Construction and validation of a 15-gene ferroptosis signature in lung adenocarcinoma

Guangxu Tu[1,2], Weilin Peng[1,2], Qidong Cai[1,2], Zhenyu Zhao[1,2], Xiong Peng[1,2], Boxue He[1,2], Pengfei Zhang[1,2], Shuai Shi[1,2], Yongguang Tao[3,4], Xiang Wang[1,2]*

[1] Department of Thoracic Surgery, The Second Xiangya Hospital, Central South University, Changsha, Hunan, China

[2] Hunan Key Laboratory of Early Diagnosis and Precision Therapy, Department of Thoracic Surgery, The Second Xiangya Hospital, Central South University, Changsha, Hunan, China

[3] Key Laboratory of Carcinogenesis and Cancer Invasion, Ministry of Education, Department of Pathology, Xiangya Hospital, Central South University, Changsha, Hunan, China

[4] NHC Key Laboratory of Carcinogenesis (Central South University), Cancer Research Institute and School of Basic Medicine, Central South University, Changsha, Hunan, China

*Corresponding Author:

Xiang Wang

Address: 139 Renmin Road, Changsha, Hunan Province, 410011, China

Email address: wangxiang@csu.edu.cn

Abstract

Background: Ferroptosis is a novel form of programmed cell death characterized by the excessive accumulation of intracellular iron and an increase in reactive oxygen species. Emerging studies have shown that ferroptosis plays a vital role in the progression of lung adenocarcinoma, but the effect of ferroptosis-related genes on prognosis has been poorly studied. The purpose of this study was to explore the prognostic value of ferroptosis-related genes.

Methods: Lung adenocarcinoma samples were downloaded from The Cancer Genome Atlas (TCGA) and Gene Expression Omnibus (GEO) databases. The least absolute shrinkage and selection operator (LASSO) Cox regression algorithm was used to establish a predictive signature for risk stratification. Kaplan-Meier (K-M) survival analysis and receiver operating characteristic (ROC) curve analysis were conducted to evaluate the signature. We further explored the potential correlation between the risk score model and tumor immune status.

Results: A 15-gene ferroptosis signature was constructed to classify patients into different risk groups. The overall survival (OS) of patients in the high-risk group was significantly shorter than

38   that of patients in the low-risk group. The signature could predict OS independent of other risk

39   factors. Single-sample gene set enrichment analysis (ssGSEA) identified the difference in

40   immune status between the two groups.

41   **Conclusions:** The 15-gene ferroptosis signature identified in this study could be a potential

42   biomarker for prognosis prediction in lung adenocarcinoma. Targeting ferroptosis might be a

43   promising therapeutic alternative for lung adenocarcinoma.

44

45   Introduction

46   Lung cancer has the highest morbidity and mortality worldwide (Bray et al. 2018). The

47   most common subtype is lung adenocarcinoma (LUAD), accounting for approximately

48   40% of all lung cancers (Wei et al. 2018). Due to the lack of obvious clinical symptoms,

49   most patients are diagnosed at relatively advanced stages and have 5-year survival

50   rates of less than 15% (Denisenko et al. 2018). In recent years, targeted therapies, such

51   as epidermal growth factor tyrosine kinase inhibitors (EGFR-TKIs), have achieved much

52   success (Li et al. 2016). However, drug resistance, which is an inevitable problem,

53   causes the prognosis of LUAD patients to be far from satisfactory, and the survival rate

54   at 5 years is still only 21% (Macheleidt et al. 2018). Therefore, it is meaningful to

55   explore novel mechanisms of therapy and identify an effective prognostic model for risk

56   stratification to improve the clinical outcomes of LUAD patients.

57   Iron is an indispensable element for human biological processes, while iron metabolism

58   plays a dual role in the proliferation and death of tumor cells (Wang et al. 2019a). An

59   increased level of iron within a limited range facilitates the proliferation of cancer cells,

60   while the excessive accumulation of iron leads to the death of cancer cells by lipid

61   peroxidation of the cell membrane, namely, ferroptosis (Mou et al. 2019; Stockwell et al.

62   2017). Ferroptosis is a novel form of regulated cell death and has a tumor suppressive

63   function (Dixon et al. 2012). Emerging studies, though limited, have shown that

64   ferroptosis plays a pivotal role in the regulation of tumor progression in non-small cell

65   lung cancer (NSCLC). For example, SLC7A11, which could negatively regulate the

66   process of ferroptosis, was reported to be overexpressed in LUAD and closely

67   associated with tumor progression (Hu et al. 2020; Ji et al. 2018; Ma et al. 2021).

68   Upregulated GPX4 was reported to promote the proliferation of cancer cells and play a

69 role in the resistance to ferroptosis in NSCLC (Ji et al. 2018). High expression of NFS1
70 in LUAD could protect cancer cells from ferroptosis (Alvarez et al. 2017). High
71 expression of FSP1, EGLN1 and STRYK1 was found to be associated with greater
72 ferroptosis resistance in lung cancer cells (Doll et al. 2019; Jiang et al. 2017; Lai et al.
73 2019). In recent years, ferroptosis was reported to interact with some immune cells to
74 influence tumor progression. For example, abnormal ferroptosis-mediated cell death
75 could induce neutrophil recruitment and the inflammatory response to cancer cell death
76 (Pentimalli et al. 2019). Additionally, Wang et al. revealed that CD8+ T cells drive the
77 ferroptosis of cancer cells and enhance the antitumor effect, indicating a vital role of
78 ferroptosis in human anticancer immunity (Wang et al. 2019b; Zhang et al. 2020). All
79 these discoveries shed light on ferroptosis as a promising target for cancer therapy.
80 However, there are still limited studies exploring the potential role of ferroptosis-related
81 genes in LUAD so far, and their effect on prognosis remains largely unknown.

82 In this study, we comprehensively analyzed the expression patterns, prognostic value,
83 biological functions and potential pathways of ferroptosis-related genes to gain a better
84 understanding of ferroptosis in LUAD. Then, we constructed a prognostic signature of
85 the ferroptosis-related genes in The Cancer Genome Atlas (TCGA) cohort and validated
86 it in two Gene Expression Omnibus (GEO) datasets. Finally, we further explored the
87 correlation between the prognostic signature and the immune status of LUAD patients.
88 Our findings may provide useful information for further studies in this field.

89

90 Methods

91 Acquisition of ferroptosis-related genes

92 We downloaded the list of ferroptosis pathway genes (map04216) from the Kyoto
93 Encyclopedia of Genes and Genomes (KEGG) pathway database
94 (https://www.genome.jp/kegg/pathway.html) and designated these genes as ferroptosis-
95 related genes. Iron metabolism-associated genes were retrieved from the R-HAS-
96 917937 pathway in the Reactome pathway database (https://reactome.org/) and the
97 cellular iron ion homeostasis pathway in the AmiGO2 database
98 (http://amigo.geneontology.org/amigo). After systematically searching and analyzing the
99 original document, we discarded the genes that do not have a modulatory effect on

100 ferroptosis. In addition, we collected and integrated newly reported ferroptosis-related

101 genes for subsequent research.

102 Data collection

103 We obtained the level 3 mRNA expression profiles and corresponding clinical data of

104 LUAD patients from the TCGA database (https://portal.gdc.cancer.gov/) and GEO

105 database (https://www.ncbi.nlm.nih.gov/geo/) (Rousseaux et al. 2013; Schabath et al.

106 2016) up to September 20, 2020. Samples with a follow-up time of less than 30 days or

107 lack of prognostic data were excluded. Since the TCGA and GEO databases are public

108 to researchers and we completely abided by the publication guidelines as well as the

109 policies of access to the database, ethical review and approval were not required.

110 Identification of differentially expressed ferroptosis genes

111 The "limma" R package was used for the normalization of gene expression matrixes.

112 Then, we matched the mRNA sequencing data with the ferroptosis-related gene list and

113 performed differential expression analysis between the tumor tissues and normal

114 tissues in the TCGA cohort by using false discovery rate (FDR)< 0.05 as the threshold.

115 Thus, ferroptosis-related differentially expressed genes (DEGs) were identified. A

116 heatmap and volcano plot to visualize the DEGs were generated by the "pheatmap" R

117 package. In addition, the protein-protein interaction (PPI) network of the ferroptosis-

118 related DEGs was analyzed in the Search Tool for the Retrieval of Interacting Genes

119 (STRING) online database and visualized in Cytoscape 3.8.2 software. We also

120 generated a correlation network of the DEGs using the "igraph" R package.

121 Establishment and validation of the prognostic model

122 DEGs with prognostic value were screened by univariate Cox regression analysis with a

123 P value less than 0.05. Then, the least absolute shrinkage and selection operator

124 (LASSO) Cox regression algorithm was conducted to establish the signature. In brief,

125 we used the normalized expression data as the independent variable and the overall

126 survival (OS) data of the patients in the TCGA cohort as the response variable to

127 perform the LASSO algorithm for the shrinkage of variables by the "glmnet" R package.

128 Tenfold cross-validation was used to narrow the number of candidate genes and identify

129 the penalty parameter (λ), which corresponds to the lowest position of the likelihood

130 deviance curve. We further evaluated the prognostic value of the LASSO genes by

131   Kaplan-Meier (K-M) survival analysis. The K-M survival analysis was conducted using

132   the optimal cutoff determined by the "surv_cutpoint" function in the "survival" R package.

133   The risk score of each patient was calculated with the following formula: risk score = Σ

134   (ExpmRNAn × βmRNAn). The patients were stratified by the median risk score into

135   high- and low-risk groups. After that, we performed principal component analysis (PCA)

136   to evaluate the discriminatory ability by the "status" R package. We evaluated the

137   predictive ability of the signature for OS through K-M survival analysis as well as time-

138   dependent receiver operating characteristic (ROC) curve analysis conducted by the

139   "survminer" and "survivalROC" R packages. Finally, we performed multivariate Cox

140   regression analysis to identify independent risk factors for OS. In this study, the TCGA

141   cohort was used as the derivation cohort, and the GSE72094 and GSE30219 cohorts

142   were used as external validation cohorts.

143   **Construction and evaluation of the predictive nomogram**

144   We constructed a nomogram to predict the survival probabilities at 1 year, 2 years and 3

145   years in the TCGA cohort by integrating all the independent risk factors using the "rms"

146   R package. The patients were stratified into different risk groups, and K-M survival

147   analysis was conducted to analyze the OS difference between the different risk groups.

148   Then, we calculated the C index and performed time-dependent ROC analysis to further

149   validate the prediction accuracy of the nomogram. Moreover, a calibration plot was used

150   to evaluate the consistency between the predicted survival probability and the real

151   observation. The GSE72094 cohort was used for the external validation of the

152   nomogram.

153   **Correlation with immune status**

154   Using the "GSVA" R package, we calculated the enrichment score of 16 immune-related

155   cells as well as 12 immune-related functions for each patient by single-sample gene set

156   enrichment analysis (ssGSEA). In brief, using a set of genes that correspond to a

157   particular immune cell or immune function, the enrichment score was calculated in the

158   gene expression matrix through the ssGSEA algorithm, and the enrichment scores were

159   normalized for subsequent analysis. Then, the enrichment scores for diverse immune

160   cells and functions of patients in different risk groups were compared to illustrate the

161   potential correlation between ferroptosis and immune status. The R script used for this

162   part   of   the   analysis   is   provided   in   the   GitHub   website

163   (https://github.com/guangxu0109/ssGSEA.git).

Functional enrichment analysis

165   To gain insight into the molecular mechanisms of these ferroptosis-related genes, we

166   performed Gene Ontology (GO) and KEGG enrichment analyses of the DEGs between

167   the high-risk group and low-risk group in the TCGA cohort, which were screened by the

168   thresholds of |log2 fold change (FC)|≥1 and FDR<0.05. GO enrichment analysis was

169   performed by the "clusterProfiler" R package, and KEGG enrichment analysis was

170   performed by gene set enrichment analysis (GSEA) using GSEA 4.0.2 software. The

171   pathways with a P value less than 0.05 were considered to be significantly enriched.

Statistical analysis

173   All statistical analyses were conducted in R software version 4.0.2. The Mann-Whitney

174   test was conducted to compare gene expression levels between tumor tissues and

175   normal tissues as well as to compare the ssGSEA enrichment scores between different

176   risk groups. A P value less than 0.05 (if not otherwise specified) was considered

177   statistically significant.

178

Results

180   **Twenty-two genes correlated with prognosis were identified in the TCGA cohort**

181   We generated a flowchart to describe the design of this study, as shown in Figure 1. A total of

182   948 LUAD patients from the TCGA database (N=477), GSE72094 cohort (N=386) and

183   GSE30219 cohort (N=85) were used for subsequent analysis in this study. Table S1 shows the

184   basic characteristics of these patients. A total of 125 ferroptosis-associated genes (as listed in

185   Table S2) were identified to intersect with the mRNA expression matrix of the TCGA and GEO

186   databases. Ninety-seven genes were confirmed to be differentially expressed between tumor

187   tissues and nontumorous tissues (62 upregulated and 35 downregulated) in the TCGA cohort

188   (Figure 2A, B and Table S3). The PPI network and the correlation network of the DEGs are

189   shown in Figure S1. Twenty-two genes were identified to be correlated with prognosis through

190   univariate Cox regression analysis (Figure 3).

191   **Establishment and assessment of the prognostic ferroptosis signature in the TCGA**

192   **cohort**

193 A signature consisting of 15 genes was established based on the minimum λ value identified by
194 the LASSO algorithm in the TCGA cohort (Figure S2). The risk score of each patient was
195 calculated with the following formula: (-0.037 * expression level of AGER) + (0.261 * expression
196 level of CISD1) + (-0.019 * expression level of DPP4) + (0.173 * expression level of EGLN1) +
197 (0.077 * expression level of FANCD2) + (-0.253 * expression level of GLS2) + (-0.233 *
198 expression level of ISCU) + (0.087 * expression level of ITGA6) + (0.024 * expression level of
199 ITGB4) + (0.118 * expression level of KRAS) + (0.109 * expression level of NEDD4) + (-0.048 *
200 expression level of PEBP1) + (-0.133 * expression level of SLC11A2) + (0.013* expression level
201 of TFAP2A) + (0.208 * expression level of VDAC1). We further performed K-M survival analysis
202 for the 15 ferroptosis-related genes in the prognostic signature. The results confirmed that all
203 these genes were significantly correlated with OS (Figure S3). Among them, AGER, DPP4,
204 GLS2, ISCU, PEBP1 and SLC11A2 were identified to be protective factors for OS, while the
205 remaining factors (CISD1, EGLN1, FANCD2, ITGA6, ITGB4, KRAS, NEDD4, TFAP2A, and
206 VDAC1) were risk factors, which was consistent with the results of univariate Cox analysis, as
207 shown in Figure 3.
208 The patients in the TCGA cohort were divided into different risk groups by the median risk score
209 (Table S4). The PCA plot in Figure 4A shows that patients in different risk groups were clearly
210 distributed in two directions, indicating that the gene signature had good discriminatory power.
211 The K-M curve showed that the OS of patients in the high-risk group was significantly lower than
212 that of patients in the low-risk group (Figure 4B, C). In addition, OS was significantly decreased
213 with increasing risk scores (Figure 4D). The area under the curve (AUC) value of the risk score
214 model to predict OS was 0.775, which was the highest among all the risk factors, indicating the
215 superior predictive ability of the prognostic signature (Figure 5E).

External validation of the ferroptosis-related gene signature

217 To further validate the robustness of the signature, we applied the 15-gene ferroptosis signature
218 to the GSE72094 and GSE30219 cohorts. The patients in the two cohorts were stratified into
219 high- and low-risk groups by the median risk score (Table S5 and Table S6). In the GSE72094
220 cohort, the PCA plot showed that patients in different risk groups were distributed in two
221 directions (Figure 5A). The K-M curve indicated that patients in the high-risk group had
222 significantly shorter OS than those in the low-risk group, and OS was significantly decreased
223 with increasing risk scores (Figure 5B-D). The AUC value of the risk score model was 0.711,
224 which was the highest among all the risk factors (Figure 5E). In the GSE30219 cohort, the PCA
225 plots showed that patients in different groups were distributed in two directions (Figure 5F). The
226 K-M survival curve confirmed a significantly worse OS for patients in the high-risk group than for

227   those in the low-risk group (Figure 5G). OS significantly decreased with increasing risk scores

228   (Figure 5H, I). The AUC value of the risk score model was 0.877, which was the highest among

229   all the risk factors (Figure 5J). All these results indicated that the 15-gene ferroptosis signature

230   had a robust predictive performance.

231   **Independent prognostic value of the ferroptosis-related gene signature**

232   To further assess the independent prognostic value of the 15-gene ferroptosis signature, we

233   performed univariate and multivariate Cox regression analyses on all characteristics, including

234   age, sex, and TNM stage, and the risk score model based on the ferroptosis signature in both

235   the derivation cohort and two validation cohorts. The results are presented in Table 1. In the

236   TCGA cohort, TNM stage and risk score model were identified to be independent prognostic

237   factors for OS. In the GSE72094 cohort, sex, TNM stage and risk score model were

238   independent prognostic factors for OS. The risk score model was the only independent

239   prognostic factor for OS in the GSE30219 cohort.

240   Given that the risk score model was not the only independent prognostic factor in the TCGA and

241   GSE72094 cohorts, we further performed subgroup survival analysis to verify whether the 15-

242   gene ferroptosis signature could be independent of other risk factors to predict prognosis. In the

243   TCGA cohort, patients in stage I-II and stage III-IV were stratified into high-risk and low-risk

244   groups by the median risk score. K-M curves showed that the OS of the high-risk group was

245   significantly worse than that of the low-risk group regardless of whether patients were stage I-II

246   or stage III-IV, indicating that the 15-gene ferroptosis signature could predict OS independent of

247   TNM stage (Figure 6A, B). Similarly, in the GSE72094 cohort, the OS of the high-risk group was

248   significantly worse than that of the low-risk group regardless of whether the patient was male or

249   female, indicating that the signature could predict OS independent of sex (Figure 6C, D). For the

250   TNM stage subgroup, the OS of patients in the high-risk group was significantly shorter than

251   that of patients in the low-risk group except for patients in stage III and stage IV, which was

252   likely due to the small sample size of patients in these groups (Figure 6E, F). Overall, the 15-

253   gene ferroptosis signature could predict OS independent of other clinical characteristics.

254   **Construction and assessment of the predictive nomogram**

255   Based on the independent risk factors identified in the TCGA cohort, we constructed a

256   nomogram model to predict the OS probabilities of patients at 1, 2 and 3 years for risk

257   assessment and earlier intervention to improve patient survival time (as shown in Figure 7A). As

258   depicted in Figure 7B, a significantly poorer prognosis was observed in the high-risk group. The

259   C index for the nomogram model to predict OS was 0.770 (95% CI: 0.724, 0.816), indicating

260   that the nomogram model had a robust predictive accuracy. We further evaluated the

261 discrimination and calibration of the nomogram by time-dependent ROC curves and calibration
262 plots for 1-year and 3-year OS. The 1-year and 3-year calibration plots indicated that the
263 predicted survival was highly consistent with the actual survival in the TCGA cohort (Figure 7D,
264 E). As shown in Figure 7H and Figure 7I, the AUC values of the nomogram for predicting 1-year
265 and 3-year OS were 0.812 and 0.757, respectively. All the AUC values of the nomogram were
266 superior to those of other independent risk factors, indicating that the nomogram had a better
267 predictive performance.
268 The nomogram was further validated in the GSE72094 cohort, while TNM stage information was
269 not given in the GSE30219 cohort. The results showed that the OS of patients in the high-risk
270 group was significantly shorter than that of patients in the low-risk group (Figure 7C). The C
271 index of the nomogram model was 0.708 (95% CI: 0.660, 0.756). The calibration plots indicated
272 great consistency between the predicted survival rate and the real observation (Figure 7F, G).
273 The AUC values of the nomogram for predicting 1-year and 3-year OS were 0.735 and 0.758,
274 respectively, which were also superior to those of all other independent risk factors (Figure 7J,
275 K).
276 **Functional analysis of the 15-gene ferroptosis signature**
277 GO and KEGG enrichment analyses were performed to elucidate the possible biological
278 functions and pathways involved in the 15-gene ferroptosis signature. The results of GO
279 enrichment analysis indicated that the DEGs between the high-risk group and the low-risk group
280 were mainly enriched in pathways of the cell cycle and immune response, such as chromosome
281 segregation, mitotic nuclear division, mitotic sister chromatid segregation, antimicrobial humoral
282 response and humoral immune response (Figure 8A and Table S7). As shown in Figure 8B and
283 Table 2, the KEGG enrichment analysis revealed that the top 5 pathways enriched in the high-
284 risk group were the cell cycle, ubiquitin-mediated proteolysis, oocyte meiosis, homologous
285 recombination and p53 signaling pathways. The top 5 pathways enriched in the low-risk group
286 were the arachidonic acid metabolism, primary bile acid biosynthesis, alpha linolenic acid
287 metabolism, asthma, and intestinal immune network for IgA production pathways.
288 **Correlation with immune status**
289 Given that pathways of the immune response were significantly enriched in the 15-gene
290 ferroptosis signature, we further sought to investigate the correlation between immune status
291 and risk score of the ferroptosis signature. The enrichment results are presented in Tables S8-
292 10. Then, we analyzed the difference in the enrichment score of immune cells and immune
293 functions between the high-risk and low-risk groups. By comparing the enrichment results of the
294 three cohorts, we found that enrichment scores of activated dendritic cells (aDCs), dendritic

295   cells (DCs), immature dendritic cells (iDCs), mast cells and neutrophils were significantly lower
296   in the high-risk group than in the low-risk group (Figure 9A, C, E). Regarding immune-related
297   functions, higher enrichment score of antigen-presenting cell (APC) inhibition was found in the
298   high-risk groups of all three cohorts, while a higher enrichment score of the type II IFN (IFN-γ)
299   response was found in the low-risk group (Figure 9B, D, F). All these results indicated a close
300   correlation between risk score of the ferroptosis signature and immune cells as well as immune
301   functions.

302

303   **Discussion**

304   Ferroptosis is a novel form of programmed cell death characterized by the excessive
305   accumulation of intracellular iron and an increase in reactive oxygen species (ROS)
306   (Dixon et al. 2012). Disturbances in iron hemostasis lead to excessive intracellular iron
307   accumulation and may induce ferroptosis (Bogdan et al. 2016). In recent years, this
308   unique pattern of cell death has been the focus of a large number of studies, and it is
309   well characterized as a promising therapeutic alternative for various cancer types
310   (Hassannia et al. 2019). However, there are still limited studies regarding the specific
311   role of ferroptosis in LUAD as well as its potential mechanism and pathways. The rapid
312   development of RNA sequencing and microarrays and large-scale public databases
313   provide an opportunity to obtain a better understanding of these ferroptosis-related
314   genes and to construct a reliable ferroptosis-based prognostic signature.

315   In the present study, we systematically analyzed the expression profile of ferroptosis-
316   related genes in LUAD, and we found that the majority of ferroptosis-related genes
317   (77.6%, 97/125) were differentially expressed between LUAD tumor tissues and normal
318   tissues. Univariate Cox regression analysis showed that 22 genes were associated with
319   OS, and LASSO Cox regression finally identified 15 genes (AGER, CISD1, DPP4,
320   EGLN1, FANCD2, GLS2, ISCU, ITGA6, ITGB4, KRAS, NEDD4, PEBP1, SLC11A2,
321   TFAP2A, and VDAC1) to construct the ferroptosis-related gene signature. Their
322   modulatory effects on ferroptosis are summarized in Table 3. Among them, CISD1,
323   DPP4, EGLN1, FANCD2, ISCU, ITGA6, ITGB4, KRAS and NEDD4 negatively regulated
324   ferroptosis, while AGER, GLS2, PEBP1, SLC11A2 and VDAC1 positively regulated
325   ferroptosis. The depletion of AGER inhibited autophagy-dependent ferroptosis (Wen et
326   al. 2019). Low CISD1 expression could contribute to the ferroptosis of hepatocellular

327 cancer cells by iron-mediated intramitochondrial lipid peroxidation (Yuan et al. 2016).
328 The accumulation of DPP4 could protect cells from ferroptosis by inhibiting lipid
329 peroxidation in human colorectal cancer (Xie et al. 2017). A previous study indicated
330 that EGLN1 could act as an oncogene by inducing LSH expression, which inhibits
331 ferroptosis in lung cancer (Jiang et al. 2017). FANCD2 was found to act as a ferroptosis
332 suppressor by decreasing lipid peroxidation (Song et al. 2016). Upregulation of GLS2
333 exhibited antitumor effects in gastric cancer by promoting ferroptosis (Niu et al. 2019).
334 ISCU, a mitochondrial protein, could regulate iron metabolism and increase the
335 expression of GSH, thus significantly alleviating ferroptosis (Du et al. 2019). Integrin
336 subunit alpha 6 (ITGA6) and integrin subunit beta 4 (ITGB4) belong to the integrin
337 family, which mainly functions in cell adhesion. Overexpression of ITGA6 and ITGB4
338 attenuated the ferroptosis induced by erastin, while knockout of ITGA6 and ITGB4
339 promoted ferroptosis in breast cancer cells (Brown et al. 2017). Activated mutant KRAS
340 could promote the expression of SCL7A11, thus inhibiting ferroptosis in lung
341 adenocarcinoma (Hu et al. 2020). The depletion of NEDD4 was found to promote the
342 ferroptosis induced by erastin by limiting the degradation of VDAC2/3 in melanoma
343 (Yang et al. 2020). PEBP1, a scaffold protein kinase cascade inhibitor, was reported to
344 promote ferroptosis by enabling lipoxygenase (Wenzel et al. 2017). SLC11A2, also
345 called DMT1, is a major iron transporter and contributes to iron uptake in most cell
346 types, and its upregulation could trigger ferroptosis in acute myocardial infarction mice
347 (Song et al. 2020). TFAP2A was reported to negatively modulate ferroptosis by
348 activating the NRF2 signaling pathway (Huang et al. 2020). Inhibition of VDAC1 could
349 significantly alleviate ferroptosis and improve cell survival by decreasing the ROS level
350 in mitochondria (Nagakannan et al. 2019). Subsequent K-M survival analyses confirmed
351 the prognostic value of the 15 ferroptosis-related genes, indicating their potential role in
352 the initiation and progression of LUAD. Unsurprisingly, the risk scores of the 15-gene
353 ferroptosis signature were demonstrated to be significantly associated with the OS of
354 LUAD patients in both the TCGA cohort and two external validation cohorts. The
355 signature was independent of other clinical characteristics in all three cohorts. In
356 addition, the nomogram integrating the independent risk factors, including the risk score
357 model, exhibited high predictive value and may help clinicians make optimal clinical

358 decisions to improve the OS rate of LUAD patients. These findings indicated the
359 important role of ferroptosis in the progression of LUAD and the possibility of the
360 ferroptosis signature as a biomarker for OS.

361 KEGG pathway analysis showed that the high-risk group was mainly enriched in
362 pathways closely associated with tumorigenesis, such as the cell cycle, oocyte meiosis
363 and homologous recombination. GO analysis of the DEGs between the high-risk group
364 and low-risk group showed that the biological functions of the DEGs were mainly
365 enriched in the regulation of the cell cycle and immune response, indicating a link
366 between ferroptosis and antitumor immunity. Cancer growth and metastasis are closely
367 related to interactions with the immune system (Matsushita et al. 2012). When
368 investigating the correlation with immune status, we interestingly found that enrichment
369 scores of aDCs, DCs, iDCs, mast cells and neutrophils were significantly lower in the
370 high-risk group. Among these differentially enriched immune cells, iDCs specialize in
371 antigen capture, and DCs are professional APCs (Lin et al. 2019; Tiberio et al. 2018).
372 Both aDCs and iDCs play crucial roles in the process of cytotoxic T cell activation and in
373 the regulation of the immune response to cancer cells (Durai & Murphy 2016).
374 Moreover, neutrophils infiltrating tumor tissues, termed tumor-associated neutrophils
375 (TANs), also play a role in antitumor immunity (Lecot et al. 2019). It was confirmed that
376 TANs could release cytotoxic substances such as ROX, thus directly inducing tumor cell
377 apoptosis (Takeshima et al. 2016). The role of mast cells in the progression of tumors is
378 controversial. For instance, mast cells could play a protumorigenic role by releasing
379 angiogenic and lymphangiogenic factors, which promote angiogenesis and
380 lymphogenesis (Detoraki et al. 2009). In contrast, mast cells could secrete tumor
381 necrosis factor α (TNF-α) and directly mediate tumor cell cytotoxicity, thus playing an
382 antitumorigenic role (Varricchi et al. 2017). In lung cancer, Bao et al. and Welsh et al.
383 reported that the low abundance of mast cell in cancer specimens was correlated with
384 worse OS (Bao et al. 2020; Welsh et al. 2005). When we analyzed the difference in
385 immune functions, a stronger inhibition of APCs was found in the high-risk groups of
386 both the derivation cohort and two validation cohorts. The synergistic effect of reduced
387 DC and stronger inhibition of APCs in the high-risk group may contribute to the great
388 suppression of the tumor antigen presentation process. Additionally, we also found that

389 enrichment score of type II IFN was significantly lower in the high-risk group. IFN-γ
390 produced by APCs, T cells, B cells, NK cells and NKT cells is the only member of the
391 type II INF family (Castro et al. 2018). It is well known that IFN-γ plays a pivotal role in
392 cancer immune surveillance, stimulating antitumor immunity and facilitating the
393 recognition and elimination of cancer cells (Benci et al. 2019; Shankaran et al. 2001;
394 Street et al. 2001). All these findings suggested that ferroptosis may be involved in
395 antitumor immune response. However, more experimental investigation is warranted to
396 confirm these findings and reveal the underlying mechanism.
397

398 **Conclusions**
399 The 15-gene ferroptosis signature identified in this study could be a potential biomarker
400 for prognosis prediction in LUAD. Targeting ferroptosis might be a promising therapeutic
401 alternative for LUAD.
402

403 **Acknowledgments**

404 The authors are truly thankful to the TCGA and GEO databases for the availability of the
405 data.

406

407

408

409 **References**
410

411 Alvarez SW, Sviderskiy VO, Terzi EM, Papagiannakopoulos T, Moreira AL, Adams S, Sabatini
412 DM, Birsoy K, and Possemato R. 2017. NFS1 undergoes positive selection in lung
413 tumours and protects cells from ferroptosis. *Nature* 551:639-643. 10.1038/nature24637
414 Bao X, Shi R, Zhao T, and Wang Y. 2020. Mast cell-based molecular subtypes and signature
415 associated with clinical outcome in early-stage lung adenocarcinoma. *Molecular*
416 *oncology* 14:917-932. 10.1002/1878-0261.12670
417 Benci JL, Johnson LR, Choa R, Xu Y, Qiu J, Zhou Z, Xu B, Ye D, Nathanson KL, June CH,
418 Wherry EJ, Zhang NR, Ishwaran H, Hellmann MD, Wolchok JD, Kambayashi T, and
419 Minn AJ. 2019. Opposing Functions of Interferon Coordinate Adaptive and Innate
420 Immune Responses to Cancer Immune Checkpoint Blockade. *Cell* 178.
421 10.1016/j.cell.2019.07.019
422 Bogdan AR, Miyazawa M, Hashimoto K, and Tsuji Y. 2016. Regulators of Iron Homeostasis:
423 New Players in Metabolism, Cell Death, and Disease. *Trends Biochem Sci* 41:274-286.
424 10.1016/j.tibs.2015.11.012

425 Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, and Jemal A. 2018. Global cancer
426   statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36
427   cancers in 185 countries. *CA Cancer J Clin* 68:394-424. 10.3322/caac.21492
428 Brown CW, Amante JJ, Goel HL, and Mercurio AM. 2017. The α6β4 integrin promotes
429   resistance to ferroptosis. *Journal of Cell Biology* 216:4287-4297. 10.1083/jcb.201701136
430   %J Journal of Cell Biology
431 Castro F, Cardoso AP, Gonçalves RM, Serre K, and Oliveira MJ. 2018. Interferon-Gamma at
432   the Crossroads of Tumor Immune Surveillance or Evasion. *Frontiers in immunology*
433   9:847. 10.3389/fimmu.2018.00847
434 Denisenko TV, Budkevich IN, and Zhivotovsky B. 2018. Cell death-based treatment of lung
435   adenocarcinoma. *Cell Death Dis* 9:117. 10.1038/s41419-017-0063-y
436 Detoraki A, Staiano RI, Granata F, Giannattasio G, Prevete N, de Paulis A, Ribatti D, Genovese
437   A, Triggiani M, and Marone G. 2009. Vascular endothelial growth factors synthesized by
438   human lung mast cells exert angiogenic effects. *The Journal of allergy and clinical*
439   *immunology* 123. 10.1016/j.jaci.2009.01.044
440 Dixon SJ, Lemberg KM, Lamprecht MR, Skouta R, Zaitsev EM, Gleason CE, Patel DN, Bauer
441   AJ, Cantley AM, Yang WS, Morrison B, and Stockwell BR. 2012. Ferroptosis: an iron-
442   dependent form of nonapoptotic cell death. *Cell* 149:1060-1072.
443   10.1016/j.cell.2012.03.042
444 Doll S, Freitas FP, Shah R, Aldrovandi M, da Silva MC, Ingold I, Goya Grocin A, Xavier da Silva
445   TN, Panzilius E, Scheel CH, Mourão A, Buday K, Sato M, Wanninger J, Vignane T,
446   Mohana V, Rehberg M, Flatley A, Schepers A, Kurz A, White D, Sauer M, Sattler M,
447   Tate EW, Schmitz W, Schulze A, O'Donnell V, Proneth B, Popowicz GM, Pratt DA,
448   Angeli JPF, and Conrad M. 2019. FSP1 is a glutathione-independent ferroptosis
449   suppressor. *Nature* 575:693-698. 10.1038/s41586-019-1707-0
450 Du J, Wang T, Li Y, Zhou Y, Wang X, Yu X, Ren X, An Y, Wu Y, Sun W, Fan W, Zhu Q, Wang
451   Y, and Tong X. 2019. DHA inhibits proliferation and induces ferroptosis of leukemia cells
452   through autophagy dependent degradation of ferritin. *Free radical biology & medicine*
453   131:356-369. 10.1016/j.freeradbiomed.2018.12.011
454 Durai V, and Murphy KM. 2016. Functions of Murine Dendritic Cells. *Immunity* 45:719-736.
455   10.1016/j.immuni.2016.10.010
456 Hassannia B, Vandenabeele P, and Vanden Berghe T. 2019. Targeting Ferroptosis to Iron Out
457   Cancer. *Cancer cell* 35:830-849. 10.1016/j.ccell.2019.04.002
458 Hu K, Li K, Lv J, Feng J, Chen J, Wu H, Cheng F, Jiang W, Wang J, Pei H, Chiao PJ, Cai Z,
459   Chen Y, Liu M, and Pang X. 2020. Suppression of the SLC7A11/glutathione axis causes
460   synthetic lethality in KRAS-mutant lung adenocarcinoma. *The Journal of clinical*
461   *investigation* 130:1752-1766. 10.1172/JCI124049
462 Huang HX, Yang G, Yang Y, Yan J, Tang XY, and Pan Q. 2020. TFAP2A is a novel regulator
463   that modulates ferroptosis in gallbladder carcinoma cells via the Nrf2 signalling axis.
464   *European review for medical and pharmacological sciences* 24:4745-4755.
465   10.26355/eurrev_202005_21163
466 Ji X, Qian J, Rahman SMJ, Siska PJ, Zou Y, Harris BK, Hoeksema MD, Trenary IA, Heidi C,
467   Eisenberg R, Rathmell JC, Young JD, and Massion PP. 2018. xCT (SLC7A11)-mediated
468   metabolic reprogramming promotes non-small cell lung cancer progression. *Oncogene*
469   37:5007-5019. 10.1038/s41388-018-0307-z
470 Jiang Y, Mao C, Yang R, Yan B, Shi Y, Liu X, Lai W, Liu Y, Wang X, Xiao D, Zhou H, Cheng Y,
471   Yu F, Cao Y, Liu S, Yan Q, and Tao Y. 2017. EGLN1/c-Myc Induced Lymphoid-Specific
472   Helicase Inhibits Ferroptosis through Lipid Metabolic Gene Expression Changes.
473   *Theranostics* 7:3293-3305. 10.7150/thno.19988

474 Lai Y, Zhang Z, Li J, Li W, Huang Z, Zhang C, Li X, and Zhao J. 2019. STYK1/NOK correlates
475   with ferroptosis in non-small cell lung carcinoma. *Biochemical and biophysical research*
476   *communications* 519:659-666. 10.1016/j.bbrc.2019.09.032
477 Lecot P, Sarabi M, Pereira Abrantes M, Mussard J, Koenderman L, Caux C, Bendriss-Vermare
478   N, and Michallet M-C. 2019. Neutrophil Heterogeneity in Cancer: From Biology to
479   Therapies. *Frontiers in immunology* 10:2155-2155. 10.3389/fimmu.2019.02155
480 Li S, Choi Y-L, Gong Z, Liu X, Lira M, Kan Z, Oh E, Wang J, Ting JC, Ye X, Reinhart C, Liu X,
481   Pei Y, Zhou W, Chen R, Fu S, Jin G, Jiang A, Fernandez J, Hardwick J, Kang MW, I H,
482   Zheng H, Kim J, and Mao M. 2016. Comprehensive Characterization of Oncogenic
483   Drivers in Asian Lung Adenocarcinoma. *Journal of thoracic oncology : official publication*
484   *of the International Association for the Study of Lung Cancer* 11:2129-2140.
485   10.1016/j.jtho.2016.08.142
486 Lin C-L, Huang H-M, Hsieh C-L, Fan C-K, and Lee Y-L. 2019. Jagged1-expressing adenovirus-
487   infected dendritic cells induce expansion of Foxp3(+) regulatory T cells and alleviate T
488   helper type 2-mediated allergic asthma in mice. *Immunology* 156:199-212.
489   10.1111/imm.13021
490 Ma L, Chen T, Zhang X, Miao Y, Tian X, Yu K, Xu X, Niu Y, Guo S, Zhang C, Qiu S, Qiao Y,
491   Fang W, Du L, Yu Y, and Wang J. 2021. The m(6)A reader YTHDC2 inhibits lung
492   adenocarcinoma tumorigenesis by suppressing SLC7A11-dependent antioxidant
493   function. *Redox biology* 38:101801-101801. 10.1016/j.redox.2020.101801
494 Macheleidt IF, Dalvi PS, Lim SY, Meemboor S, Meder L, Kasgen O, Muller M, Kleemann K,
495   Wang L, Nurnberg P, Russeler V, Schafer SC, Mahabir E, Buttner R, and Odenthal M.
496   2018. Preclinical studies reveal that LSD1 inhibition results in tumor growth arrest in lung
497   adenocarcinoma independently of driver mutations. *Mol Oncol* 12:1965-1979.
498   10.1002/1878-0261.12382
499 Matsushita H, Vesely MD, Koboldt DC, Rickert CG, Uppaluri R, Magrini VJ, Arthur CD, White
500   JM, Chen Y-S, Shea LK, Hundal J, Wendl MC, Demeter R, Wylie T, Allison JP, Smyth
501   MJ, Old LJ, Mardis ER, and Schreiber RD. 2012. Cancer exome analysis reveals a T-
502   cell-dependent mechanism of cancer immunoediting. *Nature* 482:400-404.
503   10.1038/nature10755
504 Mou Y, Wang J, Wu J, He D, Zhang C, Duan C, and Li B. 2019. Ferroptosis, a new form of cell
505   death: opportunities and challenges in cancer. *J Hematol Oncol* 12:34. 10.1186/s13045-
506   019-0720-y
507 Nagakannan P, Islam MI, Karimi-Abdolrezaee S, and Eftekharpour E. 2019. Inhibition of VDAC1
508   Protects Against Glutamate-Induced Oxytosis and Mitochondrial Fragmentation in
509   Hippocampal HT22 Cells. *Cellular and molecular neurobiology* 39:73-85.
510   10.1007/s10571-018-0634-1
511 Niu Y, Zhang J, Tong Y, Li J, and Liu B. 2019. Physcion 8-O-β-glucopyranoside induced
512   ferroptosis via regulating miR-103a-3p/GLS2 axis in gastric cancer. *Life sciences*
513   237:116893. 10.1016/j.lfs.2019.116893
514 Pentimalli F, Grelli S, Di Daniele N, Melino G, and Amelio I. 2019. Cell death pathologies:
515   targeting death pathways and the immune system for cancer therapy. *Genes and*
516   *immunity* 20:539-554. 10.1038/s41435-018-0052-x
517 Rousseaux S, Debernardi A, Jacquiau B, Vitte A-L, Vesin A, Nagy-Mignotte H, Moro-Sibilot D,
518   Brichon P-Y, Lantuejoul S, Hainaut P, Laffaire J, de Reyniès A, Beer DG, Timsit J-F,
519   Brambilla C, Brambilla E, and Khochbin S. 2013. Ectopic activation of germline and
520   placental genes identifies aggressive metastasis-prone lung cancers. *Science*
521   *translational medicine* 5:186ra166. 10.1126/scitranslmed.3005723
522 Schabath MB, Welsh EA, Fulp WJ, Chen L, Teer JK, Thompson ZJ, Engel BE, Xie M, Berglund
523   AE, Creelan BC, Antonia SJ, Gray JE, Eschrich SA, Chen DT, Cress WD, Haura EB,
524   and Beg AA. 2016. Differential association of STK11 and TP53 with KRAS mutation-

525   associated gene expression, proliferation and immune surveillance in lung
526   adenocarcinoma. *Oncogene* 35:3209-3216. 10.1038/onc.2015.375
527 Shankaran V, Ikeda H, Bruce AT, White JM, Swanson PE, Old LJ, and Schreiber RD. 2001.
528   IFNgamma and lymphocytes prevent primary tumour development and shape tumour
529   immunogenicity. *Nature* 410:1107-1111.
530 Song X, Xie Y, Kang R, Hou W, Sun X, Epperly MW, Greenberger JS, and Tang D. 2016.
531   FANCD2 protects against bone marrow injury from ferroptosis. *Biochem Biophys Res*
532   *Commun* 480:443-449. 10.1016/j.bbrc.2016.10.068
533 Song Y, Wang B, Zhu X, Hu J, Sun J, Xuan J, and Ge Z. 2020. Human umbilical cord blood-
534   derived MSCs exosome attenuate myocardial injury by inhibiting ferroptosis in acute
535   myocardial infarction mice. *Cell biology and toxicology*. 10.1007/s10565-020-09530-8
536 Stockwell BR, Friedmann Angeli JP, Bayir H, Bush AI, Conrad M, Dixon SJ, Fulda S, Gascón S,
537   Hatzios SK, Kagan VE, Noel K, Jiang X, Linkermann A, Murphy ME, Overholtzer M,
538   Oyagi A, Pagnussat GC, Park J, Ran Q, Rosenfeld CS, Salnikow K, Tang D, Torti FM,
539   Torti SV, Toyokuni S, Woerpel KA, and Zhang DD. 2017. Ferroptosis: A Regulated Cell
540   Death Nexus Linking Metabolism, Redox Biology, and Disease. *Cell* 171:273-285.
541   10.1016/j.cell.2017.09.021
542 Street SE, Cretney E, and Smyth MJ. 2001. Perforin and interferon-gamma activities
543   independently control tumor initiation, growth, and metastasis. *Blood* 97:192-197.
544 Takeshima T, Pop LM, Laine A, Iyengar P, Vitetta ES, and Hannan R. 2016. Key role for
545   neutrophils in radiation-induced antitumor immune responses: Potentiation with G-CSF.
546   *Proceedings of the National Academy of Sciences of the United States of America*
547   113:11300-11305.
548 Tiberio L, Del Prete A, Schioppa T, Sozio F, Bosisio D, and Sozzani S. 2018. Chemokine and
549   chemotactic signals in dendritic cell migration. *Cellular & molecular immunology* 15:346-
550   352. 10.1038/s41423-018-0005-3
551 Varricchi G, Galdiero MR, Loffredo S, Marone G, Iannone R, Marone G, and Granata F. 2017.
552   Are Mast Cells MASTers in Cancer? *Frontiers in immunology* 8:424-424.
553   10.3389/fimmu.2017.00424
554 Wang F, Lv H, Zhao B, Zhou L, Wang S, Luo J, Liu J, and Shang P. 2019a. Iron and leukemia:
555   new insights for future treatments. *Journal of experimental & clinical cancer research :*
556   *CR* 38:406. 10.1186/s13046-019-1397-3
557 Wang W, Green M, Choi JE, Gijon M, Kennedy PD, Johnson JK, Liao P, Lang X, Kryczek I, Sell
558   A, Xia H, Zhou J, Li G, Li J, Li W, Wei S, Vatan L, Zhang H, Szeliga W, Gu W, Liu R,
559   Lawrence TS, Lamb C, Tanno Y, Cieslik M, Stone E, Georgiou G, Chan TA, Chinnaiyan
560   A, and Zou W. 2019b. CD8(+) T cells regulate tumour ferroptosis during cancer
561   immunotherapy. *Nature* 569:270-274. 10.1038/s41586-019-1170-y
562 Wei X, Zhang K, Qin H, Zhu J, Qin Q, Yu Y, and Wang H. 2018. GMDS knockdown impairs cell
563   proliferation and survival in human lung adenocarcinoma. *BMC cancer* 18:600-600.
564   10.1186/s12885-018-4524-1
565 Welsh TJ, Green RH, Richardson D, Waller DA, O'Byrne KJ, and Bradding P. 2005.
566   Macrophage and mast-cell invasion of tumor cell islets confers a marked survival
567   advantage in non-small-cell lung cancer. *Journal of clinical oncology : official journal of*
568   *the American Society of Clinical Oncology* 23:8959-8967.
569 Wen Q, Liu J, Kang R, Zhou B, and Tang D. 2019. The release and activity of HMGB1 in
570   ferroptosis. *Biochem Biophys Res Commun* 510:278-283. 10.1016/j.bbrc.2019.01.090
571 Wenzel SE, Tyurina YY, Zhao J, St Croix CM, Dar HH, Mao G, Tyurin VA, Anthonymuthu TS,
572   Kapralov AA, Amoscato AA, Mikulska-Ruminska K, Shrivastava IH, Kenny EM, Yang Q,
573   Rosenbaum JC, Sparvero LJ, Emlet DR, Wen X, Minami Y, Qu F, Watkins SC, Holman
574   TR, VanDemark AP, Kellum JA, Bahar I, Bayır H, and Kagan VE. 2017. PEBP1

575   Wardens Ferroptosis by Enabling Lipoxygenase Generation of Lipid Death Signals. *Cell*
576    171. 10.1016/j.cell.2017.09.044
577 Xie Y, Zhu S, Song X, Sun X, Fan Y, Liu J, Zhong M, Yuan H, Zhang L, Billiar TR, Lotze MT,
578   Zeh HJ, Kang R, Kroemer G, and Tang D. 2017. The Tumor Suppressor p53 Limits
579   Ferroptosis by Blocking DPP4 Activity. *Cell reports* 20:1692-1704.
580   10.1016/j.celrep.2017.07.055
581 Yang Y, Luo M, Zhang K, Zhang J, Gao T, Connell DO, Yao F, Mu C, Cai B, Shang Y, and
582   Chen W. 2020. Nedd4 ubiquitylates VDAC2/3 to suppress erastin-induced ferroptosis in
583   melanoma. *Nature communications* 11:433. 10.1038/s41467-020-14324-x
584 Yuan H, Li X, Zhang X, Kang R, and Tang D. 2016. CISD1 inhibits ferroptosis by protection
585   against mitochondrial lipid peroxidation. *Biochemical and biophysical research*
586   *communications* 478:838-844. 10.1016/j.bbrc.2016.08.034
587 Zhang Y, Song J, Zhao Z, Yang M, Chen M, Liu C, Ji J, and Zhu D. 2020. Single-cell
588   transcriptome analysis reveals tumor immune microenvironment heterogenicity and
589   granulocytes enrichment in colorectal cancer liver metastases. *Cancer Letters* 470:84-
590   94.
591

Figure 1

Work flow of the study.

The TCGA cohort was used to construct the prognostic ferroptosis-related gene signature.
The GSE72094 cohort and GSE30219 cohort were used to further validate the prognostic
signature. TCGA, The Cancer Genome Atlas; LUAD, lung adenocarcinoma; DEGs, differentially
expressed genes; FDR, false discovery rate; OS, overall survival; PCA, principal component
analysis; ROC, the receiver operating characteristic.

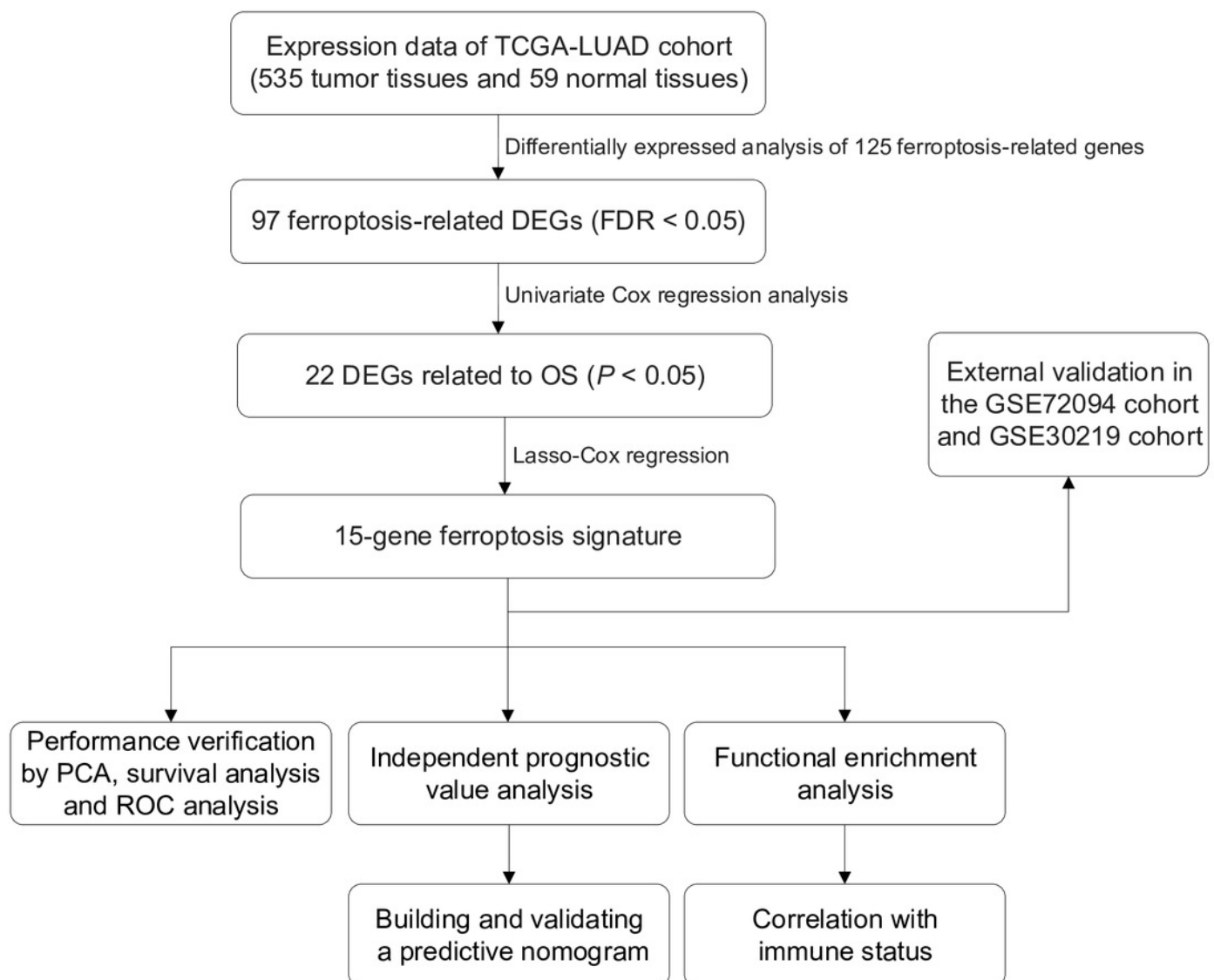



Figure 2

Heatmap and volcano plot of the ferroptosis-related DEGs.

(A) Heatmap of the top 20 upregulated and downregulated ferroptosis-related genes between normal and tumor tissues in the TCGA-LUAD cohort. (B) Volcano plot of the 97 ferroptosis-related DEGs in the TCGA-LUAD cohort. The top 5 up- and down-regulated ferroptosis-related DEGs were labeled in the volcano plot. FDR<0.05 was set as screening criteria. The blue, red and black dots represented the down-, up-regulated ferroptosis-related DEGs and genes that were not satisfied the screening criteria, respectively. DEGs, differentially expressed genes; FDR, false discovery rate; TCGA, The Cancer Genome Atlas; LUAD, lung adenocarcinoma; N, normal tissues; T, tumor tissues.

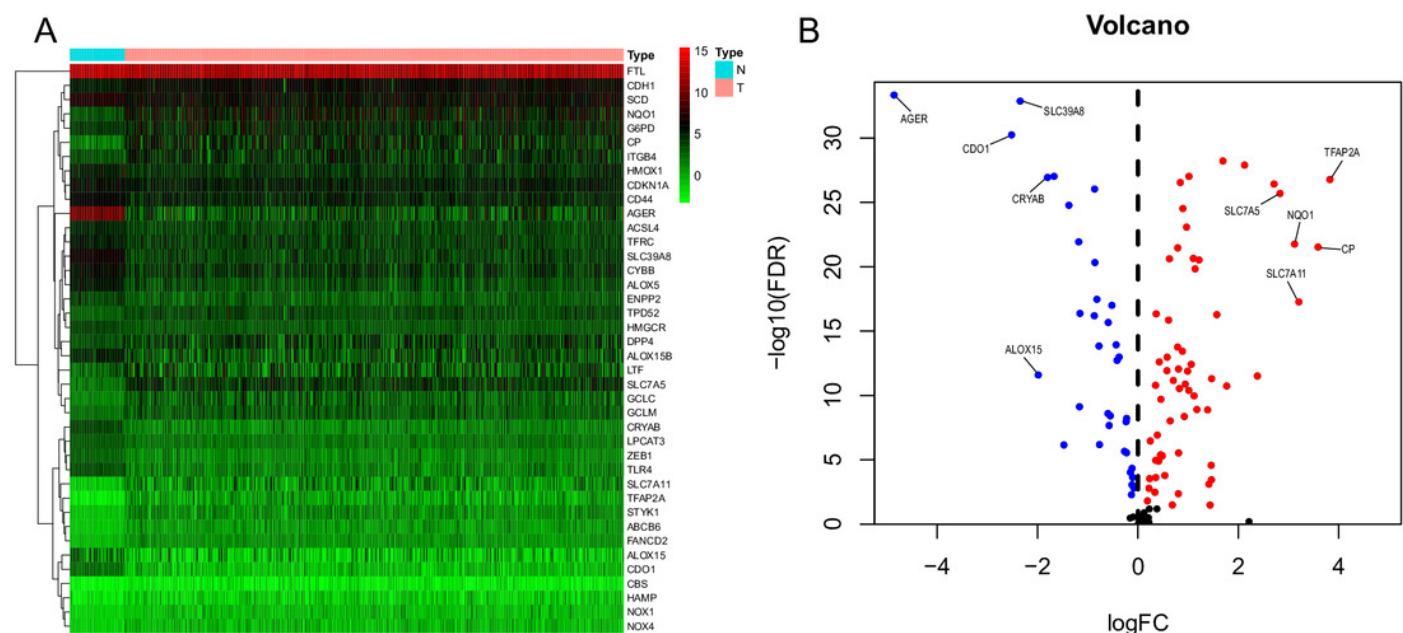



Figure 3

Forest map of 22 prognostic ferroptosis-related DEGs screened by univariate Cox regression.

DEGs, differentially expressed genes. Yellow: risk factors; blue: protective factors.

Figure 4

Performance verification of the 15-gene ferroptosis signature in the TCGA cohort.

(A) PCA plot of patients in high-risk group (red) and low-risk group (navy blue). (B) Kaplan-Meier curve of patients in high-risk group (red) and low-risk group (navy blue). (C) The distribution of risk score of each patient. (D) The distribution of OS, survival status and risk score of each patient. Patients' OS was decreased with the increase of risk score. (E) Time-dependent ROC curves of risk score and clinical characteristics. TCGA, The Cancer Genome Atlas; PCA, principal component analysis; OS, overall survival; AUC, area under curve; ROC, receiver operating characteristic.

A

B

C

D

E

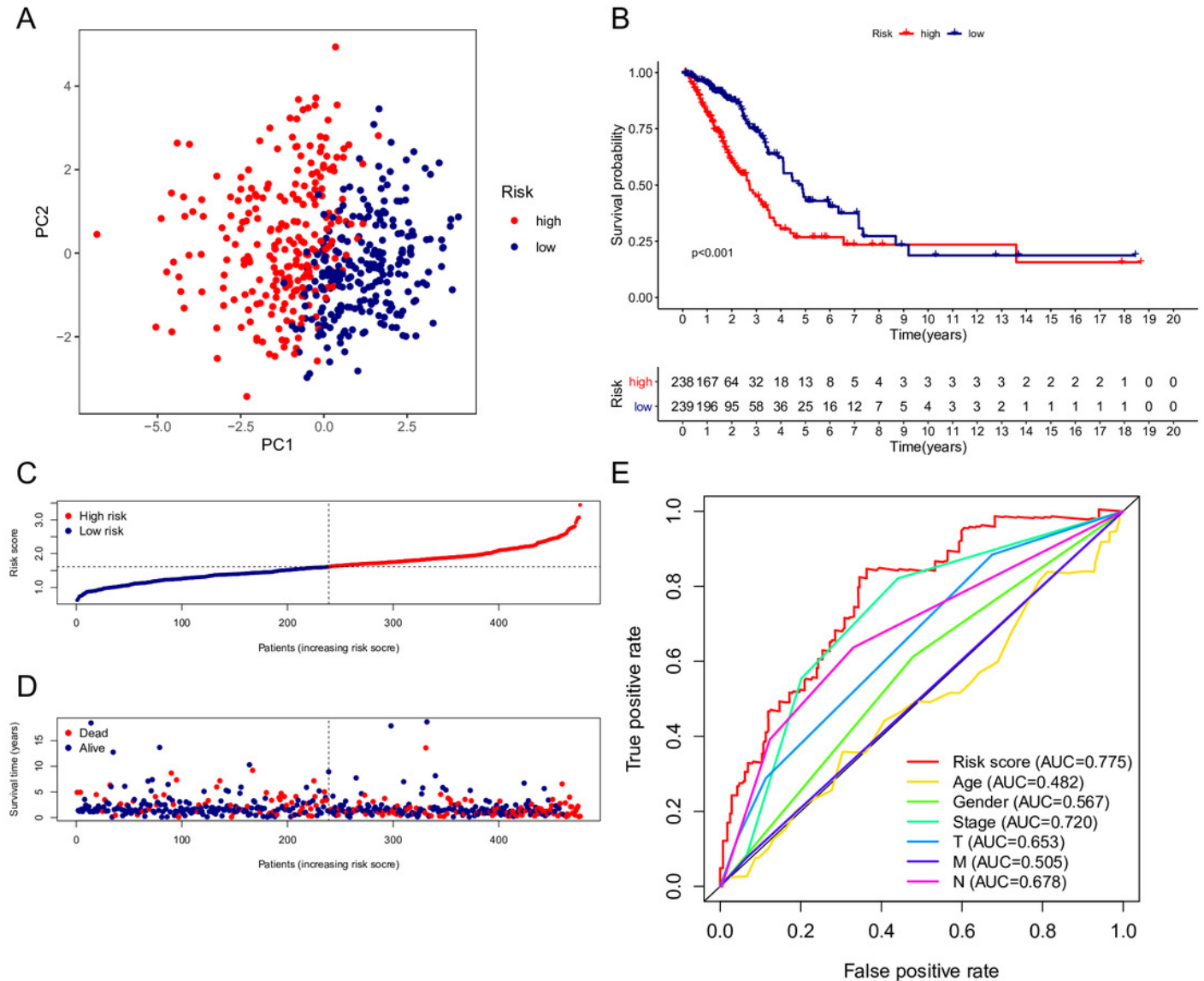



Figure 5

Validation of the 15-gene ferroptosis signature in the GSE72094 cohort (A-E) and GSE30219 cohort (F-J).

(A, F) PCA plots of patients in high-risk group (red) and low-risk group (navy blue). (B, G) Kaplan-Meier curves of patients in high-risk group (red) and low-risk group (navy blue). (C, H) The distribution of risk score of each patient. (D, I) The distribution of OS, survival status and risk score of each patient. (E, J) Time- dependent ROC curves of risk score and clinical characteristics. PCA, principal component analysis; OS, overall survival; AUC, area under curve; ROC, receiver operating characteristic.

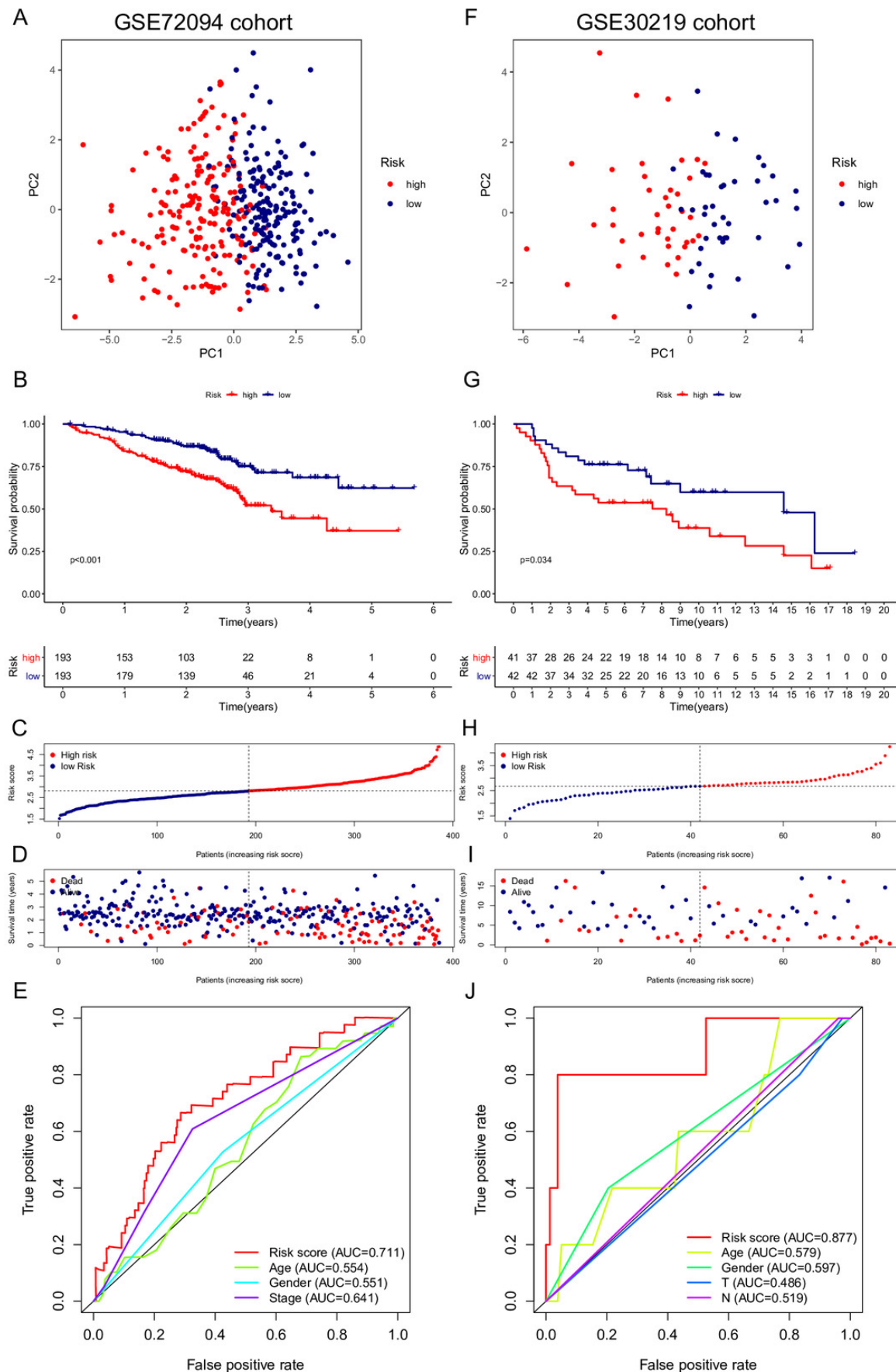



Figure 6

Stratification analyses of the TCGA cohort (A-B) and GSE72094 cohort (C-F).

(A, B) Kaplan-Meier curves of patients in high-risk group (red) and low-risk group (navy blue) stratified via stage I-II and stage III-IV in the TCGA cohort, respectively. (C, D) Kaplan-Meier curves of patients in high-risk group (red) and low-risk group (navy blue) stratified via female and male in the GSE72094 cohort, respectively. (E, F) Kaplan-Meier curves of patients in high-risk group (red) and low-risk group (navy blue) stratified via stage I-II and stage III-IV in the GSE72094 cohort. TCGA, The Cancer Genome Atlas.

TCGA cohort

A

B

GSE72094 cohort

C

D

E

F

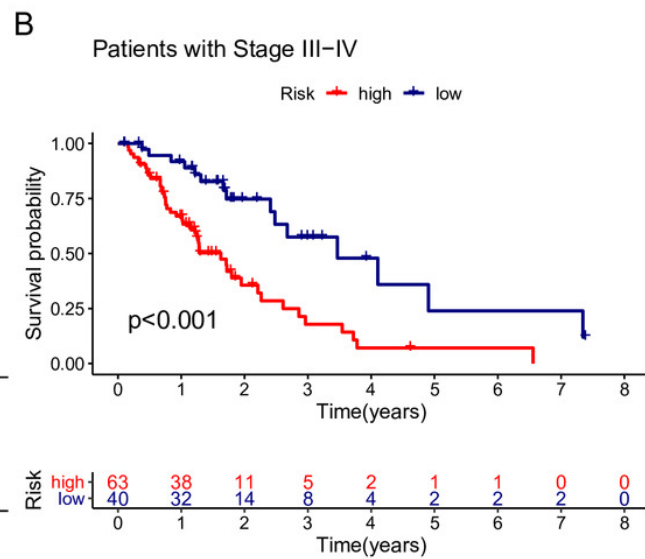
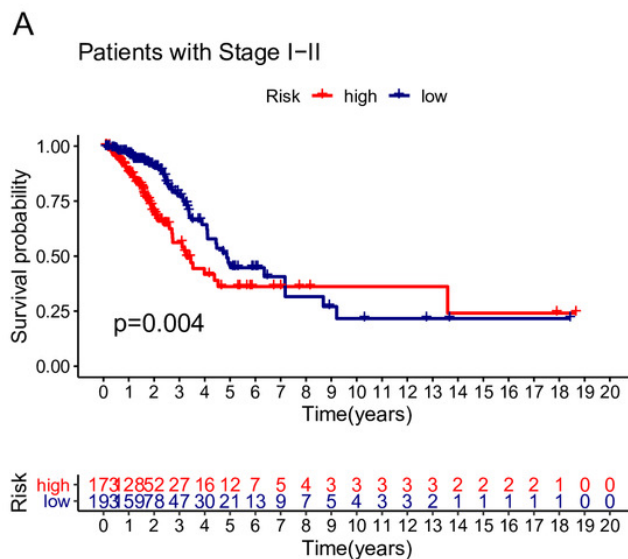
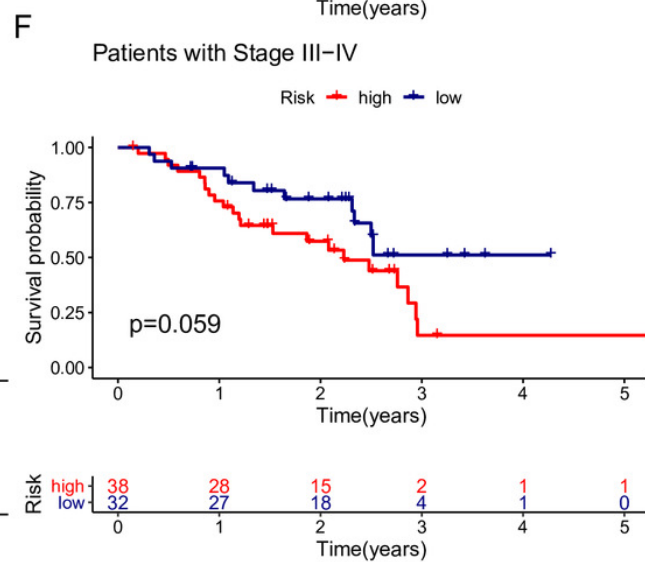
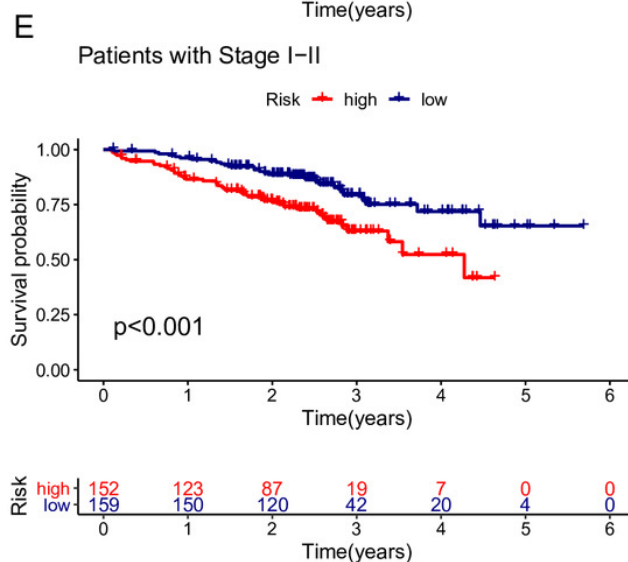
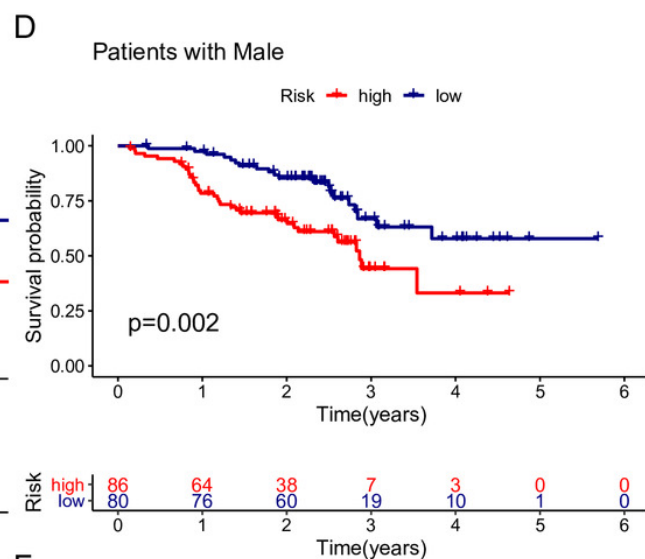
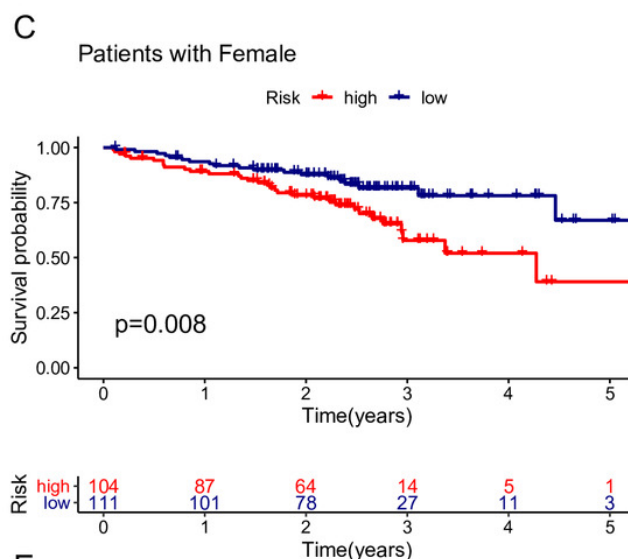



Figure 7

Construction and validation of the predictive nomogram.

(A) The nomogram for OS prediction at 1, 2 and 3 years was constructed in the TCGA cohort. (B) Kaplan-Meier curve of patients in high-risk group (red) and low-risk group (navy blue) in the TCGA cohort. (C) Kaplan-Meier curve of patients in high-risk group (red) and low-risk group (navy blue) in the GSE72094 cohort. (D, E) Calibration plots of nomogram for OS prediction at 1 year and 3 years in the TCGA cohort, respectively. (F, G) Calibration plots of nomogram for OS prediction at 1 year and 3 years in the GSE72094 cohort, respectively. (H, I) Time-dependent ROC curves to evaluate the predictive performance of nomogram at 1 year and 3 years in the TCGA cohort, respectively. (J, K) Time-dependent ROC curves to evaluate the predictive performance of nomogram at 1 year and 3 years in the GSE72094 cohort, respectively. In the calibration plot, the closer the red line (fitting line) and gray line (ideal line) are, the higher predictive accuracy of the model is. TCGA, The Cancer Genome Atlas; OS, overall survival; AUC, area under curve; ROC, receiver operating characteristic.

A

Points	0	10	20	30	40	50	60	70	80	90	100

Stage   Stage II   Stage III

Stage I   Stage IV

Riskscore   0.6 0.8 1 1.2 1.4 1.6 1.8 2 2.2 2.4 2.6 2.8 3 3.2 3.4 3.6

Total Points   0   20   40   60   80   100   120   140   160

1−year survival   0.9 0.8 0.7 0.6 0.5 0.4 0.3 0.2

2−year survival   0.9 0.8 0.7 0.6 0.5 0.4 0.3 0.2 0.1 0.05

3−year survival   0.9 0.8 0.7 0.6 0.5 0.4 0.3 0.2 0.1 0.05

B

C

D — TCGA cohort

E — TCGA cohort

F — GSE72094 cohort

G — GSE72094 cohort

H — ROC at 1-year
Nomogram (AUC=0.812)
Stage (AUC=0.720)
Riskscore (AUC=0.775)

I — ROC at 3-year
Nomogram (AUC=0.757)
Stage (AUC=0.684)
Riskscore (AUC=0.720)

J — ROC at 1-year
Nomogram (AUC=0.735)
Stage (AUC=0.641)
Riskscore (AUC=0.711)

K — ROC at 3-year
Nomogram (AUC=0.758)
Stage (AUC=0.672)
Riskscore (AUC=0.689)

Figure 8

GO and KEGG pathway enrichment analyses.

(A) Representative GO pathways enriched in the DEGs between the high- and low-risk groups. (B) The top 5 enriched KEGG pathways identified by GSEA in the high- and low-risk groups. |log2FC|≥1 and FDR<0.05 were set as the criteria to screen DEGs between the high- and low-risk groups. The pathways with a P value less than 0.05 were considered to be significantly enriched. DEGs, differentially expressed genes; GO, Gene Ontology; BP, Biological Process; CC, Cellular Component; MF, Molecular Function; KEGG, Kyoto Encyclopedia of Genes and Genomes; GSEA, Gene Set Enrichment Analysis.

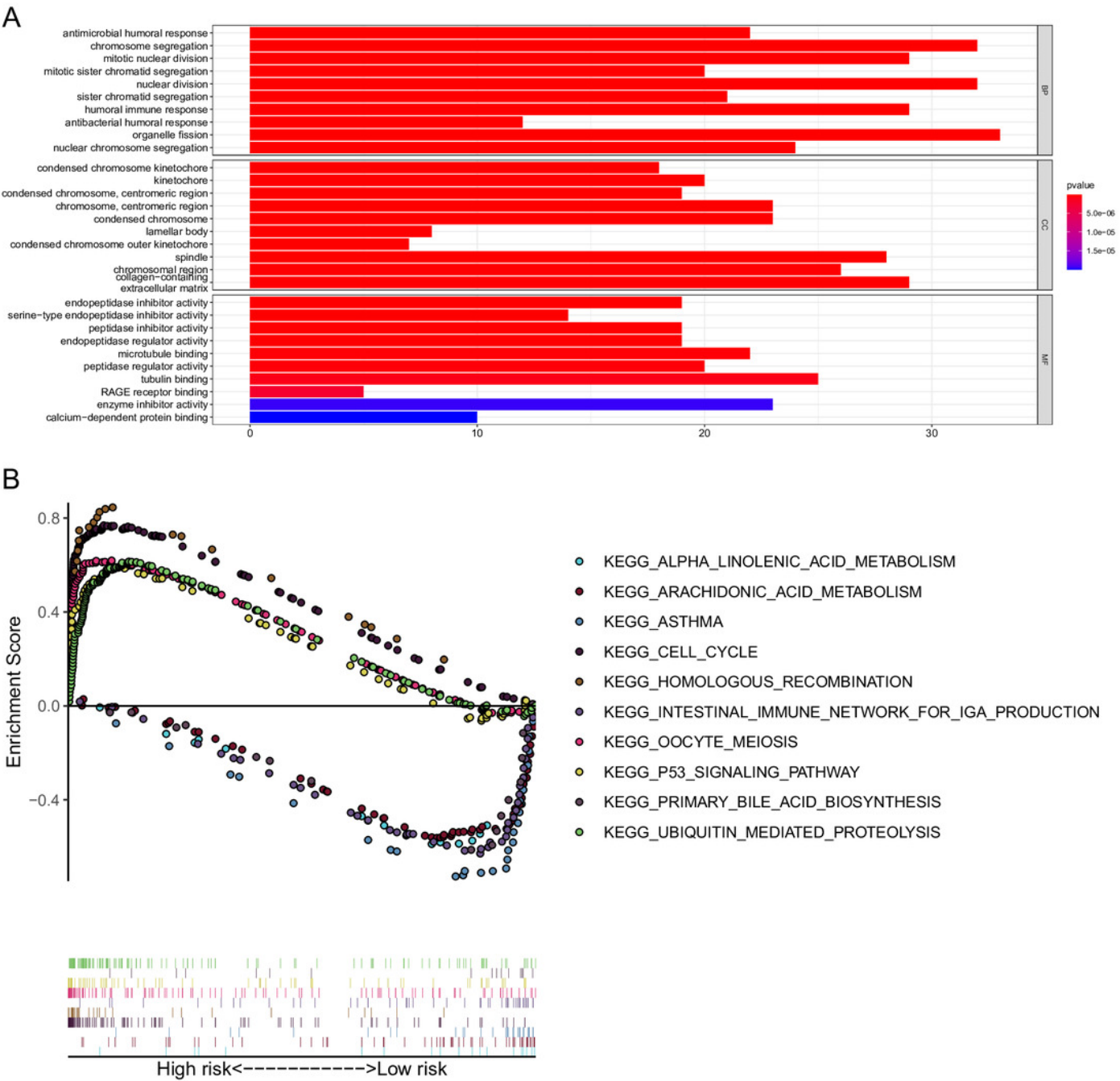
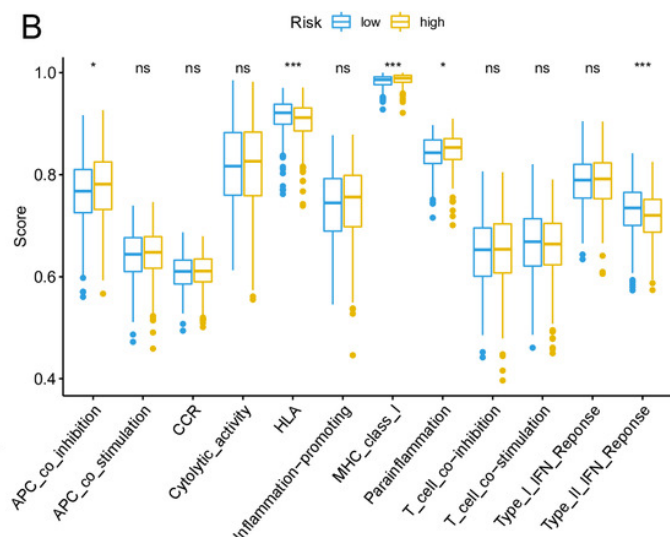
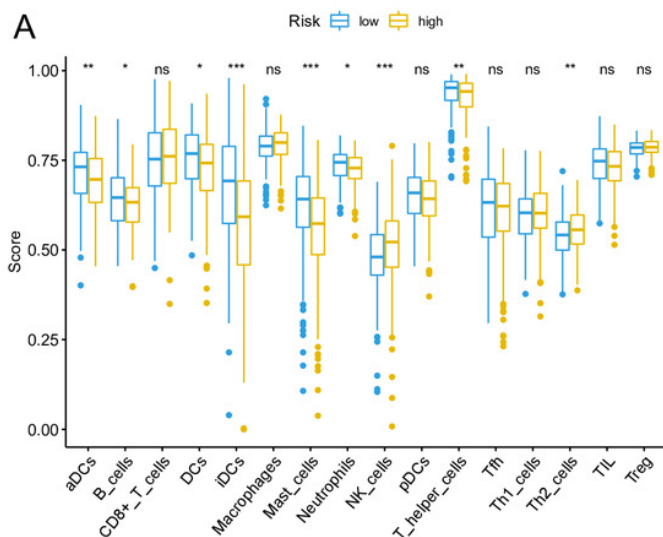
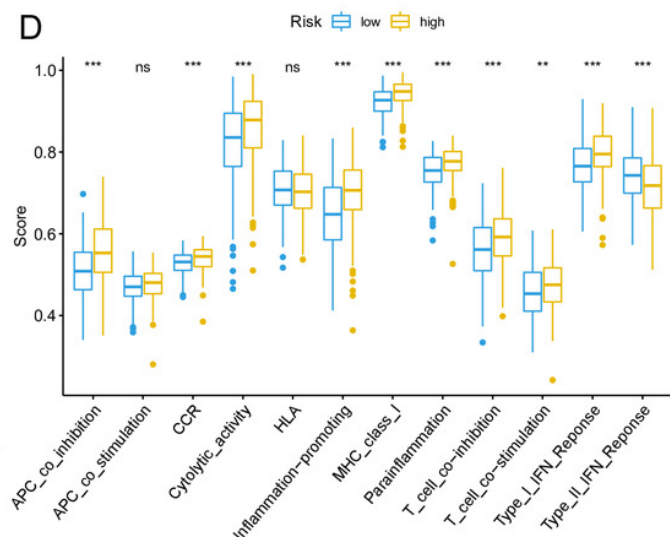
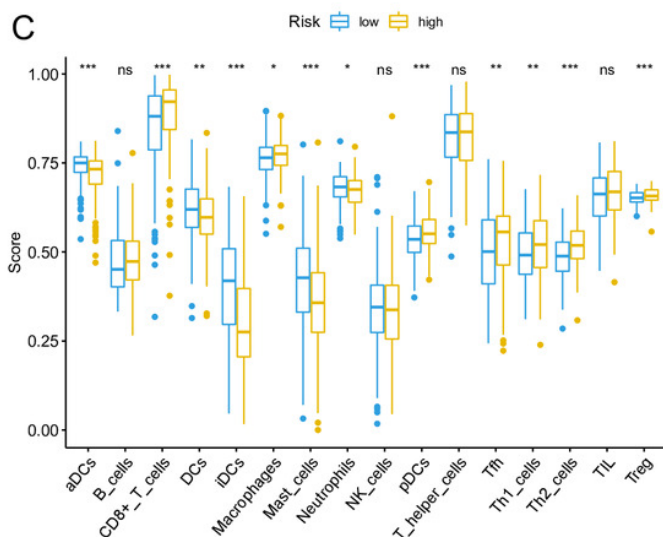
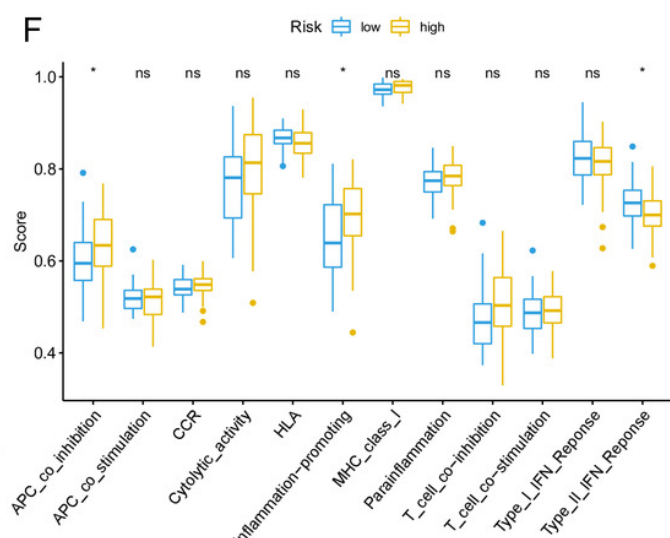
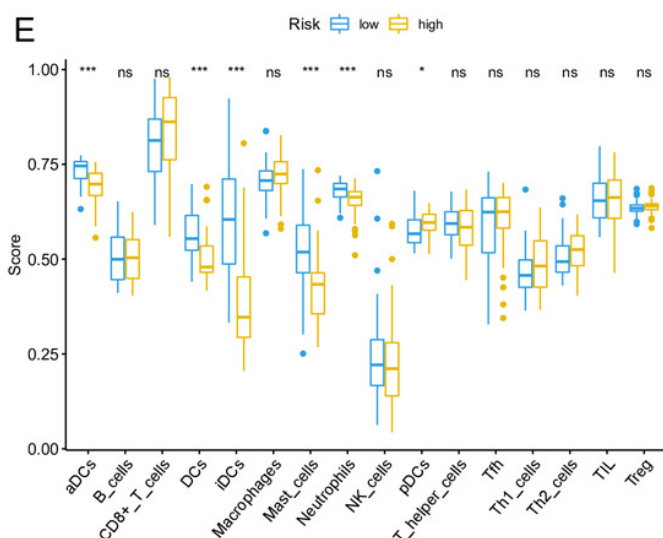



Figure 9

Comparison of ssGSEA scores of different risk groups in the TCGA cohort (A, B), GSE72094 cohort (C, D) and GSE30219 cohort (E, F).

(A, C, E) Boxplots to display the enrichment scores of 16 immune cells of different risk groups in the TCGA cohort, GSE72094 cohort and GSE30219 cohort, respectively. (B, D, F) Boxplots to display the enrichment scores of 12 immune functions of different risk groups in the TCGA cohort and GSE72094 cohort and GSE30219 cohort, respectively. Adjusted P values were showed as: ns, not significant; *, P< 0.05; **, P< 0.01; ***, P< 0.001. ssGSEA, single-sample gene set enrichment analysis; TCGA, The Cancer Genome Atlas; DC, Dendritic cells; APC, Antigen-presenting cells; Type II INF, IFN-γ.

Univariate and multivariate analysis of OS in the TCGA, GSE72094 and GSE30219 cohorts.

Variables	Univariate analysis			Multivariate analysis		
	HR	95% CI	P value	HR	95% CI	P value
TCGA cohort						
Age	0.997	0.978-1.015	0.718			
Gender	1.000	0.694-1.441	1.000			
Stage	1.648	1.396-1.946	<0.001	1.591	1.064-2.380	0.024
T	1.600	1.285-1.994	<0.001	1.064	0.830-1.364	0.626
N	2.765	1.911-4.001	<0.001	1.316	0.723-2.397	0.369
M	1.748	0.959-3.187	0.068			
Risk score	3.670	2.521-5.341	<0.001	2.918	1.972-4.318	<0.001
GSE72094 cohort						
Age	1.010	0.990-1.030	0.344			
Gender	1.501	1.027-2.194	0.036	1.547	1.050-2.280	0.027
Stage	1.628	1.358-1.951	<0.001	1.589	1.351-1.920	<0.001
Risk score	2.607	1.918-3.544	<0.001	2.135	1.722-3.208	<0.001
GSE30219 cohort						
Age	1.031	0.996-1.067	0.088			
Gender	1.114	0.516-2.403	0.784			
T	1.463	0.856-2.499	0.164			
N	1.247	0.299-5.202	0.762			
Risk score	2.881	1.516-5.474	0.001	6.751	2.400-18.785	<0.001

1 Abbreviations: TCGA, The Cancer Genome Atlas; HR, hazard ratio; CI, confidence interval.

Top 5 enriched KEGG pathways of the high-risk and low-risk groups analysed by GSEA.

Enriched pathways	Size	Es	NES	NOM p-value	FDR q-value
High-risk group					
KEGG_CELL_CYCLE	125	0.769	2.495	<0.001	<0.001
KEGG_UBIQUITIN_MEDIATED_PROTEOLY-SIS	135	0.615	2.458	<0.001	<0.001
KEGG_OOCYTE_MEIOSIS	113	0.619	2.342	<0.001	<0.001
KEGG_HOMOLOGOUS_RECOMBINATION	28	0.845	2.291	<0.001	<0.001
KEGG_P53_SIGNALING_PATHWAY	68	0.589	2.270	<0.001	<0.001
Low-risk group					
KEGG_ARACHIDONIC_ACID_METABOLISM	58	0.571	1.988	0.002	0.103
KEGG_PRIMARY_BILE_ACID_BIOSYNTHE-SIS	16	0.661	1.883	<0.001	0.144
KEGG_ALPHA_LINOLENIC_ACID_METABO-LISM	19	0.621	1.823	0.006	0.156
KEGG_ASTHMA	28	0.745	1.820	0.017	0.119
KEGG_INTESTINAL_IMMUNE_NETWORK_FOR_IGA_PRODUCTION	46	0.650	1.740	0.040	0.177

1 Abbreviations: ES, enrichment score; NES, normalized enrichment score; NOM p-value, nominal p

2 value; FDR q-valve, false discovery rate.

Modulatory effect of the signature genes on ferroptosis.

Gene symbol	Protein	Modulatory effect on ferroptosis	References
AGER	advanced glycosylation end product-specific receptor	depletion attenuates the autophagy-dependent ferroptosis	Wen et al. 2019
CISD1	CDGSH iron-sulfur domain-containing protein 1	down-expression contributes to ferroptosis by iron-mediated intramitochondrial lipid peroxidation	Yuan et al. 2016
DPP4	dipeptidyl peptidase 4	overexpression protects cell from ferroptosis by inhibiting lipid peroxidation	Xie et al. 2017
EGLN1	egl nine homolog 1	overexpression inhibits ferroptosis by the induction of LSH	Jiang et al. 2017
FANCD2	Fanconi anemia group D2 protein	knockout promotes ferroptosis though increasing iron accumulation and lipid peroxidation	Song et al. 2016
GLS2	glutaminase liver isoform	upregulation promotes ferroptosis in gastric cancer	Niu et al. 2019
ISCU	iron-sulfur cluster assembly enzyme ISCU	overexpression attenuates ferroptosis by increasing the level of GSH	Du et al. 2019
ITGA6	integrin alpha-6	overexpression inhibits ferroptosis by suppressing the expression of ACSL4	Brown et al. 2017
ITGB4	integrin beta-4	overexpression inhibits ferroptosis by suppressing the expression of ACSL4	Brown et al. 2017
KRAS	GTPase KRas	inhibits ferroptosis by upregulating the expression of SLC7A11	Hu et al. 2020
NEDD4	E3 ubiquitin-protein ligase NEDD4	upregulation inhibits ferroptosis induced by erastin through the degradation of VDAC2/3	Yang et al. 2020
PEBP1	phosphatidylethanolamine-binding protein 1	overexpression increases sensitivity to ferroptosis in HAEC and HT22 cells	Wenzel et al. 2017
SLC11A2	natural resistance-associated macrophage protein 2	upregulation contributes to ferroptosis by increasing iron uptake	Song et al. 2020
TFAP2A	transcription factor AP-2-alpha	negatively regulates ferroptosis by increasing the expression of NRF2	Huang et al. 2020
VDAC1	voltage-dependent anion-selective channel protein 1	inhibition alleviates ferroptosis by decreasing the ROS level in mitochondria	Nagakannan et al. 2019