

Signature of immune infiltration-related ferroptosis genes to predict the prognosis of patients with osteosarcoma

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Abstract. Osteosarcoma (OS) is the most prevalent malignant bone tumor among children and adolescents, and ferroptosis has been implicated in tumor immune infiltration. The present study aimed to explore the role of ferroptosis-related genes in immune infiltration and to develop a prognostic signature for osteosarcoma (OS). In the present study, two immune subtypes of OS were identified and demonstrated to be reproducible and effective. Building upon the distinction between high and low immunity, six common differentially expressed immune infiltration-related ferroptosis genes (DEIRFGs) were identified. After which, univariate Cox regression (UCR) analysis was employed to identify DEIRFGs associated with overall survival. Next, using Gene Ontology analysis, it was revealed that the six mRNAs were enriched in the immune-associated functions and pathway. Subsequently, using UCR analysis and the iterative Least Absolute Shrinkage and Selection Operator Cox regression analysis based on two ferroptosis genes (interferon- γ and toll-like receptor 4), a model was established. Additionally, it was confirmed that the risk model was an independent prognostic factor for overall survival. Furthermore, the results were verified using a Gene Expression Omnibus dataset. Finally, the content of tumor-infiltrating immune cells

demonstrated that the high-risk group was significantly associated with immune cell infiltration, compared with the low-risk group. Moreover, the expression of immune checkpoint inhibitors was significantly upregulated in the low-risk cohort compared with in the high-risk cohort. Finally, using gene set enrichment analysis, it was demonstrated that the risk score was associated with the toll-like receptor signaling pathway, antigen processing and presentation, and cytokine-cytokine receptor interaction. In summary, a signature of DEIRFGs to predict prognosis in patients with OS was developed. Moreover, it was demonstrated that the molecular mechanisms responsible in the high-risk group may influence immune infiltration-related biological processes.

Introduction

Osteosarcoma (OS), a typical malignant bone tumor, predominantly affects children and young adults. Notably progress in the prevention and treatment of OS has been achieved through the introduction of potential treatment approaches. However, the global annual incidence of OS is ~3.4 cases per million population, with a slight male predominance. Despite advancements in treatment, the 5-year survival rate for patients with localized disease is 60-70%, which drops to 15-30% for those with metastatic or recurrent disease. Mortality trends have shown an annual increase of approximately 1.4% in certain populations (1-4). It has also been reported that ~80% of patients with OS have high programmed cell death ligand 1 (PD-L1) mRNA expression (5). Programmed cell death protein 1 (PD-1)/PD-L1 pathway inhibitors have been evaluated in clinical trials for the treatment of bone and soft-tissue sarcomas; however, there has been no notable responses recorded in patients with advanced OS (6). The success of immunotherapy is highly dependent on the level of immune cells within the microenvironment of the host (7,8). Therefore, assessing the immunoreactivity of OS is crucial in making decisions about individualized treatment.

Ferroptosis, a novel type of cell death, is typically accompanied by high levels of lipid peroxidation and iron build-up (9). It serves a role in numerous key pathological processes, including the development and incidence of cancer (10). Moreover, a previous study reported that

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Abbreviations: OS, osteosarcoma; DEG, differentially expressed gene; UCR, univariate Cox regression; MCR, multivariate Cox regression; ssGSEA, single-sample Gene Set Enrichment Analysis; TARGET, Therapeutically Applicable Research to Generate Effective Treatments; ROC, receiver operating characteristic; KEGG, Kyoto Encyclopedia of Genes and Genomes; GO, Gene Ontology; TIIC, tumor-infiltrating immune cell

Key words: osteosarcoma, tumor immune infiltration, ferroptosis, prognostic model

ferroptosis serves a core role in modulating genes involved in tumor immune escape (11). For example, the absence of glutathione peroxidase 4, a key regulator of ferroptosis, prevents an increase in the levels of CD8⁺ and CD4⁺ T cells (11). Hence, determination of immune infiltration-related ferroptosis genes for cancer immunoreactivity could reveal novel insights into OS treatment.

In the present study, a single-sample Gene Set Enrichment Analysis (ssGSEA) was used to assign patients with OS into two clusters. A risk model was constructed using two immune infiltration-related ferroptosis genes.

Materials and methods

Data abstraction. Clinical and pathological data from 87 patients with OS were retrieved from the Therapeutically Applicable Research to Generate Effective Treatments (TARGET) database (accession no. phs000468.v22.p8) (<https://www.cancer.gov/ccg/research/genome-sequencing/target>) (12). Furthermore, the expression profile of the GSE21257 dataset (13) was downloaded from the Gene Expression Omnibus (GEO; <https://www.ncbi.nlm.nih.gov/geo/>), which contains data from 53 human OS tissues. The gene probes of the platform were transformed to gene names by referencing the GPL10295 platform (14).

OS subgroup determination based on immune gene sets. The 29 immune-related gene sets encompass several types of immune cells, functions and pathways, were obtained from a previously published study by Nguyen *et al* (15). For each overall survival dataset (TARGET-OS database, phs000468.v22.p8), the level of immune-cell infiltration in cancers was estimated using ssGSEA with the GSVA package (bioconductor.org/packages/release/bioc/html/GSVA.html) in R language (R 4.2.2 with Bioconductor 3.16.). Subsequently, tumor purity, immune score and stromal score were inferred using the ESTIMATE R package [v1.0.13 Kosuke Yoshihara Lab (Academic)]. Consensus clustering and molecular subtyping were performed using the ‘ConsensusClusterPlus’ R package [bioconductor.org/packages/release/bioc/html/ConsensusClusterPlus.html v1.68.0 (Bioconductor release 3.19)], based on the ssGSEA scores. K-means clustering, spectral clustering and PCA-k-means clustering were each replicated 50 times, and the optimal clustering outcome was utilized.

Differential expression analysis. Gene expression data from RNA-sequencing (RNA-seq) were normalized using the voom transformation prior to differential expression analysis. The ‘limma’ R package was used to screen the differentially expressed genes (DEGs) between high immunity (Immunity_H) and low immunity (Immunity_L) samples. DEGs were identified based adjusted $P < 0.05$ and $(\log_2\text{FCI}) > 0.5$. This threshold was selected to capture moderate transcriptional changes relevant to immune modulation in OS, where subtle gene expression variations may markedly impact biological pathways. Whilst stringent cutoffs (such as $\log_2\text{FCI} > 1$) are common, studies indicate that immune-related genes often exhibit smaller yet functionally critical expression shifts in tumor microenvironments (16-18).

Moreover, the ‘pheatmap’ R package was used to plot heatmaps, whilst ‘ggpubr’ was used to generate boxplots. FerrDb (<http://www.zhounan.org/ferrdb/>) and PubMed (<https://pubmed.ncbi.nlm.nih.gov/>) were used to obtain data on the 283 ferroptosis genes. For each of the DEGs and ferroptosis genes, a Venn diagram was used for overlapping.

Development of a prognostic model based on ferroptosis genes. Prognosis-related ferroptosis genes were identified using the univariate Cox regression (UCR) model. Subsequently, the expression levels of these genes were combined, and the estimated regression coefficients were used to calculate prognostic scores for each patient. The following formula was utilized to determine the risk score for each patient: Risk score = $\beta_1x_1 + \beta_2x_2 + \dots + \beta_nx_n$. Subsequently, based on the median risk score as the cut-off value, samples were divided into high- and low-risk groups. The Kaplan-Meier (K-M) method was employed to determine the survival rates in each group. Variables were included in both UCR and multivariate Cox regression (MCR) analyses to assess the independence of the risk variables. Receiver operating characteristic (ROC) curves were used to evaluate the accuracy and specificity of the signature associated with immune infiltration-related ferroptosis genes.

Gene ontology (GO) annotation and pathway enrichment analysis. RStudio software (version 4.2; Posit Software, PBC; [rstudio.com/](https://www.rstudio.com/)) was utilized to perform mRNA GO enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) functional analyses.

CIBERSORT estimation. CIBERSORT estimates the abundance ratios based on gene expression data from several cell types within a mixed cell population (19,20). In the present study, the CIBERSORT algorithm was utilized to measure the percentage of immune cells. Subsequently, patients were categorized into low- and high-risk groups based on prognostic risk score for overall survival.

Gene set enrichment analysis (GSEA). GSEA was used to elucidate the different signaling pathways between the two risk subgroups, and all datasets were used for a GSEA using $c2.cp.kegg.v7.0.symbols.gmt$. Nominal $P < 0.05$, $(\text{normalized ESI Enrichment Score}) > 1$ and false Discovery Rate) $q < 0.25$ were selected out.

Drug sensitivity analysis. Drug sensitivity data were retrieved from the CellMiner database (<https://discover.nci.nih.gov/cellminer/>). The R packages, ‘impute’, ‘limma’, ‘ggplot2’ and ‘ggpubr’ were used for data processing, statistical analysis and result visualization.

Quantification of gene expression using reverse transcription-quantitative PCR (RT-qPCR). The relative mRNA expression levels of IFNG and TLR4 were measured in human OS cells lines, MG-63, 143B and U2OS, and in the normal human osteoblast hFOB1.19 cell line. The hFOB1.19 cell line was maintained in DMEM/F12 medium (Gibco; Thermo Fisher Scientific, Inc.) at 34°C with 5% CO₂ in a humidified atmosphere. Moreover, The human OS cell lines

MG-63 and 143B cells (iCell Bioscience Inc.) were cultured in MEM (Gibco; Thermo Fisher Scientific, Inc.), while U2OS cells (iCell Bioscience Inc.) were cultured in McCoy's 5A medium (cat. no. 16600082; Gibco; Thermo Fisher Scientific, Inc.). Both culture media were supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 U/ml streptomycin (100 µg/ml) at 37°C with 5% CO₂ in a humidified environment.

Total RNA was extracted using TRIzol reagent (Thermo Fisher Scientific, Inc.) and subsequently used to synthesize cDNA with SuperScript™ II Reverse Transcriptase (Invitrogen™; Thermo Fisher Scientific, Inc.), incorporating 5 µg oligo (dT) primers per sample. RT was performed under the following temperature conditions: 25°C for 10 min (primer annealing), followed by 42°C for 50 min (cDNA synthesis), and 70°C for 15 min (enzyme inactivation). qPCR was performed using SYBR® Green PCR Master Mix (Applied Biosystems®; Thermo Fisher Scientific, Inc.) in a total volume of 20 µl with the 7500 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermocycling conditions were set at 95°C for 5 min, followed by 40 cycles of 95°C for 30 sec and 60°C for 45 sec. Melt-curve analysis was used to confirm the specificity of the amplification, and GAPDH served as the endogenous control for normalization of the amount of total RNA in each group. The relative gene expression was calculated in fold change according to the 2^{-ΔΔC_q} method (21), repeated independently in triplicate. The primer sequences were designed as follows: Interferon γ (IFNG) forward, 5'-TGCCTGCAATCTGAGCCAGT-3' and reverse, 5'-TGG AAGCACCAGGCATGAAA-3'; toll-like receptor 4 (TLR4) forward, 5'-TAGCGAGCCACGCATTCACA-3' and reverse, 5'-TAGGAACCACCTCCACGCAG-3'; and GAPDH forward, 5'-GACCTGACCTGCCGTCTA-3' and reverse, 5'-AGGAGT GGGTGTCTGCTGT-3'.

Authentication testing of the MG-63, 143B, U2OS and hFOB1.19 cell lines was performed (Shanghai Biowing Applied Biotechnology Co., Ltd.) via short tandem repeat (STR) profiling. The STR profiles matched the standards recommended for the authentication of the MG-63, 143B, U2OS and hFOB1.19 cell lines.

Semi-quantification of gene expression using western blot analysis. Total proteins extracted from human OS (MG-63, 143B and U2OS) and the normal human osteoblast hFOB1.19 cell line were harvested using ice-cold radioimmunoprecipitation lysis buffer (Thermo Fisher Scientific, Inc.) supplemented with phenylmethanesulfonyl fluoride for 1 h. Following the manufacturer's instructions, a bicinchoninic acid protein assay kit (Sigma-Aldrich; Merck KGaA) was used to determine the amount of protein. A total of 20 µg protein/lane was separated on 12% SDS-PAGE (Beyotime Biotechnology) and transferred onto PVDF membranes (MilliporeSigma; Merck KGaA). The membranes were blocked with 5% skimmed milk at room temperature for 1 h, and washed in Tris-buffered saline Tween-20 [150 mmol/l NaCl (pH 7.5), 20 mmol/l Tris-HCl and 0.1% Tween 20] at room temperature three times. Primary monoclonal antibodies against TLR4 (1:1,000; cat. no. BA1717; Wuhan Boster Biological Technology, Ltd.) and the loading control, GAPDH (1:10,000; cat. no. AB0037; Shanghai Abways Biotechnology Co., Ltd.) were incubated with the membranes

overnight at 4°C. Membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (1:10,000 dilution; cat. no. BE0101; bioeasy) for 1 h at room temperature. Protein bands were visualized using ECL reagent (cat. no. T4600; Tianneng Technologies) according to the manufacturer's instructions.

Detection of IFNG expression using ELISA. The levels of the anti-fibrotic factor IFNG in MG-63, 143B, U2OS and normal human osteoblasts were assessed using the Human Interferon γ ELISA Kit (cat. no. H0108c; Wuhan Elabscience Biotechnology Co., Ltd.) according to the manufacturer's instructions.

Statistical analysis. Statistical analyses were performed using GraphPad Prism (version 8.0; Dotmatics) or SPSS (version 24.0; IBM Corp.). Data are presented as the mean ± standard deviation of ≥3 independent experimental repeats. For linear correlation, Pearson's correlation coefficient was used. Comparisons between multiple groups were performed using one-way analysis of variance followed by Tukey's post hoc test. Overall survival was determined using K-M survival curves along with the log-rank test. P<0.05 was considered to indicate a statistically significant difference. For comparisons between two groups, statistical significance was assessed using the Mann-Whitney U test for non-normally distributed data (immune cell infiltration proportions and immune checkpoint gene expression). P<0.05 was considered to indicate a statistically significant difference.

Results

Immune subtypes identified in OS. A total of 87 patients with OS were included in the present study. ssGSEA was performed on each sample using gene sets comprised of mRNA transcripts specific to most subpopulations of both innate and adaptive immune cells. All tumor samples were categorized into k subtypes, with k-values of 2-9. Based on the consensus score of the cumulative distribution function curve, k=2 was determined to be the optimal number of subtypes (referred to as Immunity_H and Immunity_L; Fig. 1).

Immune features of the three immune subtypes. The ssGSEA clustering analysis indicated that the extent of immune infiltration was higher in the Immunity_H group than in the Immunity_L group (Fig. 2A). The overall survival cases in the Immunity_H group exhibited significantly lower tumor purity and higher ESTIMATE, stromal and immune scores compared with those in the Immunity_L group (Fig. 2A; P<0.005). Additionally, the expression of most human leukocyte antigens was significantly higher in the high immune cell infiltration cluster (Immunity_H group) compared with the low immune cell infiltration cluster (Immunity_L group). (P<0.05; Fig. 2B). The Immunity_H subtype showed significantly increased infiltration of CD8⁺ T cells, memory activated CD4⁺ T cells, M2 macrophages and M1 macrophages compared with the Immunity_L subtype (P<0.05; Fig. 2C). However, the Immunity_H subtype exhibited significantly lower infiltration of M0 macrophages and natural killer (NK) cells resting compared with the Immunity_L subtype (P<0.05;

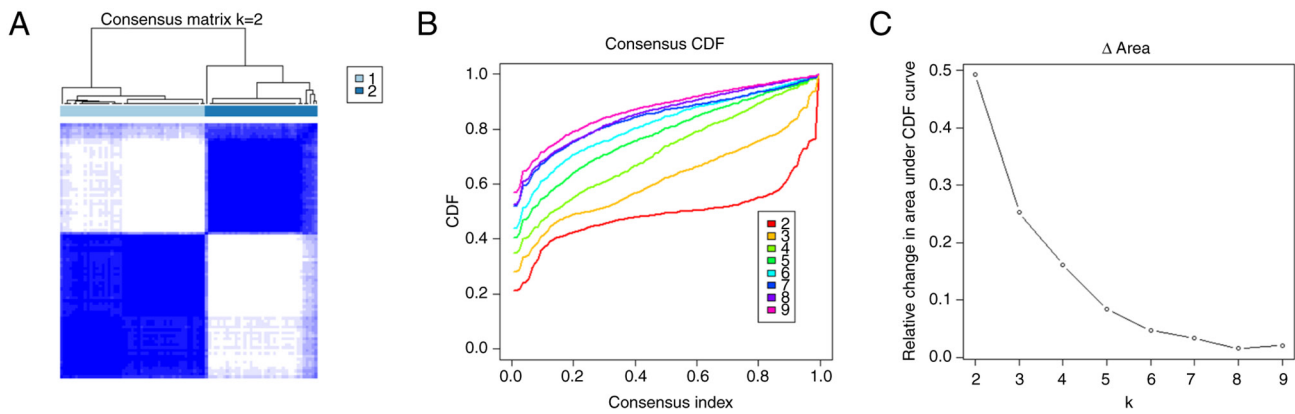


Figure 1. Consensus clustering of osteosarcoma. (A) The Therapeutically Applicable Research to Generate Effective Treatments database samples were divided into two distinct clusters when $k=2$. (B) Relative change in the area under the CDF curve for $k=2-9$, (C) Consensus clustering CDF curve for $k=2-9$. CDF, cumulative distribution function.

Fig. 2C). Furthermore, the expression of the following eight immune checkpoint genes associated with immune escape were assessed: lymphocyte-activation gene 3 (LAG3), V-set immunoregulatory receptor, CD274, hepatitis A virus cellular receptor 2 (HAVCR2), killer cell immunoglobulin like receptor (KIR)-two Ig domains and long cytoplasmic tail 1, killer cell lectin like receptor C1, 5'-nucleotidase ecto, cytotoxic T-lymphocyte associated protein 4 (CTLA4), T cell immunoreceptor with Ig and ITIM domains (TIGIT), KIR-three Ig domains and long cytoplasmic tail 2, KIR-two Ig domains and long cytoplasmic tail 3 and programmed cell death 1 (PDCD1). The expression levels of these genes were significantly higher in the Immunity_H group than in Immunity_L group ($P<0.05$; Fig. 2D). Finally, K-M analysis indicated that patients with OS in the Immunity_H cluster had a significantly improved survival rate compared with those in the Immunity_L cluster ($P=0.0171$; Fig. 2E).

Identification of DEGs and functional enrichment analyses.

The results revealed that Immunity_H represents an immune-infiltration cluster, whilst Immunity_L signifies a cluster with low immune cell infiltration. Consequently, differentially expressed immune infiltration-related genes were identified from both Immunity_H and Immunity_L groups. In total, 1,240 DEGs were identified, comprising 159 upregulated genes and 1,081 downregulated genes (Fig. 3).

Immune infiltration-related ferroptosis genes and functional enrichment analyses.

Immune infiltration-related genes that were differentially expressed were intersected with 283 ferroptosis genes. A total of six overlapping genes were screened for subsequent analyses (Fig. 4). Additional investigations were performed into the potential functions of the genes by employing GO and KEGG analyses. The GO terms were mainly enriched in positive regulation of the tumor necrosis factor biosynthetic process, reactive oxygen species metabolic process, regulation of tumor necrosis factor biosynthetic process and positive regulation of interleukin-12 production (Fig. 5A). Additionally, the KEGG pathway analysis (Fig. 5B) demonstrated that the hypoxia-inducible factor 1 signaling pathway, nucleotide-binding and oligomerization

domain-like receptor signaling pathway in cancer, PD-L1 expression and PD-1 checkpoint pathway were also enriched in the PD-L1-positive and toll-like receptor-signaling pathways (Fig. 5B). The results indicate that the inflammatory and immunological responses may be involved in the development of OS tumorigenesis.

Construction of the diagnostic and prognosis model based on two ferroptosis genes. UCR analysis was performed with OS prognosis as the dependent variable, and $P<0.05$ was considered to indicate a statistically significant difference. TLR4 was revealed to have a notable association with overall survival ($P<0.05$; Fig. 6A). Subsequently, Least Absolute Shrinkage and Selection Operator (LASSO) regression analysis identified two genes, IFNG and TLR4 (Fig. 6B and C). After which, a prognostic signature was developed based on the following formula: Risk score = $(-0.3623 \times \text{TLR4 expression value}) + (-0.8072 \times \text{IFNG expression value})$. Patients were stratified into high- ($n=43$) and low- ($n=44$) risk groups. The high-risk group exhibited a significantly worse prognosis compared with the low-risk group ($P=7.443 \times 10^{-3}$; Fig. 7A). Moreover, ROC curve analysis demonstrated that the Cox prediction model achieved an area under the curve (AUC) of 0.683 for survival prediction at 3 years (Fig. 7I), showing improved accuracy and suggesting moderate predictive capability. The riskScore plot, status plot and the expression of the two signature genes are presented in Fig. 7C, E and G. The GEO dataset was then used to assess the robustness of the model. Compared with patients in the low-risk group, patients in the higher risk group had a shorter survival period, which was in-line with the previous results ($P=7.443 \times 10^{-3}$; Fig. 7B); the AUC of ROC was 0.577 (Fig. 7J). The detailed risk scores, status plot and ferroptosis-related gene expression are presented in Fig. 7B, D, F and H.

Additionally, to assess whether the signature was independent of other clinical characteristics, UCR and MCR analyses were performed in two cohorts. UCR indicated that the metastasis status and risk score had a significant influence on prognosis in both datasets (Fig. 8A and B). In MCR analysis, the risk score derived from the signature was an independent prognostic factor in the TARGET cohort [hazard ratio (HR), 76.630; 95% confidence interval (CI), 5.448-1077.884;

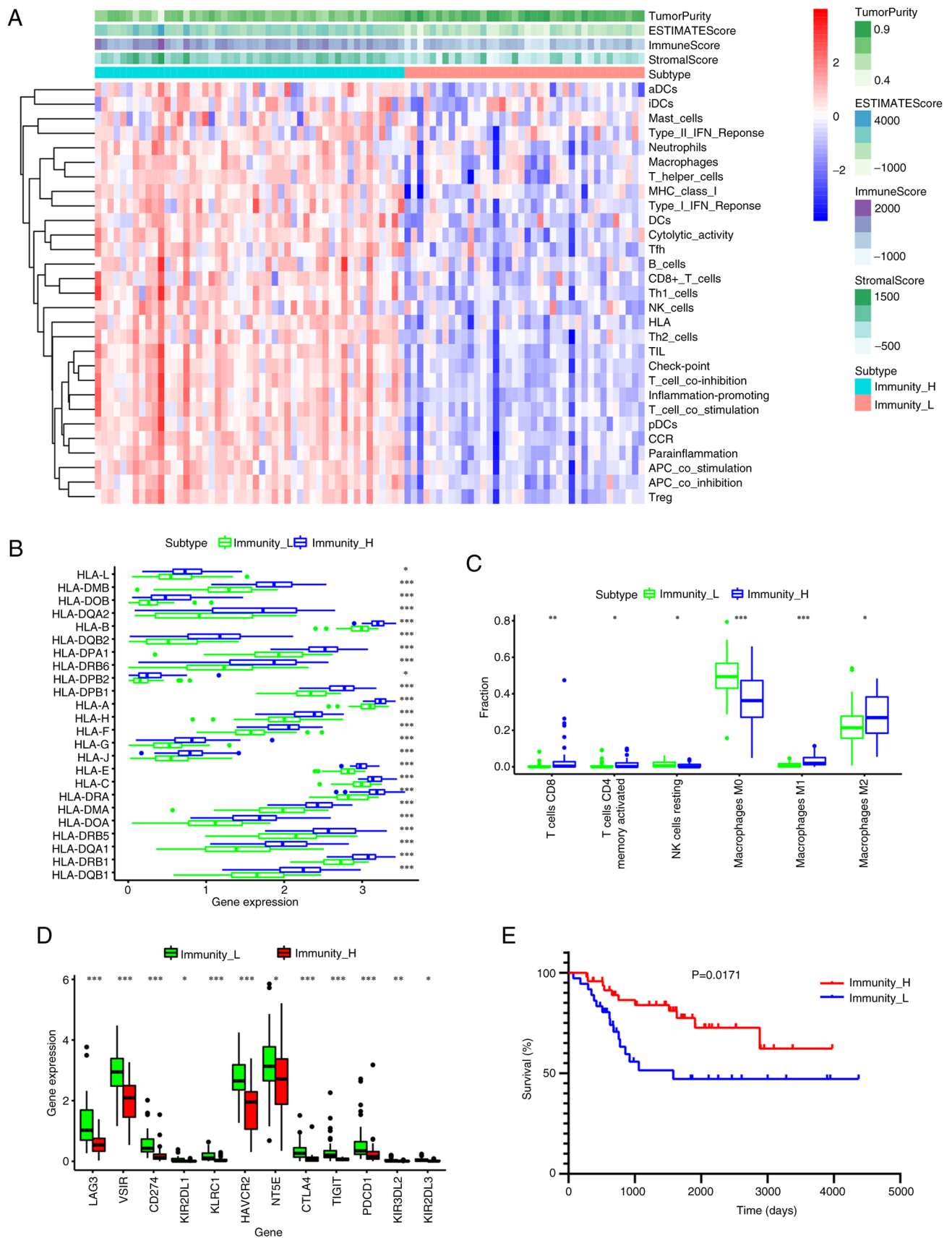


Figure 2. Determination of the two immune subtypes in osteosarcoma. (A) Heatmap of single-sample Gene Set Enrichment Analysis scores. (B) There was a significant difference in the expression of most HLAs between the high- and low-immune cell infiltration clusters. (C) Difference in the expression levels of infiltrating immune cells between the two clusters. (D) Difference in the expression levels of the immune checkpoint genes, LAG3, VSIR, CD274, HAVCR2, NT5E, CTLA4, TIGIT and PDCD1, among the two subtypes. (E) Patients in low immune cell infiltration cluster showed worse overall survival than those in high immune cell infiltration cluster. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$. LAG3, lymphocyte-activation gene 3; VSIR, V-set immunoregulatory receptor; HAVCR2, hepatitis A virus cellular receptor 2; NT5E, 5'-nucleotidase ecto; CTLA4, cytotoxic T-lymphocyte associated protein 4; TIGIT, T cell immunoreceptor with Ig and ITIM domains; PDCD1, programmed cell death 1.

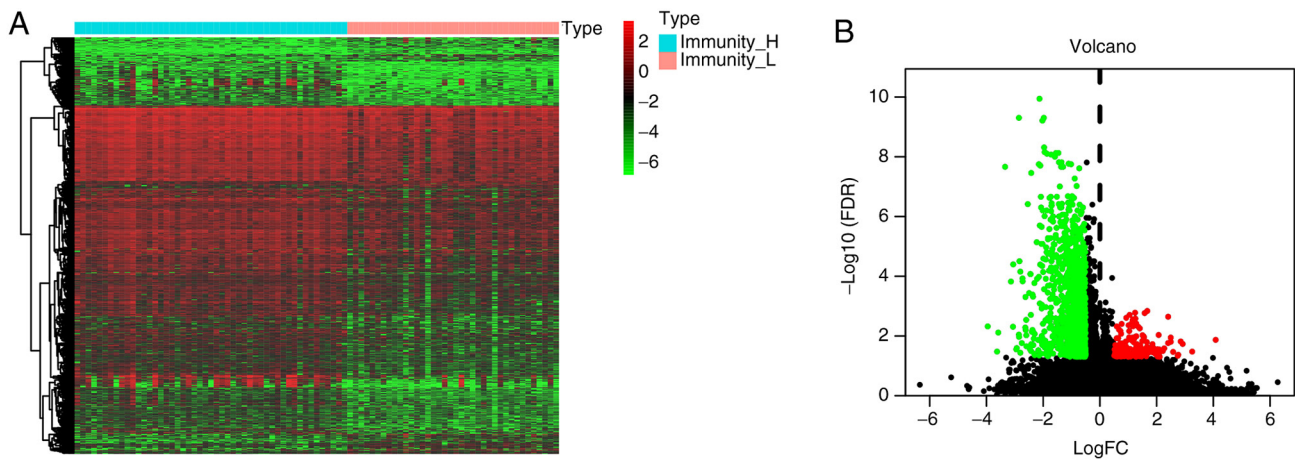


Figure 3. Determination of differentially expressed immune infiltration-related ferroptosis genes. (A) Heatmap(green, negative) Z-score; red color indicates a high (positive) Z-score; black, expression levels at or near the mean.) and (B) volcano plot of differentially expressed immune infiltration-related ferroptosis genes between the high- and low-immunity clusters. H, high; L, low; FC, fold change; FDR, false discovery rate.

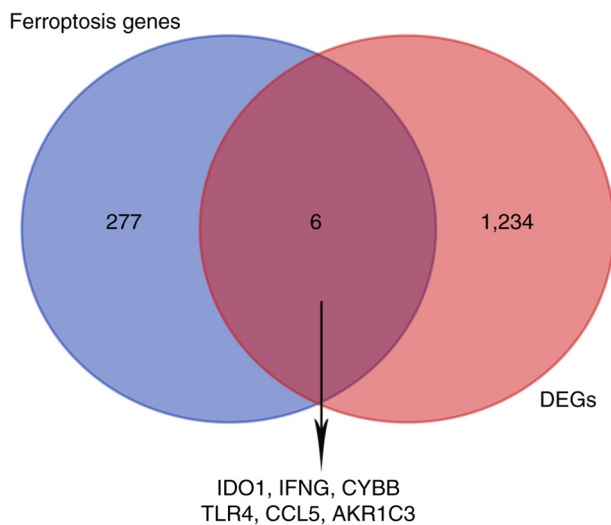


Figure 4. Overlap between immune infiltration-related DEGs and the ferroptosis gene set. DEGs, differentially expressed genes; IDO1, indoleamine 2,3-dioxygenase 1; IFNG, interferon γ ; CYBB, cytochrome B-245 β chain; TLR4, toll-like receptor 4; CCL5, CC motif chemokine ligand 5; AKR1C3, aldo-keto reductase family 1 member C3.

$P < 0.001$]. However, the risk score was not an independent prognostic factor in the GEO cohort (HR, 1.332; 95% CI, 0.406-4.368; $P = 0.636$; Fig. 8C and D); however, this may be due to the limited number of samples used ($n = 53$).

Assessment of the diverse tumor-infiltrating immune cell (TIIC) types between low- and high-risk cohorts. Using CIBERSORT, the immune infiltration percentage of each cell type was calculated using the gene matrix as a basis. In patients in the high-risk group, there was a significantly increased number of M0 macrophages ($P = 0.00074$; Fig. 9A). By contrast, the low-risk group exhibited significantly higher levels of M1 macrophages ($P = 0.006$; Fig. 9B) and M2 macrophages ($P = 0.017$; Fig. 9C). Furthermore, the expression of immune checkpoint genes in the low-risk group, including LAG3, CD274, CTLA4, HAVCR2, TIGIT, HAVCR2 and

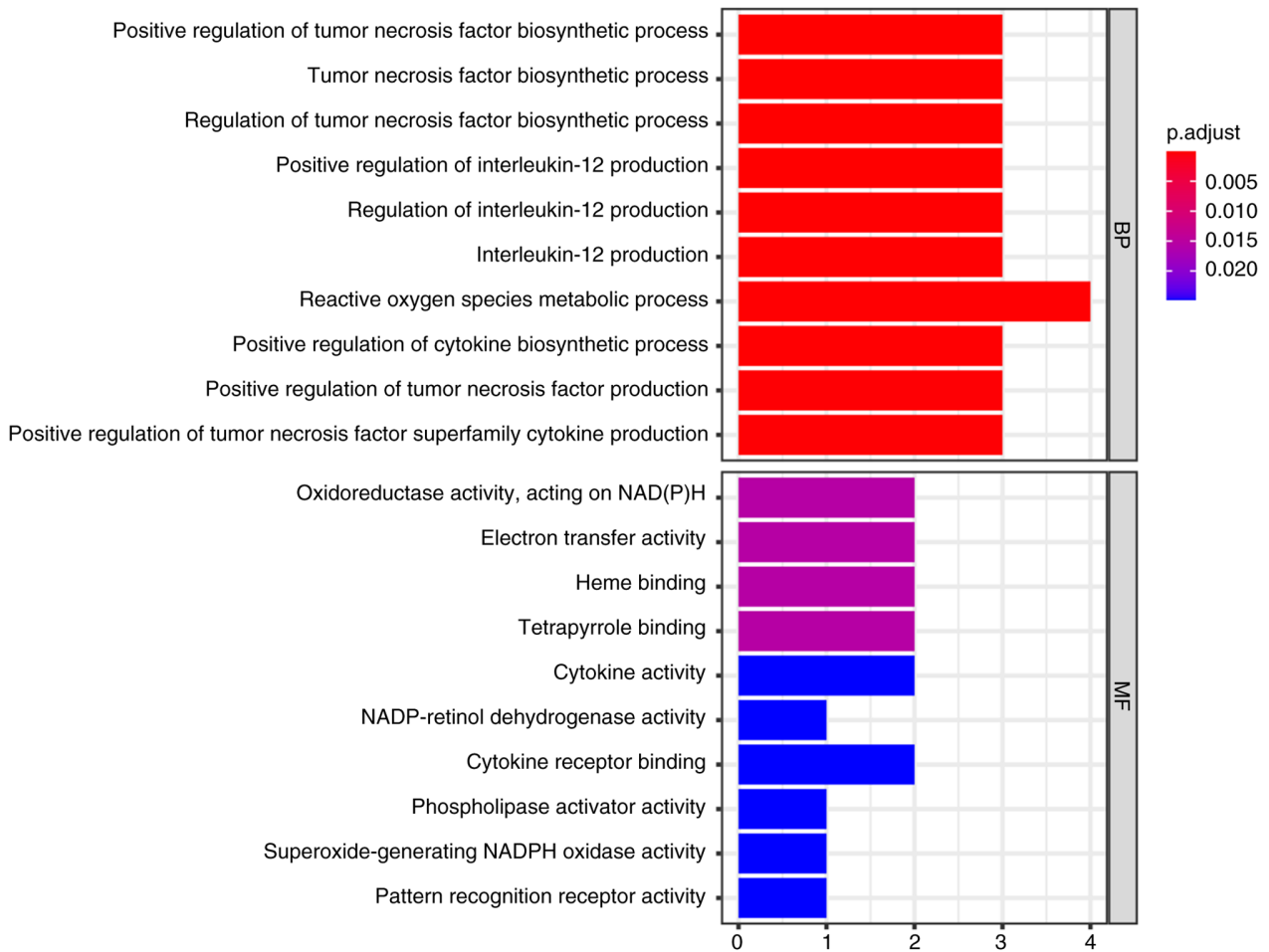
PDCD1 was higher than that in the high-risk group ($P < 0.05$; Fig. 9G). Additionally, consistent results were obtained using the GEO cohort (Fig. 9D-F and H; $P < 0.005$).

Drug sensitivity analysis of IFNG and TLR4. To assess the underlying correlation between IFNG and TLR4 gene expression and drug sensitivity in several human cancer cell lines from the CellMiner database, a correlation analysis was performed. TLR4 was demonstrated to be significantly correlated with the drug sensitivity of entinostat (correlation coefficient = 0.303; $P = 0.019$; Fig. 10A), lapatinib (correlation coefficient = -0.285; $P = 0.028$; Fig. 10B) and acetalax (correlation coefficient = -0.275; $P = 0.034$; Fig. 10C). Additionally, IFNG expression was significantly correlated with the drug sensitivity of decitabine (correlation coefficient = 0.343; $P = 0.007$; Fig. 10D), mitomycin (correlation coefficient = 0.308; $P = 0.017$; Fig. 10E) and cisplatin (correlation coefficient = 0.281; $P = 0.030$; Fig. 10F).

Association between risk and immune-related pathways. GSEA indicated that, compared with the low-risk group, the high-risk group was negatively associated with immune-related pathways, such as antigen processing and presentation, the Toll-like receptor signaling pathway and cytokine-cytokine receptor interaction (Fig. 11).

Validation of the expression level of immune infiltration-related ferroptosis genes (IFNG and TLR4) in hFOB1.19, MG-63, 143B and U2OS cell lines. To confirm that the two immune infiltration-related ferroptosis genes, IFNG and TLR4, are specifically expressed in OS cells, RT-qPCR was performed using the MG-63, 143B, U2OS and normal human osteoblast (hFOB1.19) cell lines. Both IFNG and TLR4 were expressed in MG-63, 143B and U2OS cells, with TLR4 expression significantly higher in these OS cell lines than in hFOB1.19 cells. By contrast, IFNG expression in the MG-63, 143B and U2OS cells was significantly lower than in hFOB1.19 cells than in the OS cell lines (Fig. 12). The same pattern was observed in the results of the western blot analysis and ELISA (Figs. 13 and 14). Consequently, this cellular

A



B

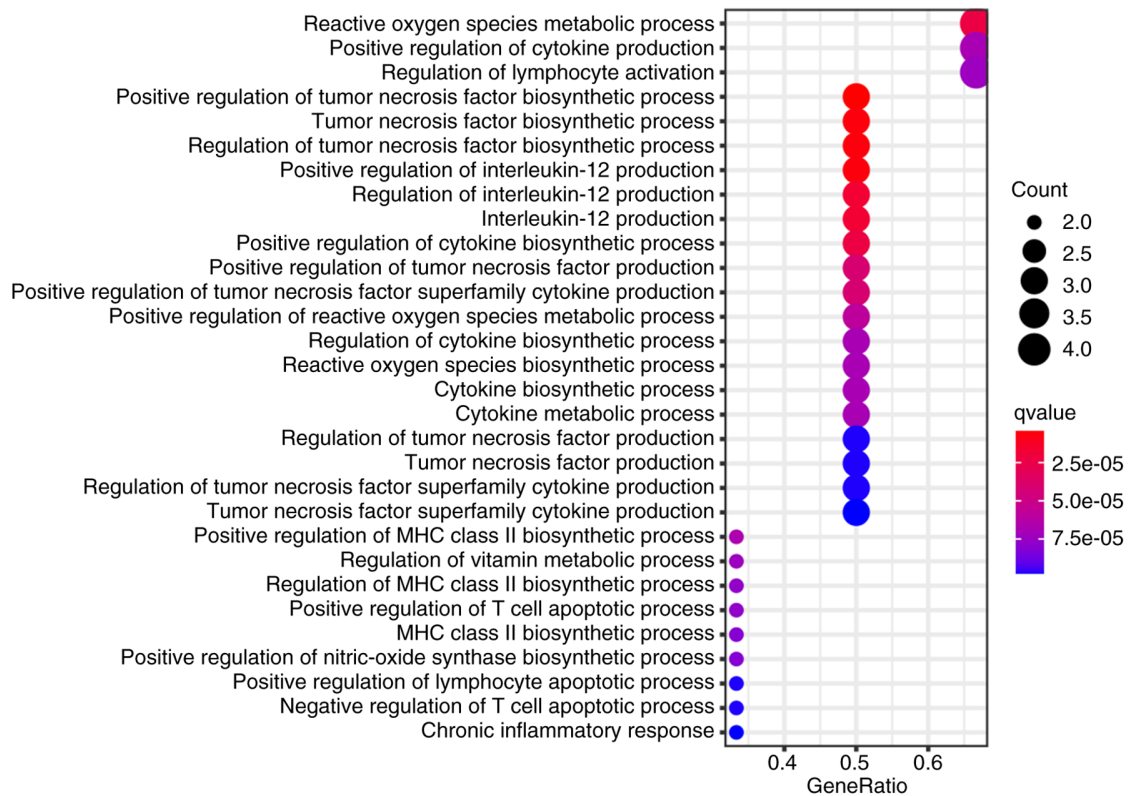


Figure 5. GO and KEGG enrichment analysis for the differentially expressed immune infiltration-related ferroptosis genes in osteosarcoma. (A) GO enrichment result. (B) KEGG enrichment result. GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

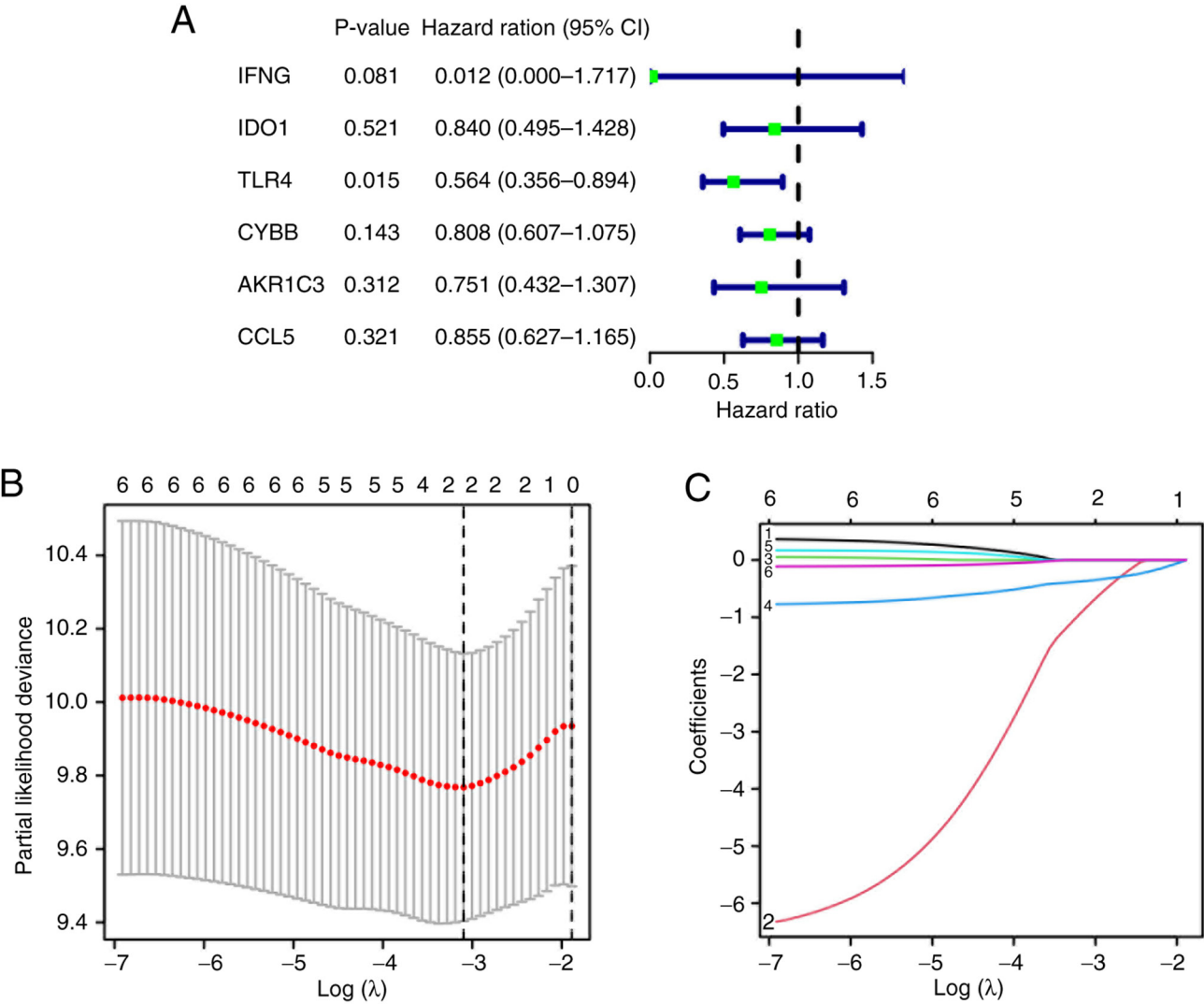


Figure 6. Development of the immune infiltration-related ferroptosis genes prognostic signature. (A) Univariable Cox regression analysis of selected genes. (B) Optimal values of the penalty parameter, defined by 1,000-round cross-validation. (C) Least Absolute Shrinkage and Selection Operator Cox analysis identified two genes associated with prognosis. IDO1, indoleamine 2,3-dioxygenase 1; IFNG, interferon γ ; CYBB, cytochrome B-245 β chain; TLR4, toll-like receptor 4; CCL5, CC motif chemokine ligand 5; AKR1C3, aldo-keto reductase family 1 member C3.

experiment corroborates the reliability of the bioinformatics analysis results.

Discussion

Human OS, a primary malignant bone tumor, is most prevalent during adolescence and in children (22). With the rapid development of molecular biology technology, there has been a growing interest in anticancer immunotherapies, including immune modulators, immune checkpoint inhibitors and genetically modified T cells (23,24). Patients with OS lacking immune cell infiltration present with high rates of metastasis and poor clinical outcomes (25). Notably, immune reconstitution has been reported to suppress OS recurrence and improve survival in metastatic OS (26).

Recent studies have reported that ferroptosis-related genes exhibit cell-type-specific expression in immune cells, directly influencing their functional roles. TLR4 was reported to be predominantly expressed in myeloid cells (macrophages and dendritic cells). Moreover, its upregulation

in M2 macrophages was reported to promote iron retention via *ferritin heavy chain 1* and suppress lipid peroxidation, enhancing anti-inflammatory functions (27,28). IFNG is secreted by activated CD8⁺ T cells and NK cells. It induces tumor ferroptosis by downregulating the solute carrier family 7 member 11/glutathione peroxidase 4 pathway whilst upregulating *acyl-CoA synthetase long-chain family member 4 (ACSL4)* (29,30). In OS, TLR4 likely stabilizes iron homeostasis in stromal macrophages, whilst IFNG from infiltrating T cells sensitizes tumor cells to ferroptosis. This spatial crosstalk creates a feed-forward loop where ferroptotic cells release damage-associated molecular patterns [DAMPs; such as high-mobility group box 1 (HMGB1)], further recruiting dendritic cells and amplifying antitumor immunity (30–32).

Based on the immune signature score, patients with OS were categorized into two clusters. Significant differences were observed in tumor purity, ESTIMATE scores, stromal scores, immune scores and the expression of immune checkpoint markers between the Immunity_H and Immunity_L

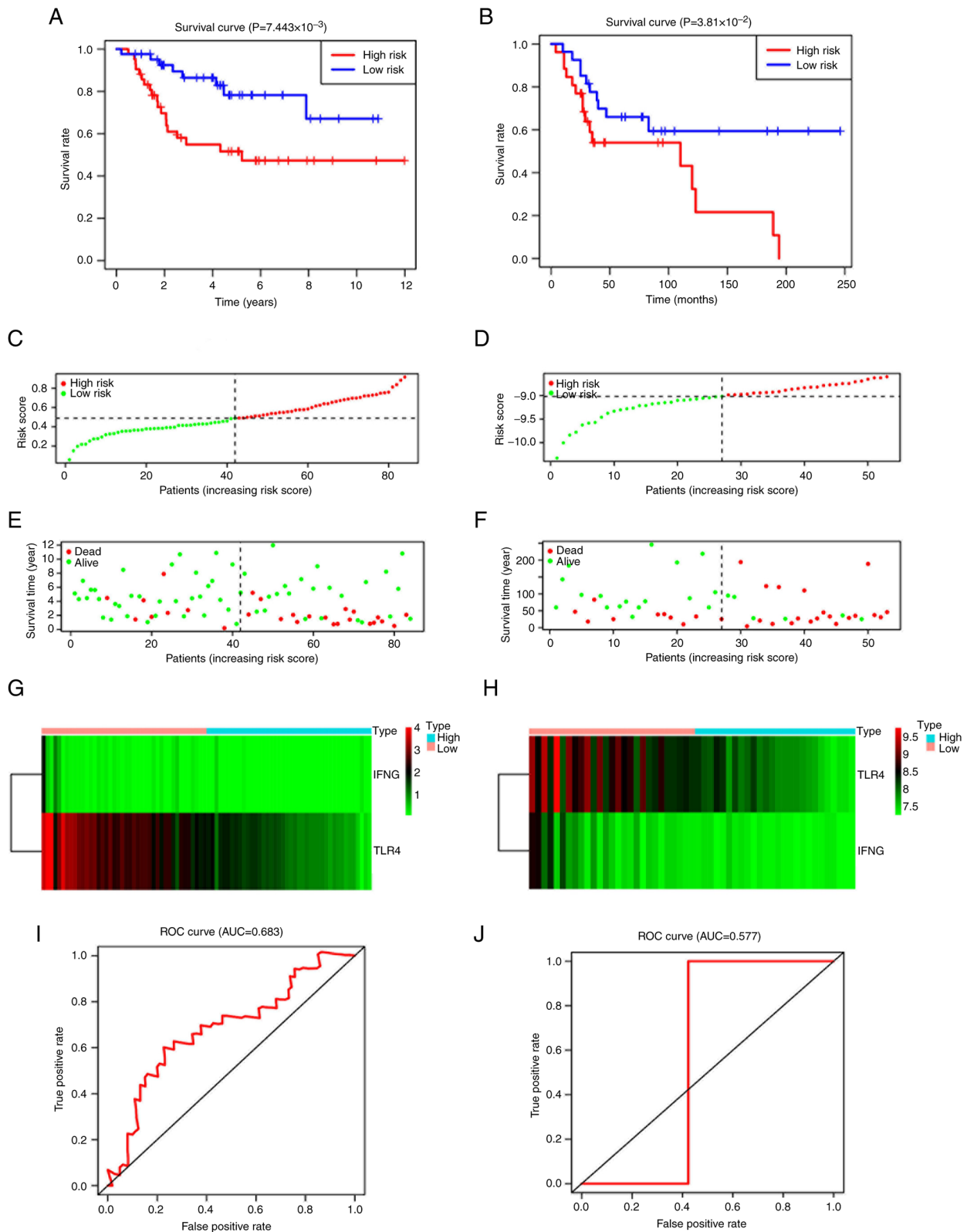


Figure 7. Robustness of the two-gene signature model. Kaplan-Meier survival curves of patients with OS in the high- and low-risk cohorts in the (A) TARGET and (B) GEO datasets. Risk score distribution in the (C) TARGET and (D) GEO datasets. Survival status of patients with OS in the (E) TARGET and (F) GEO datasets. Heatmap of the two signature genes in the (G) TARGET and (H) GEO datasets. ROC curve for overall survival of patients with OS in the (I) TARGET and (J) GEO datasets. OS, osteosarcoma; TARGET, Therapeutically Applicable Research to Generate Effective Treatments; GEO, Gene Expression Omnibus; ROC, receiver operating characteristic; AUC, area under the curve; IFNG, interferon γ ; TLR4, toll-like receptor 4.

groups. Patients in the Immunity_H group exhibited greater immune infiltration compared with those in the Immunity_L group.

A prognosis model incorporating two ferroptosis-related genes was utilized to develop a risk model for predicting the overall survival of patients with OS. The risk score was then

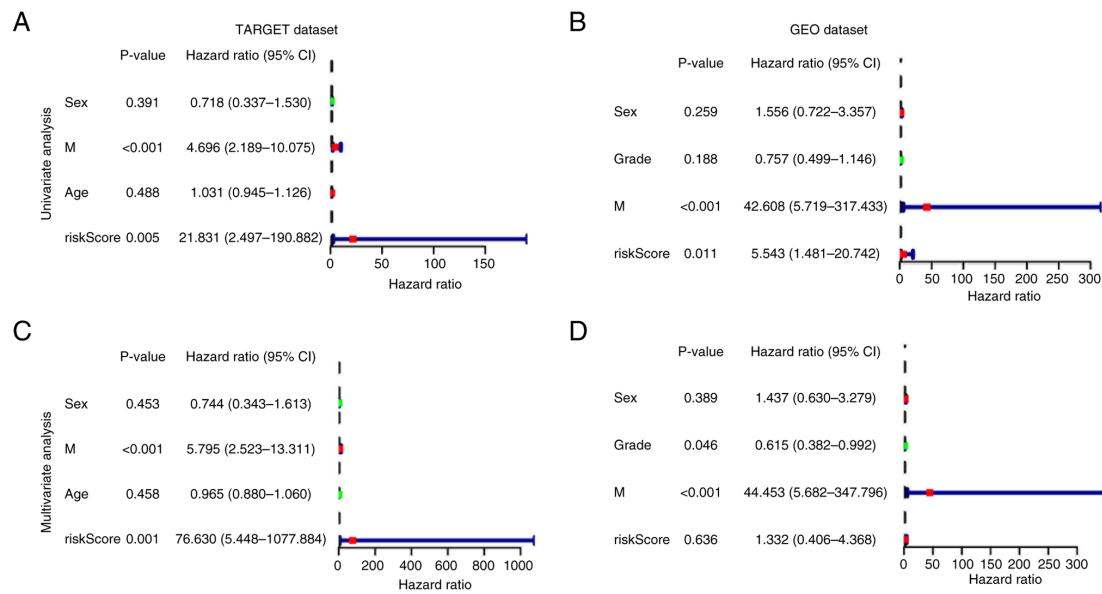


Figure 8. Univariate and multivariate Cox analyses of clinicopathological factors associated with osteosarcoma prognosis. Univariate Cox analysis of risk score, sex, age and metastasis using the (A) TARGET and (B) GEO datasets. Multivariate Cox analysis using the (C) TARGET and (D) GEO datasets. TARGET, Therapeutically Applicable Research to Generate Effective Treatments; GEO, Gene Expression Omnibus; M, metastasis.

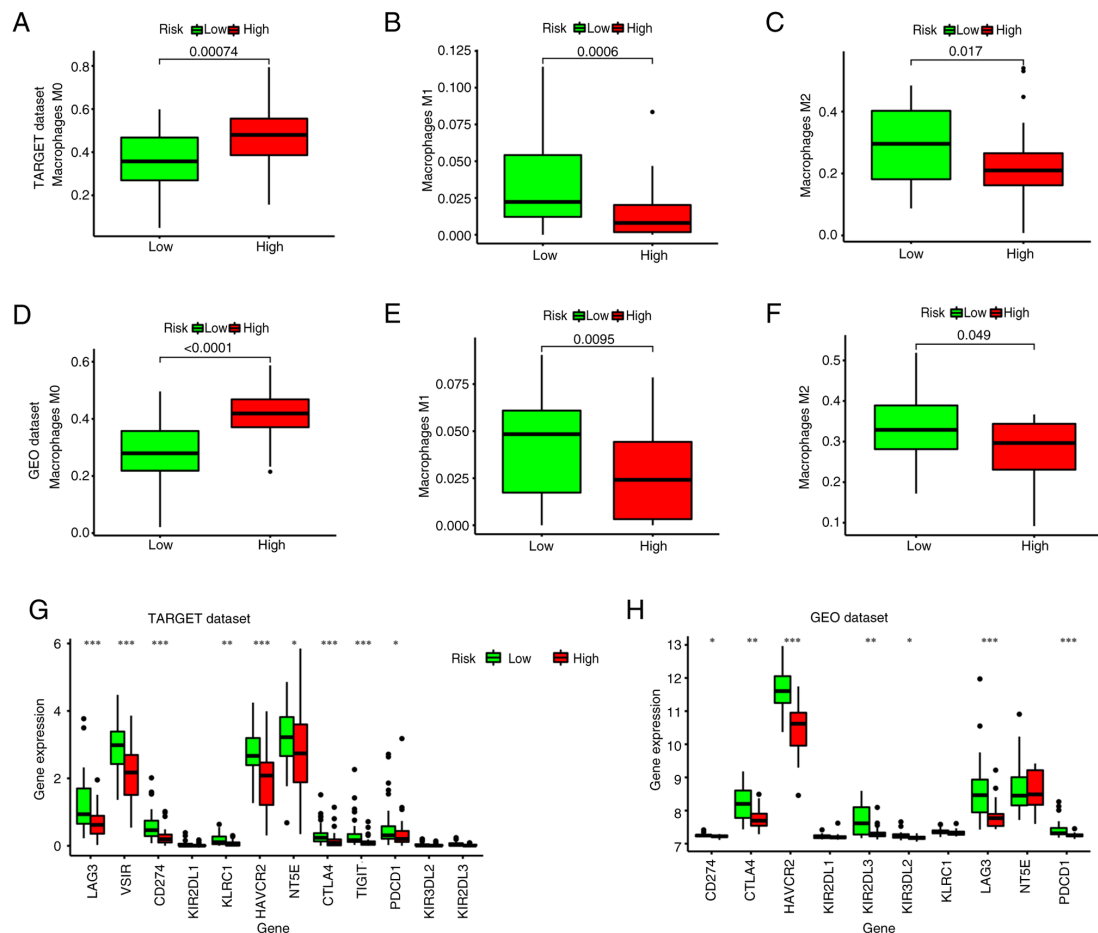


Figure 9. Analysis of immune cell infiltration in patients with osteosarcoma. Differences in the number of (A) M0, (B) M1 and (C) M2 macrophages between the high- and low-risk groups in the TARGET dataset. Differences in the number of (D) M0, (E) M1 and (F) M2 macrophages between the high- and low-risk groups in the GEO dataset. Differences in the expression of immune checkpoint genes among the high- and low-risk groups in the (G) TARGET and (H) GEO datasets. *P<0.05; **P<0.01; ***P<0.005. TARGET, Therapeutically Applicable Research to Generate Effective Treatments; GEO, Gene Expression Omnibus; LAG3, lymphocyte-activation gene 3; VSIR, V-set immunoregulatory receptor; KIR2DL1, killer cell Immunoglobulin-like Receptor, two Ig domains, Long cytoplasmic tail, inhibitory receptor 1; KLRC1, Killer cell Lectin-like Receptor subfamily C member 1; HAVCR2, hepatitis A virus cellular receptor 2; NT5E, 5'-nucleotidase ecto; CTLA4, cytotoxic T-lymphocyte associated protein 4; TIGIT, T cell immunoreceptor with Ig and ITIM domains; PDCD1, programmed cell death 1.

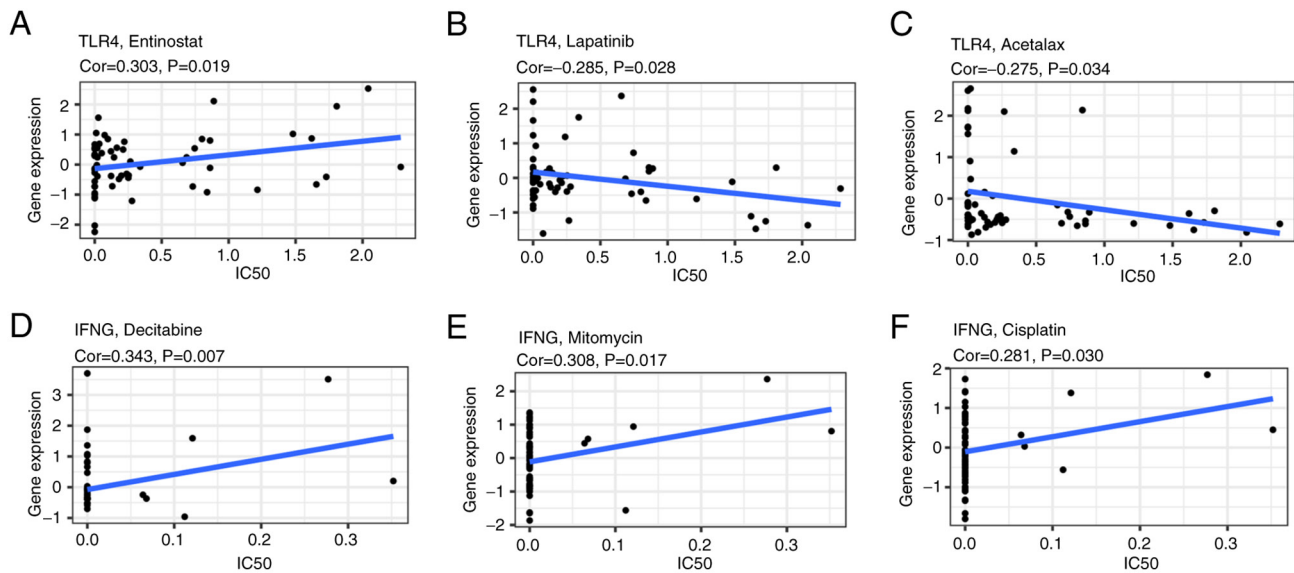


Figure 10. Drug sensitivity analysis of two signature genes expression. Correlation between TLR4 expression and the drug sensitivity of (A) Entinostat, (B) Lapatinib and (C) Acetaxol. Correlation between IFNG expression and the drug sensitivity of (D) Decitabine, (E) Mitomycin and (F) Cisplatin. TLR4, toll-like receptor 4; IFNG, interferon γ ; cor, correlation.

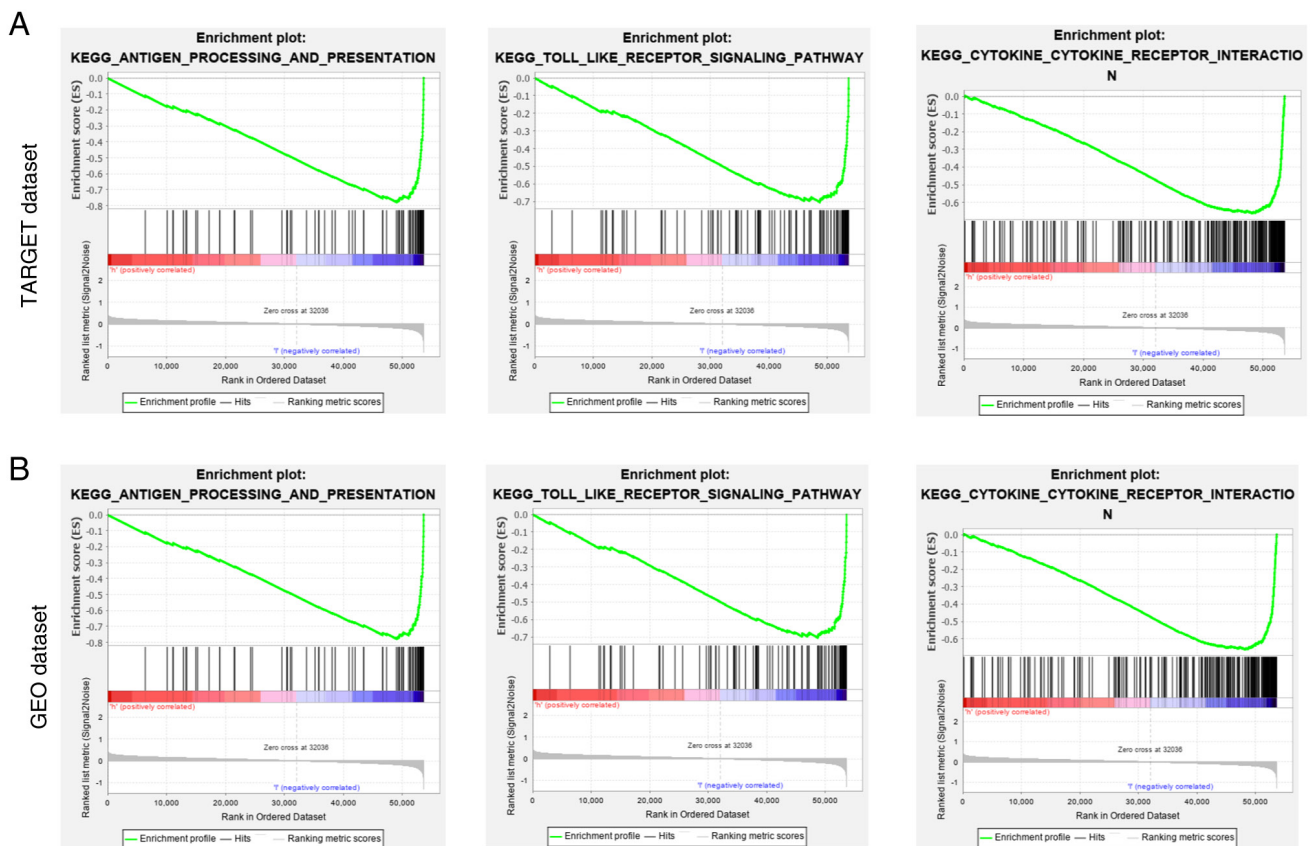


Figure 11. Gene set enrichment analysis of the high- and low-risk groups. (A) TARGET and (B) GEO datasets. TARGET, Therapeutically Applicable Research to Generate Effective Treatments; GEO, Gene Expression Omnibus; KEGG, Kyoto Encyclopedia of Genes and Genomes.

used to classify patients into two distinct risk groups. The reliability of the identified immune subtypes (Immunity_H/L) was supported by the internal consensus clustering stability within the TARGET cohort (Fig. 1). Whilst the identical immunotyping procedure was not replicated on the external GEO

validation cohort due to platform heterogeneity, the successful validation of the downstream immunotype-derived ferroptosis prognostic model (IFNG/TLR4 risk score) in this independent dataset (Fig. 7F-J) provides indirect support for the biological relevance captured by the initial subtyping approach.

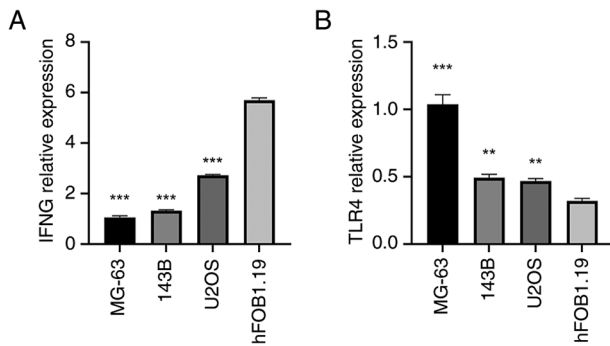


Figure 12. Reverse transcription-quantitative PCR analysis. Relative expression levels of (A) IFNG and (B) TLR4 in hFOB1.19, MG-63, 143B and U2OS cells. ** $P < 0.01$ and *** $P < 0.001$ vs. hFOB1.19 cells. TLR4, toll-like receptor 4; IFNG, interferon γ .

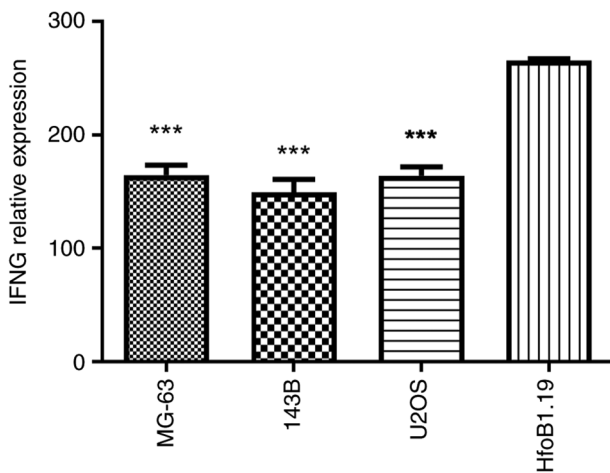


Figure 13. ELISA was used to determine the relative IFNG expression levels in hFOB1.19, MG-63, 143B and U2OS cells. *** $P < 0.001$ vs. hFOB1.19 cells. IFNG, interferon γ .

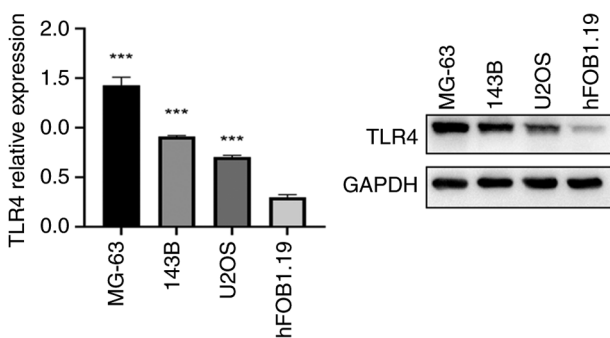


Figure 14. Western blot analysis was used to determine the relative TLR4 expression levels in hFOB1.19, MG-63, 143B and U2OS cells. *** $P < 0.001$ vs. hFOB1.19 cells. TLR4, toll-like receptor 4.

Furthermore, the K-M curve, score plot, survival status plot and ROC curve indicated that the two immune-related risk levels had a favorable predictive potential for prognosis.

Notably, the multivariate Cox regression analysis, incorporating clinical factors such as age, sex and metastasis status, demonstrated that the ferroptosis-based risk score remained

an independent prognostic predictor in the primary cohort (TARGET), suggesting its value beyond these potential confounders. However, in the smaller GEO cohort, the risk score did not reach independent significance in the multivariate model ($P = 0.636$), which may be due to the limited sample size ($n = 53$).

To address how ferroptosis influences the tumor immune microenvironment, the following mechanistic insights are proposed based on recent studies: i) Ferroptosis modulates myeloid-derived suppressor cells (MDSCs) and T-cell activity; Lnk deficiency in MDSCs triggers ferroptosis via the FMS-like tyrosine kinase 3/STAT1/interferon regulatory factor-1/arachidonate (12S)-lipoxygenase axis, reducing Arginase-1 expression and increasing TNF- α , thereby weakening MDSC-mediated immunosuppression (33). This aligns with the results of the Immunity_H cluster (enriched in CD8⁺ T cells/M1 macrophages) and improved survival rates; ii) with a dual role in T-cell function, CD8⁺ T cells promote tumor ferroptosis via IFN- γ -mediated xCT suppression, but are themselves vulnerable to ferroptosis due to ROS accumulation (34,35). This may explain the protective role of IFNG in the model in the present study, and its downregulation in OS cells; iii) macrophage polarization: M1 macrophages exhibit ferroptosis sensitivity via *ACSL4*, whilst M2 macrophages resist it via *ferroptosis suppressor protein 1* (35). The elevated M1/M2 infiltration in the low-risk group in the present study may reflect ferroptosis-driven plasticity, supported by enriched HIF-1/TLR pathways in KEGG; iv) immunogenic cell death: Ferroptotic cells release DAMPs (such as HMGB1) that activate dendritic cells and T-cell priming (36,37). This may have driven the compensatory immune checkpoint upregulation (PD-L1/CTLA4/LAG3) in the low-risk group; and v) TLR4/IFNG bridge ferroptosis and immunity: TLR4 inhibits ferroptosis via ROS/p38-MAPK suppression, potentially explaining its high-risk association. Conversely, IFNG enhances tumor ferroptosis but risks T-cell depletion (38), creating a feedback loop evident in the cellular data in the present study.

IFNG was excessively expressed in the low-risk group. Moreover, the circulating concentrations of IFNG have been reported to be markedly associated with overall survival in patients with OS (39). Furthermore, IFNG serves a crucial role in the antitumor immune response by enhancing antigen presentation, T-lymphocyte differentiation and maturation across several cancers (40). For example, IFN- γ , encoded by the IFNG gene, acts on tumor cells, enhancing their recognition by CD8⁺ T cells as well as by CD4⁺ T cells. IFN- γ can promote tumor cell apoptosis by upregulating the expression of caspase-1, -3 and -8. An important aspect is the ability of IFN- γ to induce PD-L1 expression in cancer, stromal and myeloid cells, which impairs effector tumor immunity (41,42). The aforementioned research indicates that IFNG is involved in tumor immunity, which aligns with the findings of the present study.

TLR4 is known for its function in recognizing pathogen-associated antigens and activating the production of pro-inflammatory factors (43). It has previously been reported to serve a role in lung tumorigenesis induced by chemically induced pulmonary inflammation (44). Furthermore, the inhibition of TLR4 has been reported to mitigate oxygen-glucose

deprivation-induced ferroptosis by suppressing oxidative stress and p38-MAPK signaling, ultimately enhancing neuronal cell viability (38). Moreover, the present study investigated the association between IFNG/TLR4 expression and drug sensitivity using publicly available pharmacogenomic databases (GDSC or CTRP). Spearman correlation analysis revealed that higher IFNG expression was significantly associated with increased sensitivity to entinostat ($r=0.32$, $P<0.05$) and decitabine ($r=0.28$, $P<0.05$), both epigenetic modulators. Conversely, elevated TLR4 expression correlated with resistance to cisplatin ($r=-0.35$, $P<0.05$), a conventional chemotherapeutic agent commonly used in OS treatment. These findings suggest that our ferroptosis-based risk signature may not only have prognostic value but also potential implications for predicting treatment response.

Considering the pivotal role of TIICs in the progression of cancers, the diverse types of TIICs were explored between low- and high-risk cohorts in the present study. A previous investigation reported that resting memory CD4⁺ T cells are among the most enriched TIICs in gastric cancer samples (45). In addition, studies have reported the presence of follicular helper T cells in tertiary lymphoid structures of numerous tumors, suggesting that they serve a role in generating effective and sustained antitumor immune responses (46). Macrophages are considered to serve as both antitumor agents (M1 macrophages) and protumor agents (M2 macrophages) (47). In the present study, patients in the high-risk group exhibited an elevated level of M0 macrophages. By contrast, patients in the low-risk group showed increased proportions of both M0 and M2 macrophages. Immune checkpoint molecules, including CD274, CTLA4 and LAG3, have been demonstrated to serve a role in tumor immunology (48). The targeted blockade of two molecules, CD274 and CTLA-4, resulted in clinical benefits for individuals with several solid tumors (49,50). The present study revealed that the expression of immune checkpoint genes in the low-risk group was higher than in the high-risk group. Furthermore, GSEA based on RNA-Seq data revealed that the risk score was associated with the antigen processing and presentation pathway, the toll-like receptor signaling pathway and the cytokine-cytokine receptor interaction. In the tumor microenvironment, numerous chemokines are released to facilitate the migration of immune cells, thereby mediating the balance between protumor and antitumor responses (51). The toll-like receptor pathway was originally recognized as a critical mediator regulating immune responses (52). Therefore, the toll-like receptor signaling pathway could potentially be used to predict responses to immunotherapy. In summary, the results of the present study indicate that a high risk of mortality (poor overall survival), as predicted by ferroptosis-based prognostic signature, may influence immune infiltration-related biological processes.

Several limitations of the present study warrant consideration. Firstly, the analysis was retrospective and relied on publicly available datasets (TARGET and GEO). Whilst the prognostic model was validated in an independent cohort (GEO), the inherent biases and unmeasured confounders associated with retrospective data cannot be fully eliminated. Prospective validation in larger, uniformly treated cohorts is essential. Secondly, data heterogeneity between the TARGET (RNA-seq) and GEO (microarray) datasets,

including differences in platform technology, normalization methods and sample processing, poses challenges for direct integration and validation. Mitigation of this was attempted using standardized methods (such as ssGSEA, CIBERSORT and consistent bioinformatic pipelines) and focusing on relative risk stratification rather than absolute expression cut-offs. However, this heterogeneity likely contributed to the reduced statistical significance of the risk score as an independent prognostic factor in the smaller GEO cohort during MCR (Fig. 8D). Thirdly, the statistical models have inherent limitations. The LASSO Cox regression used for model construction helps prevent overfitting but may not capture all relevant biological complexity. The sample size, particularly for subgroup analyses and the GEO validation cohort, limits statistical power. Furthermore, whilst standard significance thresholds ($P<0.05$) were used for certain comparative analyses (such as immune cell infiltration and checkpoint expression), formal adjustment for multiple testing was not applied in all exploratory comparisons, potentially increasing the risk of Type I errors. Fourthly, whilst the cellular experiments demonstrated differential expression of IFNG and TLR4 in OS cell lines compared with in normal osteoblasts (Figs. 12-14), these *in vitro* models cannot fully recapitulate the complexity of the *in vivo* tumor microenvironment and its immune interactions. Therefore, functional studies are needed to definitively establish the causal roles of these genes in modulating ferroptosis and immune infiltration within OS. Finally, the precise mechanistic links between ferroptosis (as defined by the specific genes IFNG and TLR4) and the observed immune cell profiles remain correlative based on the bioinformatic and cellular expression data. Dedicated experiments investigating how modulation of IFNG or TLR4 affects ferroptosis sensitivity and immune cell behavior in OS models are crucial next steps.

In conclusion, in the present study, two immune subtypes in OS (Immunity_H and Immunity_L) with differing immune infiltration and patient outcomes were identified. A prognostic model based on ferroptosis genes (IFNG and TLR4) stratified patients into high- and low-risk groups, where high-risk was characterized by increased M0 macrophages and reduced immune checkpoint expression. The findings highlight the interplay between ferroptosis and immune regulation in OS. Future studies should validate these genes as therapeutic targets and explore their mechanisms in larger cohorts and preclinical models.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

LRZ and GY conceived and designed the study. LRZ collected data and wrote the manuscript. GHZ and HBM analyzed data and constructed the figures. GY revised the manuscript. LRZ and GY confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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