applied in clinic as a novel signature for prognostic diagnosis and therapeutic management of LUSC patients (Fig. 3.C). Those results verified FerRLSig was an independent prognostic factor characterized by stability and accuracy for OS in patients with LUSC.

3.4 Immunity-related status and functions responsible for different OS in patients with LUSC

To assess the correlations between the LUSC prognosis and immune system function, gene set enrichment analyses (GSEA) were applied to compare between the high-risk and the low-risk, revealing that the majority of the novel FerRLSig-regulated immune responses and the immune system processes were enriched in the high-risk group, such as hematopoietic cell lineage, cell adhesion molecules, leukocyte transendothelial migration, antigen processing and presentation, natural killer cell mediated cytotoxicity, intestinal immune network for IgA production, chemokine signaling pathway, JAK stat signaling pathway, and Toll like receptor signaling pathway ($P < 0.05$, FDR < 0.05 ; Fig. 4).

Based on TIMER, CIBERSORT, CIBERSORT-ABS, QUANTISEQ, MCPOUNTER, XCELL, EPIC, the immune-related status heatmap confirmed the differences of T cell CD8+, Myeloid dendritic cell, Monocyte, XCELL, Monocyte, MCPOUNTER and Mast cell resting, CIBERSORT-ABS between high-risk and low-risk group (Fig. 5.A). The correlation analysis of single-sample gene set enrichment analysis (ssGSEA) based on TCGA-HNSCC data in immune cell subsets and functions showed that there were significant differences between two risk groups except for APC_co_inhibition (Fig. 5.B). In addition, the difference was further found in the expression of immune checkpoints, such as CD86, CD48, HAVCR2 and LAIR1 (Fig. 5.C), suggesting that FerRLSig may involve in immune regulation of tumor microenvironment via affecting immune cells. m6A related mRNA expression was significant including FTO, YTHDC2, METTL3, YTHDC1, HNRNPC and YTHDF1 by comparing high and low risk groups (Fig. 5.D), which means genes that regulate methylation may be involved in the regulation of the immune system.

4. Discussion

The incidence and mortality rate of LUSC show an increasing trend internationally, causing serious harm to human health (Torre et al., 2015). A good prognostic model is contributed to the clinical survival and treatment strategy in patients with LUSC. There have been studies on distinct FerRLSig to LUAD (Y. Guo et al., 2021; Lu, Liu, Zhao, Gui, & Zhao, 2021; Z. Zheng et al., 2021), but no LUSC. In this study, a novel FerRLSig based on the TCGA dataset was constructed to predict survival and immune infiltration of LUSC patients, and possesses the specificity and sensitivity, compared with traditional clinical factors. FerLncRNAs in this FerRLSig may regulate immune infiltration and tumor microenvironment in the prognosis of LUSC.

102 FerDEGs were discovered mainly involved in chemical carcinogenesis reactive oxygen species, HIF-1 signaling pathway *et al.*, which affect cancer cells growth and metabolism (Hua et al., 2020). Among them, 8 identified FerLncRNAs were established as a FerRLSig including LUCAT1, AL161431.1, AL122125.1, AC104248.1, AC016924.1, MIR3945HG, C10orf55 and AP006545.2. LUCAT1 increases the viability of colorectal cancer (CRC) cells and promotes the tumorigenicity by inhibiting DNA damage (Huan et al., 2020). AL161431.1 plays a carcinogenesis role in endometrial carcinoma via de-repressing MAPK (mitogen-activated protein kinase) signaling pathway (Gu & Liu, 2020). Plasminogen activator urokinase (PLAU) related gene C10orf55 is a key factor in tumorigenesis of headneck squamous cell carcinoma (HNSCC) by activation of epithelial-to-mesenchymal transition (EMT) and cancer cells growth (Chen et al., 2021). Although these FerLncRNAs have been demonstrated to be related to malignancy, their roles in the prognosis of LUSC has still been unclear.

Previous studies have found lncRNAs are associated with tumor immunity. lncRNA MIR155 host gene (MIR155HG) is relevant with the level of cytotoxic T lymphocyte-associated antigen 4 (CTLA4), and high expressed MIR155HG prompts a better OS of patient with lung cancer (Peng, Chen, Chen, Wang, & Tang, 2019). In NSCLC, as a potential therapeutic target, lncRNA MEG3 suppresses cancer cell proliferation and invasion ability via decreasing expression of IDO1 (C. Wang, Tao, & Wei, 2021). lncRNA SNHG3 in hepatocellular carcinoma (HCC) cells is proved to participate in the regulation of immune infiltration by impacting the expression of CD86 (Zhan et al., 2021). For exploring the potential functions of FerLncRNAs in LUSC, the expression of FerLncRNAs between high-risk and low-risk groups were compared. And the analysis indicated most of ICIs were high-expressed in high-risk groups. In addition, the GSEA analyses of the high-risk and low-risk groups exhibited that the immune response and immune system processes were significantly enriched in the high-risk group, and thus, the roles of FerLncRNAs had been more confirmed in the regulation of tumor immune infiltration.

Currently, few studies have explored the relevance between N6-methyladenosine (m6A) and ferroptosis. From the m6A analysis, YTHDC2 and ALKBH5 were rich in low-risk group. YTHDC2, an m6A methylase, is regarded as a powerful endogenous ferroptosis inducer via suppressing SLC3A2, which is essential for anti-oxidation as a subunit of system XC^- in cell (Ma et al., 2021). Suppression of ALKBH5 is a vital process to promote sorafenib(SF)-induced ferroptosis in HCC (Z. Liu et al., 2020). It may speculate that FerLncRNAs mediate methylation in LUSC to facilitate ferroptosis.

The signature of 8 FerLncRNAs has an independent prognostic value for patient with LUSC and exhibits a promising prospect of ferroptosis and immunity in tumor therapy. Therefore, this novel FerRLSig is significant for improving the clinical prediction of prognosis and management among LUSC patients.

Declarations

Conflict of Interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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Confirm Statement

I confirm that all methods were carried out in accordance with Bioinformatics guidelines and regulations. The data used to support the findings of this study are available from The Cancer Genome Atlas (TCGA, <https://portal.gdc.cancer.gov/>), and the code would be supplied from the corresponding author upon request. Conflicts of Interest: The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Tables

Table 1. Clinical features of Lung squamous cell carcinoma (LUSC) patients in TGCA database.

Variable	Number of samples
Age[years]	
≤ 65	190
> 65	305
Unknown	9
Vital status	
Alive	218
Dead	286
Gender	
Female	131
Male	373
TNM stage	
Stage I	245
Stage II	163
Stage III	85
Stage IV	7
Unknown	4
T stage	
T1	114
T2	295
T3	71
T4	24
N stage	
N0	320
N1	133
N2	40
N3	5
Unknown	6
M stage	
M0	414
M1	7
M2	0
M3	0
Unknown	83

Figures

A



Figure 2

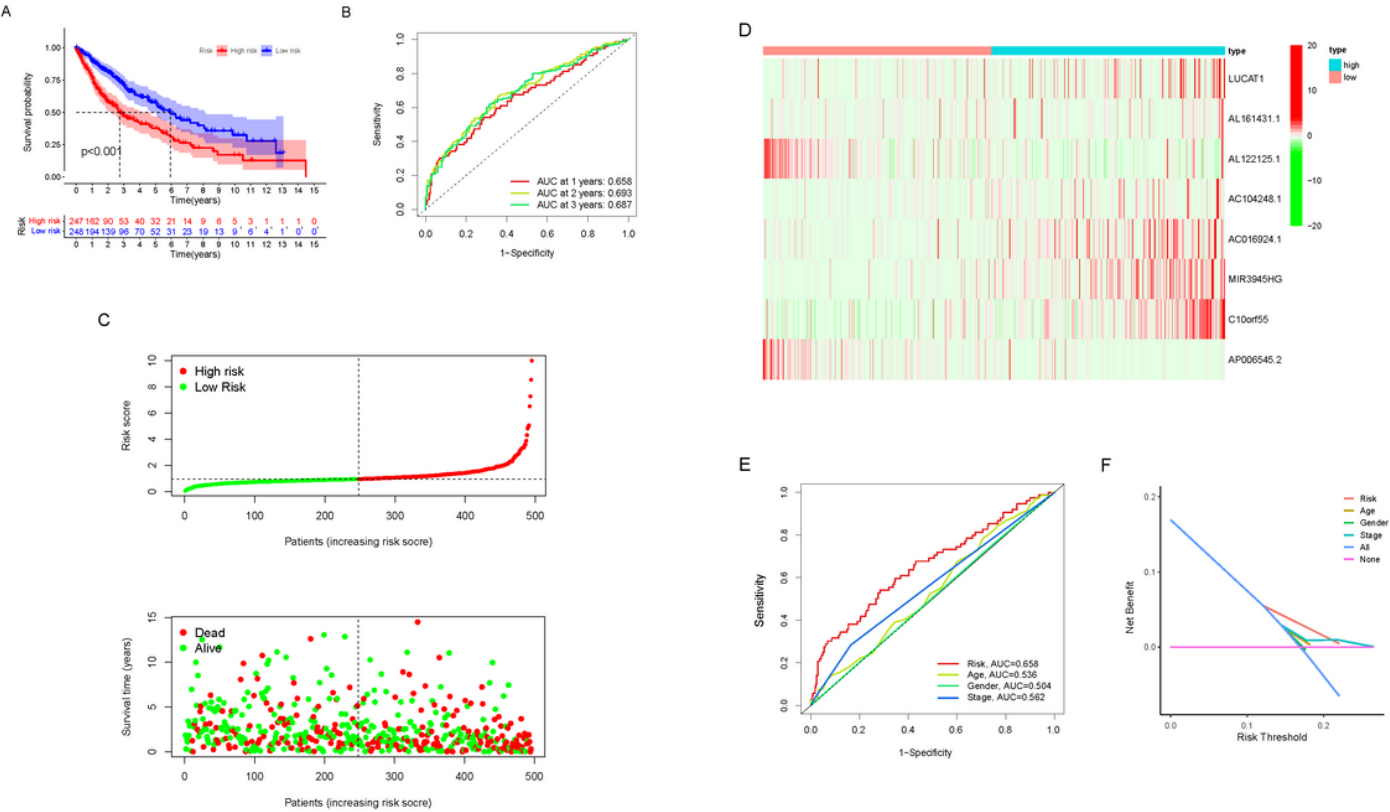


Figure 2

Ferroptosis-related lncRNAs signature based on TCGA. (A) Kaplan-Meier curves result. (B) The AUC for the prediction of 1, 2, 3-year survival rate of LUSC. (C) Risk survival status plot. (D) Heatmap of 8-FerLncRNAs. (E) The AUC values of the risk factors.(F) The DCA of the risk factors.

Figure 3

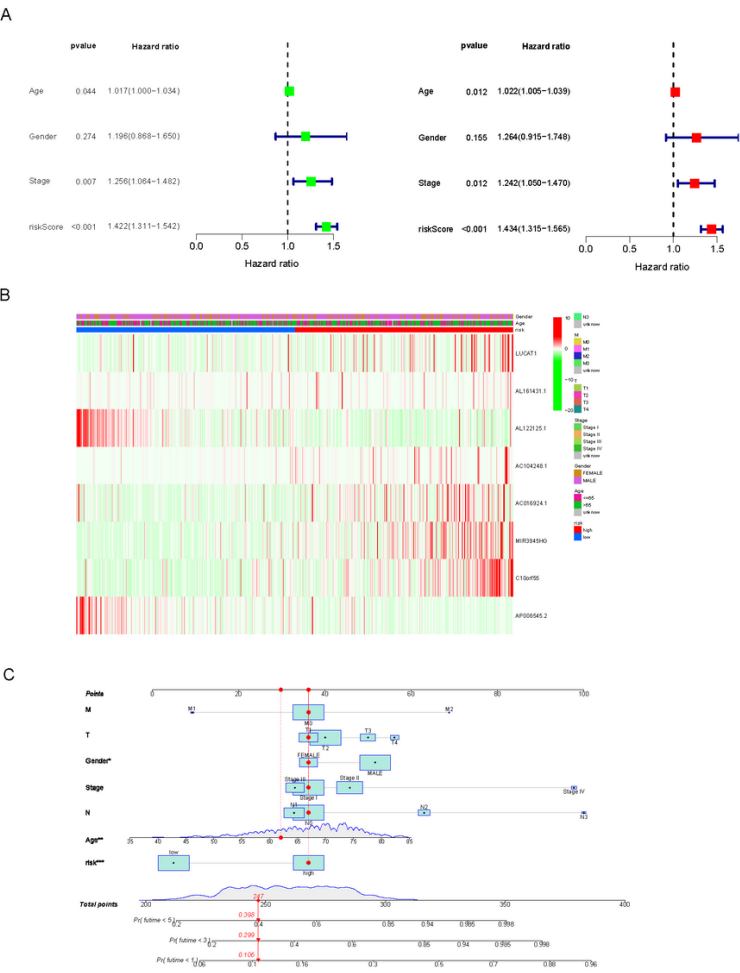


Figure 3

Independent prognostic analysis and clinical application of FerRLSig (A)Univariate and multivariate COX analysis for the expression of ferroptosis-related lncRNAs. (B) Heatmap for ferroptosis-related lncRNAs prognostic signature and clinicopathological manifestations. (C) Nomogram of Ferroptosis-related lncRNAs associated with clinicopathological factors.

Figure 4

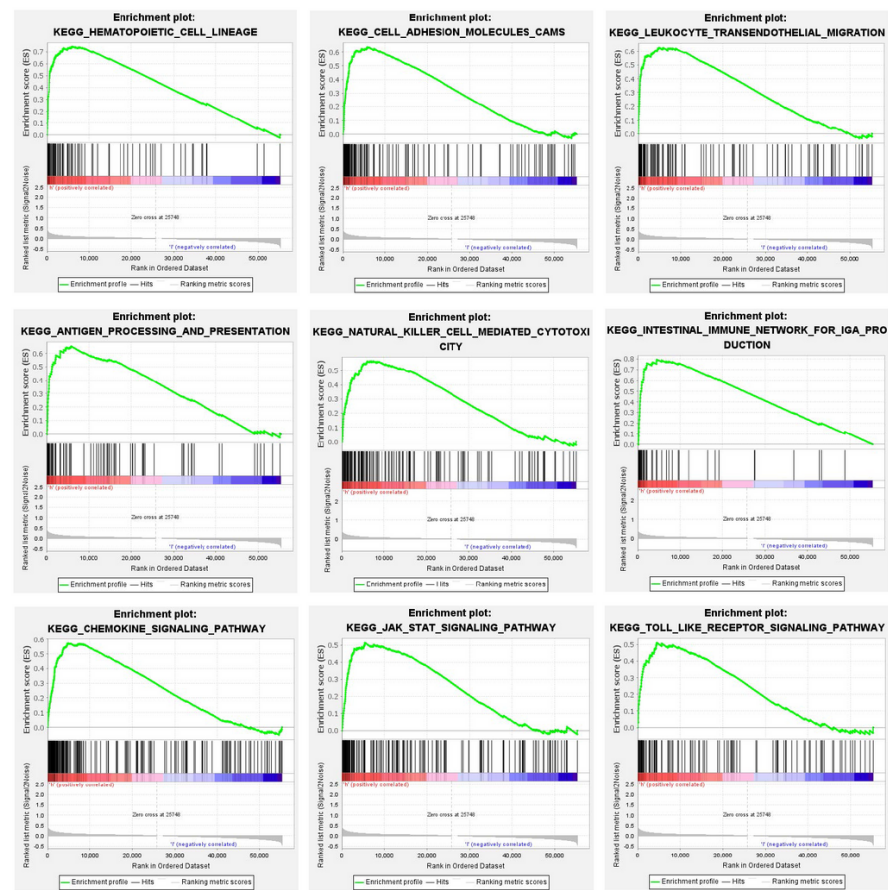


Figure 4

Gene enrichment analysis for 8-FerLncRNAs based on TCGA.

Figure 5

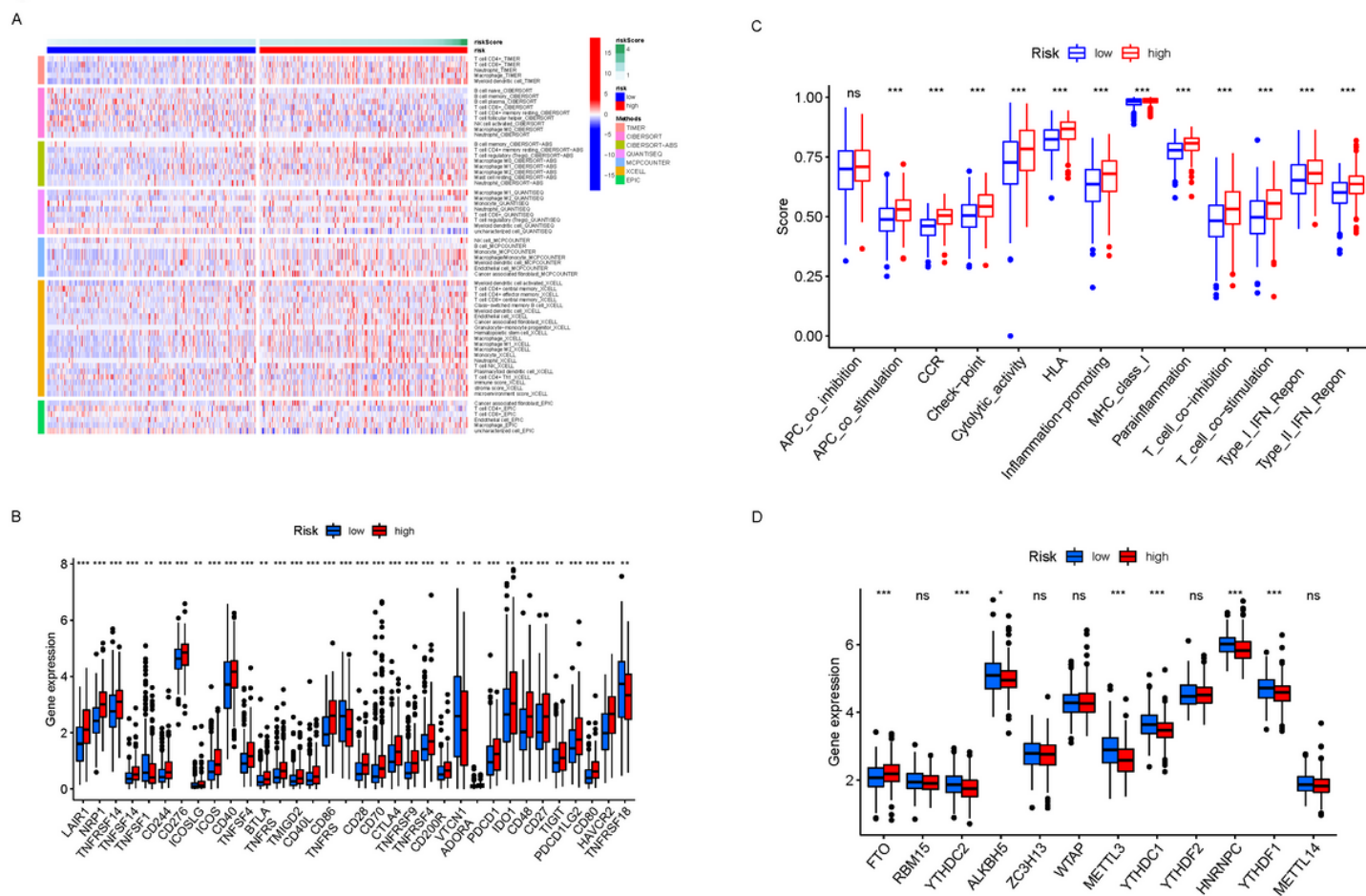


Figure 5

Comparison of the immune responses, immune checkpoints and m6A with LUSC between the high-risk and low-risk groups. (A) Heatmap for immune responses based on TIMER, CIBERSORT, CIBERSORT-ABS, QUANTISEQ, MCPOUNTER, XCELL and EPIC algorithms among high and low risk group. (B) ssGSEA for the association between immune cell subpopulations and related functions. (C) Expression of immune checkpoints among high and low LUSC risk. (D) The expression of m6A-related genes between high and low LUSC risk group.

Supplementary Files

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

- tables1.xls
- tables2.xls
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- tables4.xls