

Original Article

Single-cell analysis identifies a proliferative osteoblastic subpopulation and machine learning-derived prognostic signatures in osteosarcoma

Jianzhi Wang^{1*}, Qiaoling Luo^{2*}, Peiliang Xie³, Yihao Liu⁴, Haoling Zhang⁵, Wenjun Meng⁶, Tao Deng⁷

¹Department of Orthopedic Surgery and Orthopedic Research Institute, West China Hospital, Sichuan University, Chengdu 610041, Sichuan, China; ²Central Sterile Supply Department, West China Hospital, Sichuan University/West China School of Nursing, Sichuan University, Chengdu 610041, Sichuan, China; ³West China School of Medicine, Sichuan University, Chengdu 610041, Sichuan, China; ⁴Section of Esophageal and Mediastinal Oncology, Department of Thoracic Surgery, National Cancer Center/National Clinical Research Center for Cancer/Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100021, China; ⁵Department of Biomedical Sciences, Advanced Medical and Dental Institute, Universiti Sains Malaysia, Jalan Tun Hamdan Sheikh Tahir, 13200 Kepala Batas, Pulau Pinang, Malaysia; ⁶Department of Pain Management, West China Hospital, Sichuan University, No. 37 Guoxue Alley, Wuhou District, Chengdu 610041, Sichuan, China; ⁷The Fourth Stationed OPD, The General Hospital of Western Theater Command, Chengdu 610036, Sichuan, China.
*Equal contributors.

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Abstract: Osteosarcoma (OS) shows marked cellular heterogeneity, and patients often have very different clinical outcomes despite similar clinicopathological features. This variability remains difficult to explain using conventional risk factors alone. Here, we combined single-cell transcriptomic analysis, bulk transcriptomic profiling and machine-learning modeling to identify prognostic signatures related to tumor proliferation and the immune microenvironment. Single-cell RNA sequencing data from GSE152048 (n = 11) were used to map the cellular composition of OS. Within the osteoblastic compartment, we identified a proliferative subpopulation enriched for chromosome segregation, mitotic division and microtubule-related processes. We then used the TCGA-SARC cohort (n = 261) for model development and the GSE39058 osteosarcoma cohort (n = 41) for external validation. Weighted gene co-expression network analysis, differential expression analysis and univariate Cox regression were used to screen genes associated with immune features, proliferative activity and prognosis. These genes were defined as immune-proliferation-related signatures (IPRS). Among 117 machine-learning models, the random survival forest model performed best overall, with a 10-fold cross-validated C-index of 0.755 in the TCGA training cohort, an external validation C-index of 0.782 in GSE39058 and an average C-index of 0.769. The IPRS score separated patients into distinct prognostic groups and remained independently associated with overall survival in multivariate Cox analysis. It was also related to immune and stromal scores, immune-cell infiltration patterns and predicted drug sensitivity. We further selected ALG6 for experimental validation. Knockdown of ALG6 reduced proliferation and colony formation and increased apoptosis in 143B and U2OS cells. Overall, these results support the prognostic relevance of IPRS in osteosarcoma and suggest that ALG6 warrants further mechanistic investigation.

Keywords: Osteosarcoma (OS), multi-omics, single-cell RNA sequencing, immune-proliferation-related signatures, machine learning, prognostic model, ALG6

Introduction

Osteosarcoma (OS) is a relatively uncommon but highly aggressive primary malignant bone tumor [1]. It mainly affects children, adolescents and young adults [2]. With the use of neo-

adjuvant chemotherapy, wide surgical resection and postoperative adjuvant chemotherapy, the prognosis of patients with localized disease has improved. This benefit, however, is not shared by all patients. Some patients already have pulmonary metastasis at diagnosis,

whereas others develop relapse, distant metastasis or chemoresistance after treatment [3, 4]. Long-term survival in these settings remains unsatisfactory. At present, risk assessment for OS still depends largely on clinicopathological factors, such as tumor stage, metastatic status, tumor necrosis rate and surgical margin status. These variables are useful, but they do not fully explain why patients with similar clinical features may have very different treatment responses and survival outcomes [5]. A better understanding of the cellular basis of OS progression, together with more stable and interpretable molecular prognostic tools, is therefore needed for risk stratification and individualized treatment.

OS is highly heterogeneous at both the cellular and molecular levels. Tumor tissues contain malignant osteoblastic cells at different differentiation states, along with immune cells, fibroblasts, endothelial cells and other stromal components [6-8]. These cell populations interact within a complex tumor microenvironment and contribute to tumor growth, invasion, immune escape and therapeutic resistance [9, 10]. Proliferation is one of the central biological features of OS progression. Dysregulated cell-cycle control, abnormal mitosis and chromosomal instability may promote tumor-cell expansion and increase the chance of metastatic or drug-resistant clone formation. Proliferation, however, is not an isolated process. Rapidly dividing tumor cells may also reshape the local microenvironment through nutrient competition, cytokine secretion and immune-modulatory signaling [11].

The immune microenvironment of OS has attracted increasing attention because of its potential relevance to immunotherapy [2, 12]. Programmed cell death protein 1 (PD-1) or programmed death-ligand 1 (PD-L1) blockade can relieve suppression of T-cell function, while chimeric antigen receptor T (CAR-T) cells, tumor vaccines and other adoptive cellular therapies are designed to enhance immune recognition and tumor-cell killing. In clinical practice, single-agent immunotherapy has shown limited efficacy in OS. Even so, combinations with anti-angiogenic agents, chemotherapy or dual immune checkpoint blockade have shown disease-control potential in selected patients

with recurrent or metastatic disease [13, 14]. Treatment response is likely influenced not only by the therapeutic strategy itself, but also by the composition and functional state of the tumor immune microenvironment. Tumor-associated macrophages, T cells, natural killer cells, dendritic cells and myeloid-derived suppressor cells can all shape immune responses and affect OS progression, either through tumor-promoting or antitumor effects [15]. Immune and stromal cells may also regulate tumor-cell proliferation, invasiveness and drug sensitivity through cytokines, chemokines and direct intercellular communication [2, 16].

For this reason, evaluating OS only from the perspective of cell-cycle activity or immune infiltration may miss part of its biological complexity. Considering tumor proliferative state and immune contexture together may provide a more informative way to identify patients with distinct clinical risks and potential therapeutic vulnerabilities. In the present study, we first used single-cell transcriptomic data to map the cellular composition of OS. We then subclustered malignant osteoblastic cells and identified a proliferative osteoblastic subpopulation characterized by active cell division and chromosome segregation. Next, we constructed immune-proliferation-related signatures (IPRS) to evaluate prognosis and therapeutic potential across patients. Among the IPRS-related genes, several representative genes have been reported to be associated with tumor proliferation, immune regulation, or cellular homeostasis. *HMGA1* functions as a chromatin-associated architectural factor involved in transcriptional regulation and has been implicated in tumor progression. *IFIT1* and *CX3CR1* represent immune-associated components, with *IFIT1* participating in interferon-mediated innate immune responses and *CX3CR1* contributing to immune-cell recruitment and regulation of the tumor immune microenvironment. *NUDT18* is involved in nucleotide metabolism and cellular homeostasis, whereas *ALG6* participates in N-linked glycosylation and endoplasmic reticulum-associated protein processing. These findings support that the IPRS integrates diverse biological processes, including proliferation-related and immune-related features, rather than representing a single molecular pathway.

Among these genes, *ALG6* was selected for further functional validation because of its potential role in glycosylation-mediated regulation of protein stability and signaling pathways relevant to tumor biology. Previous studies have suggested that aberrant glycosylation can influence the expression and activity of multiple tumor-associated receptors and immune regulatory molecules. Therefore, *ALG6* was further investigated to explore its potential biological contribution to osteosarcoma progression. Through this approach, our study provides further evidence for proliferative heterogeneity in OS and supports the use of integrated molecular features for prognostic stratification.

Materials and methods

Data sources and preprocessing

Bulk RNA expression profiles and clinical data were obtained from the TCGA-SARC cohort ($n = 261$) and the GSE39058 osteosarcoma cohort ($n = 41$), respectively [17]. Single-cell RNA sequencing (scRNA-seq) data were obtained from GSE152048 ($n = 11$) [18]. The scRNA-seq dataset was used to define cellular subpopulations and to further identify proliferative subgroups among OS cells. To ensure comparability, TCGA and GSE39058 expression data were normalized to transcripts per million (TPM). Samples with complete gene-expression data and available sex, age, overall survival time and survival-status information were retained for subsequent analyses.

scRNA-seq analysis

The GSE152048 dataset was processed and analyzed using the Seurat package in R. Each sample was normalized individually during the initial analysis. The top 3,000 highly variable genes were selected, followed by scaling and principal component analysis. Doublet removal and quality control were performed for each sample. The 11 Seurat objects were then merged into a single object. Cells were retained when they expressed more than 500 genes and had less than 10% mitochondrial gene expression. Harmony was used for batch correction. Nearest-neighbor graph construction, clustering analysis and t-distributed stochastic neighbor embedding (t-SNE) visualization were performed based on the corrected low-dimensional embeddings. Major cell populations

were identified, and OS cell subclusters were extracted for analysis of proliferation-associated subpopulations.

Weighted gene co-expression network analysis for immune-proliferation-related features

Weighted gene co-expression network analysis (WGCNA) was performed using the WGCNA package in R to construct a gene co-expression network from the transcriptomic matrix of the TCGA-SARC cohort [19]. Lowly expressed genes and genes with low variability were removed before analysis. A soft-thresholding power was selected according to the scale-free topology fit index. The adjacency matrix and topological overlap matrix were then calculated. Modules with similar expression patterns were identified and merged using dynamic tree cutting.

The ImmuneScore and proliferation score were calculated prior to the module-trait correlation analysis. Briefly, ImmuneScore was calculated using the Estimation of Stromal and Immune cells in Malignant Tumours using Expression data (ESTIMATE) algorithm based on bulk transcriptomic profiles. The proliferation score was calculated using single-sample gene set enrichment analysis (ssGSEA). Specifically, the proliferation-associated genes listed in [Supplementary Table 1](#) were derived from differentially expressed genes between the proliferative osteoblastic subpopulation and the non-proliferative osteoblastic cluster identified from the scRNA-seq dataset. These genes were used as the proliferation signature gene set, and ssGSEA was applied to the bulk expression profiles of the TCGA-SARC cohort to generate a proliferation score for each patient.

Pearson correlation analysis was then performed between module eigengenes and the ImmuneScore or proliferation score. The blue module showed the strongest association with ImmuneScore, whereas the green module showed the strongest association with proliferation score. These two modules were therefore defined as key IPRS-related modules.

To further evaluate the cross-platform relationship, the 100 marker genes identified from the proliferative osteoblastic subpopulation were compared with the genes from the WGCNA blue and green modules. This analysis identi-

fied 88 shared genes. The single-cell-derived marker genes were then combined with the WGCNA blue and green module genes, generating a union of 3,573 genes. This union was subsequently intersected with bulk differentially expressed genes to identify candidate genes for IPRS construction.

Screening of differentially expressed IPRS candidates

Differential expression analysis was performed using the limma package in R. Genes with $|\log_2$ fold change > 1.5 and an adjusted P value < 0.05 were defined as differentially expressed genes. Genes were classified according to fold change and statistical significance, and volcano plots were generated to visualize upregulated, downregulated and non-significant genes. The union of the single-cell-derived proliferative marker genes and the WGCNA blue and green module genes was intersected with bulk differentially expressed genes. The resulting overlap was visualized using a Venn diagram.

Univariate Cox regression analysis

Univariate Cox proportional hazards regression was performed using the survival package in R. Overall survival time and survival status were used as outcome variables. Hazard ratios, 95% confidence intervals and P values were calculated for each candidate gene. Genes with $P < 0.05$ were considered to be associated with prognosis. Genes with hazard ratios greater than 1 were regarded as risk factors, whereas those with hazard ratios less than 1 were considered protective factors. The results were visualized using a forest plot.

Machine learning-based construction of the IPRS prognostic model and Shapley additive explanations (SHAP) interpretation

The TCGA-SARC cohort was used as the training set and GSE39058 was used as the external validation set. Ten machine-learning algorithms were integrated, including least absolute shrinkage and selection operator (LASSO), elastic net, ridge regression, random survival forest (RSF), stepwise Cox regression, CoxBoost, partial least squares Cox regression, gradient boosting machine, supervised principal components (SuperPC) and survival support vector machine. A total of 117 prognostic models

were constructed using individual algorithms and algorithm combinations [19]. The concordance index (C-index) was calculated for each model in the training and validation cohorts. During model development, 10-fold cross-validation was performed within the TCGA-SARC training cohort to optimize model-specific parameters, including the regularization parameters for penalized Cox models (LASSO, Ridge, and Elastic Net), the optimal boosting step for CoxBoost, and the optimal component or threshold parameters for other machine-learning algorithms. The cross-validated C-index was used to evaluate internal model performance, and the optimized models were subsequently validated in independent external cohorts to assess their predictive generalizability. For the final random survival forest model, the 10-fold cross-validated C-index in the TCGA training cohort was 0.755. SHAP analysis was then performed to interpret the model. SHAP values and mean absolute SHAP values were calculated to assess the contribution of each candidate gene to risk prediction. SHAP summary plots, feature-importance plots and dependence plots were generated.

Prognostic evaluation of IPRS

A risk score was generated for each patient using the optimal model. Patients were divided into high- and low-risk groups according to the median risk score in each cohort. Kaplan-Meier survival analysis was used to compare overall survival between the two groups. Statistical significance was assessed using the log-rank test.

Independent prognostic analysis and nomogram construction

Univariate Cox proportional hazards regression was used to assess associations between sex, age, IPRS risk score and overall survival. Variables with statistical significance in univariate analysis were included in a multivariate Cox regression model to identify independent prognostic factors. $P < 0.05$ was considered statistically significant. A nomogram integrating age and IPRS risk score was constructed using the rms or regplot package in R to estimate 1-, 3- and 5-year overall survival probabilities. Calibration curves for 1-, 3-, 5- and 10-year survival were generated to compare predicted survival with observed survival. Time-dependent receiver operating characteristic curves (ROC)

and the area under the curve (AUC) were used to assess the accuracy of the nomogram at different time points.

Tumor immune microenvironment analysis

CIBERSORT was used to estimate the relative infiltration proportions of 22 immune-cell types in each sample [19]. Stacked bar plots were used to display immune-cell composition. Patients were divided into high- and low-risk groups according to the median IPRS risk score, and Wilcoxon rank-sum tests were used to compare immune-cell infiltration between groups. The ESTIMATE algorithm was used to calculate the ImmuneScore, StromalScore and ESTIMATEScore for each sample. Pearson correlation analysis was used to evaluate associations between the IPRS risk score and tumor microenvironment scores or immune-cell infiltration levels. Correlation scatterplots were generated. Correlation structures among immune cells were visualized using a correlation matrix, and Mantel tests were used to assess associations between the IPRS risk score and immune-cell infiltration features.

Drug sensitivity analysis

Drug sensitivity was predicted from patient transcriptomic profiles using the oncoPredict package in R. Pearson correlation analysis was used to assess relationships between IPRS risk score, model-gene expression and drug sensitivity. Heatmaps were generated to display correlation coefficients and statistical significance. Patients were divided into high- and low-risk groups according to the median IPRS risk score, and Wilcoxon rank-sum tests were used to compare predicted drug sensitivity between the two groups. Boxplots were used for visualization.

Human cell culture and transfection

The human OS cell lines 143B and U2OS were obtained from the Cell Bank of the Chinese Academy of Sciences. Cells were cultured and passaged in RPMI-1640 medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Cells were maintained at 37.0°C in a humidified incubator containing 5% CO₂. Small interfering RNA (siRNA) targeting human ALG6 was used to silence ALG6 expression. A non-specific negative control

siRNA (si-NC; RiboBio, China) was used as the control. The siRNA sequences are listed in [Supplementary Table 2](#). 143B and U2OS cells were seeded in six-well plates. When cell confluence reached 50-60%, transfection was performed using Lipofectamine 2000 (Thermo Fisher Scientific, USA). No contamination was observed during the procedure. Both cell lines were authenticated by short tandem repeat (STR) profiling and tested negative for mycoplasma contamination prior to experiments.

RNA extraction and quantitative real-time PCR

Total RNA was extracted from 143B and U2OS cells 48 h after transfection with si-NC or si-ALG6 using a rapid RNA extraction kit (Sparkjade, Shandong, China). RNA concentration and purity were assessed using a NanoDrop spectrophotometer. Equal amounts of total RNA were reverse-transcribed into complementary DNA (cDNA) using the PrimeScript reverse transcription kit. Quantitative real-time PCR (qRT-PCR) was then performed using SYBR Green premix to detect ALG6 mRNA expression. The amplification program was 95°C for 30 s, followed by 40 cycles of 95°C for 10 s and 60°C for 30 s. β -ACTIN was used as the internal reference gene. Relative ALG6 expression was calculated using the 2^{-DeltaDeltaCt} method. Primer sequences are provided in [Supplementary Table 2](#). Each experiment was independently repeated three times.

CCK-8 cell proliferation and colony-formation assays

Cell proliferation after ALG6 knockdown was assessed using the Cell Counting Kit-8 (CCK-8) assay. At 48 h after transfection with si-ALG6 or si-NC, 143B and U2OS cells were seeded into 96-well plates at 5 × 10³ cells per well. At 0, 24, 48 and 72 h after seeding, 80 microliters of complete medium and 20 microliters of CCK-8 reagent (APExBIO, K1018) were added to each well. Cells were incubated at 37°C with 5% CO₂ for 2 h. Absorbance at 450 nm was measured using a microplate reader to evaluate cell proliferation. CCK-8 measurements were analyzed using a two-way repeated-measures ANOVA to evaluate the effects of treatment group, time, and the group-by-time interaction, followed by Sidak's multiple-comparisons test.

For the colony-formation assay, 143B and U2OS cells were transfected with si-ALG6 or si-NC for 48 h. Cells were digested with trypsin and prepared as single-cell suspensions. Cells were seeded into six-well plates at 2×10^3 cells per well and cultured in complete medium for 10 days. Colonies were washed with phosphate-buffered saline, fixed with 4% paraformaldehyde for 15 min, stained with crystal violet, rinsed and air-dried. Each group included three replicate wells, and all experiments were repeated three times.

Immunofluorescence staining

Processed 143B and U2OS cells were seeded onto sterile coverslips in 24-well plates and allowed to adhere for 24 h. Cells were fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.5% Triton X-100 for 10 min, and blocked with 1% bovine serum albumin for 40 min. After blocking, the cells were incubated overnight at 4°C with a rabbit polyclonal anti-ALG6 antibody (Abcam; ab80873; dilution, 1:200). After washing with phosphate-buffered saline (PBS), the cells were incubated with an Alexa Fluor® 594-conjugated goat anti-rabbit IgG H&L secondary antibody (Abcam, Cambridge, UK; Cat. No. ab150080; dilution, 1:500) for 1 h at room temperature in the dark. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI), and the coverslips were mounted using an antifade mounting medium. Images were acquired under identical imaging parameters using a confocal fluorescence microscope to evaluate ALG6 localization and changes in fluorescence intensity following ALG6 knockdown.

Flow cytometric analysis of apoptosis

Cell apoptosis was assessed using the fluorescein isothiocyanate (FITC) Annexin V Apoptosis Detection Kit I (BD Pharmingen, BD Biosciences, USA; Cat. No. 556547) according to the manufacturer's instructions. Briefly, cells were collected, washed twice with cold PBS, and resuspended in 1× Annexin V binding buffer at a concentration of approximately 1×10^6 cells/mL. Subsequently, 100 µL of the cell suspension containing approximately 1×10^5 cells was incubated with 5 µL of FITC Annexin V and 5 µL of propidium iodide (PI) for 15 min at room temperature in the dark. After addition of 400 µL of 1× binding buffer, the samples were ana-

lyzed within 1 h using a BD FACSCanto II flow cytometer (BD Biosciences, USA). The apoptotic rate was calculated as the sum of the percentages of early apoptotic cells (Annexin V-FITC⁺/PI⁻) and late apoptotic cells (Annexin V-FITC⁺/PI⁺).

Statistical analysis

All cellular experiments were independently repeated at least three times. Experimental data are presented as mean ± standard deviation. Comparisons between two groups were performed using two-sided unpaired Student's t tests or Wilcoxon rank-sum tests, depending on data distribution. Correlations between variables were assessed using Pearson or Spearman correlation analysis. Kaplan-Meier analysis was used to generate survival curves, and survival differences between groups were compared using log-rank tests. Univariate and multivariate Cox proportional hazards regression analyses were used to evaluate prognostic value, with hazard ratios and 95% confidence intervals calculated. Bioinformatics analyses were performed in R software (v4.3.5). Cellular experimental data were processed using GraphPad Prism 9.5.1. All tests were two-sided, and $P < 0.05$ was considered statistically significant.

Results

Single-cell transcriptomic analysis identified a proliferative osteoblastic subpopulation in osteosarcoma

We first analyzed the single-cell transcriptomic dataset to characterize the cellular composition and functional states of the osteosarcoma tumor microenvironment. After quality control, dimensionality reduction and clustering, t-SNE visualization separated the cells into multiple clusters with distinct transcriptional profiles (**Figure 1A, 1B**). Using canonical marker genes, we annotated these clusters as osteoblasts, myeloid cells, fibroblasts, chondrocytes, T cells and natural killer (T/NK) cells, endothelial cells, osteoclasts, myocytes and pericytes (**Figure 1C, 1D**).

The osteoblastic compartment was not transcriptionally uniform. When osteoblast-related subclusters were compared, cluster 3 and cluster 0 showed marked differences in gene

Immune-proliferation signatures in osteosarcoma

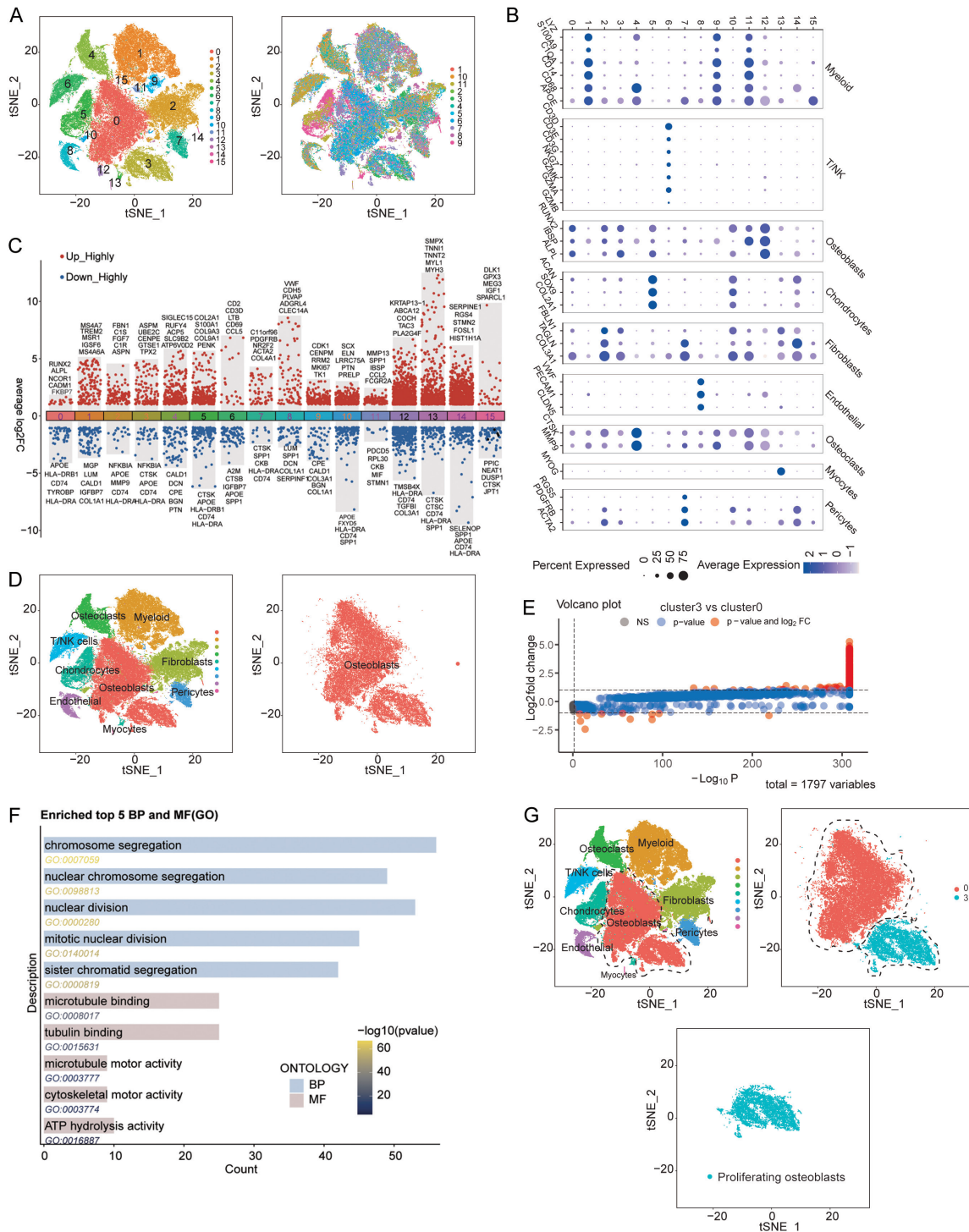


Figure 1. Single-cell transcriptomic analysis identifies a proliferative osteoblastic subpopulation in osteosarcoma. A. t-distributed stochastic neighbor embedding (t-SNE) visualization of osteosarcoma single-cell RNA sequencing data showing unsupervised cell clusters. B. Dot plot showing the expression of canonical marker genes used for cell-type annotation. C. Differentially expressed marker genes across single-cell clusters. D. t-SNE visualization of major annotated cell populations in the osteosarcoma microenvironment, including osteoblasts, myeloid cells, fibroblasts, chondrocytes, T cells and natural killer (T/NK) cells, endothelial cells, osteoclasts, myocytes and pericytes. E. Volcano plot showing differentially expressed genes between osteoblastic cluster 3 and cluster 0. F. Gene Ontology enrichment analysis of genes associated with cluster 3. G. t-SNE visualization showing cluster 3 was defined as the proliferative osteoblastic subpopulation.

expression (**Figure 1E**). Genes upregulated in cluster 3 were mainly enriched in chromosome segregation, nuclear chromosome segregation, nuclear division, mitotic nuclear division, sister chromatid segregation, microtubule binding, tubulin binding and cytoskeletal motor activity (**Figure 1F**). These features pointed to an actively dividing cell state. On this basis, cluster 3 was defined as a proliferative osteoblastic subpopulation (**Figure 1G**).

Integrated WGCNA and prognostic analysis screened immune-proliferation-related signatures

We next asked whether the proliferative osteoblastic state identified at the single-cell level could be linked to immune and prognostic information in bulk transcriptomic data. WGCNA identified multiple co-expression modules (**Figure 2A**). In the module-trait correlation analysis, the blue module showed the strongest positive correlation with ImmuneScore ($r = 0.94$, $P = 2 \times 10^{-108}$), while the green module showed the strongest positive correlation with proliferation score ($r = 0.91$, $P = 5 \times 10^{-87}$) (**Figure 2B**). The associations were further supported by module membership and gene significance analyses (**Figure 2C, 2D**).

We integrated the single-cell-derived proliferative marker genes with the bulk transcriptomic features used for IPRS construction. The 100 marker genes identified from the proliferative osteoblastic subpopulation were compared with the WGCNA blue and green module genes, and 88 shared genes were identified. The marker genes were then combined with the WGCNA blue and green module genes, generating a union of 3,573 genes. This union was intersected with 4,397 bulk differentially expressed genes, resulting in 1,264 candidate genes for further model development (**Figure 2E, 2F**). These genes were subsequently used for IPRS construction. Because these genes were related to immune infiltration, proliferative state and patient survival, we defined them as immune-proliferation-related signatures (IPRS). Univariate Cox regression analysis identified several survival-associated IPRS genes, including *ALG6*, *CFLAR*, *GBP4*, *SYNGR2*, *IFIT1*, *AMBN*, *HMGA1*, *DLEC1*, *NUDT18*, *RNF125* and *CX3CR1* (**Figure 2G**).

An IPRS-based machine-learning model effectively predicted prognosis in osteosarcoma

Based on the IPRS candidates, we compared a series of machine-learning algorithm combinations for prognostic prediction. Model performance varied across the TCGA-SARC training cohort and the GSE39058 external validation cohort. Among the 117 models evaluated, the RSF model showed the most stable overall performance, with a 10-fold cross-validated C-index of 0.755 in the TCGA training cohort, an external validation C-index of 0.782 in the GSE39058 cohort and an average C-index of 0.769 (**Figure 3A**). The RSF model was therefore selected as the final IPRS-based prognostic model.

We used SHAP analysis to examine feature contributions. The IPRS score made the largest contribution to model prediction (**Figure 3B-F**). Patients were then stratified into high- and low-risk groups according to the calculated IPRS score. The survival curves separated clearly. Kaplan-Meier analysis showed significant differences in overall survival between the two groups in both the TCGA and GSE39058 cohorts (**Figure 3G-J**). Thus, the IPRS score was able to distinguish patients with different survival outcomes in both the training and external validation cohorts.

The IPRS score was an independent prognostic factor for overall survival in osteosarcoma

We further examined whether the prognostic value of the IPRS score was independent of conventional clinical variables. In univariate Cox regression analysis, age was significantly associated with overall survival (HR = 1.021, 95% CI: 1.005-1.036, $P = 0.008$). The IPRS risk score also showed a significant association with survival (HR = 1.913, 95% CI: 1.046-2.779, $P < 0.001$). Sex was not significantly related to prognosis (**Figure 4A**). After adjustment in the multivariate Cox model, the IPRS score remained an independent risk factor for overall survival (HR = 1.368, 95% CI: 1.051-1.685, $P < 0.001$) (**Figure 4B**).

To facilitate individualized survival estimation, we constructed a nomogram incorporating

Immune-proliferation signatures in osteosarcoma

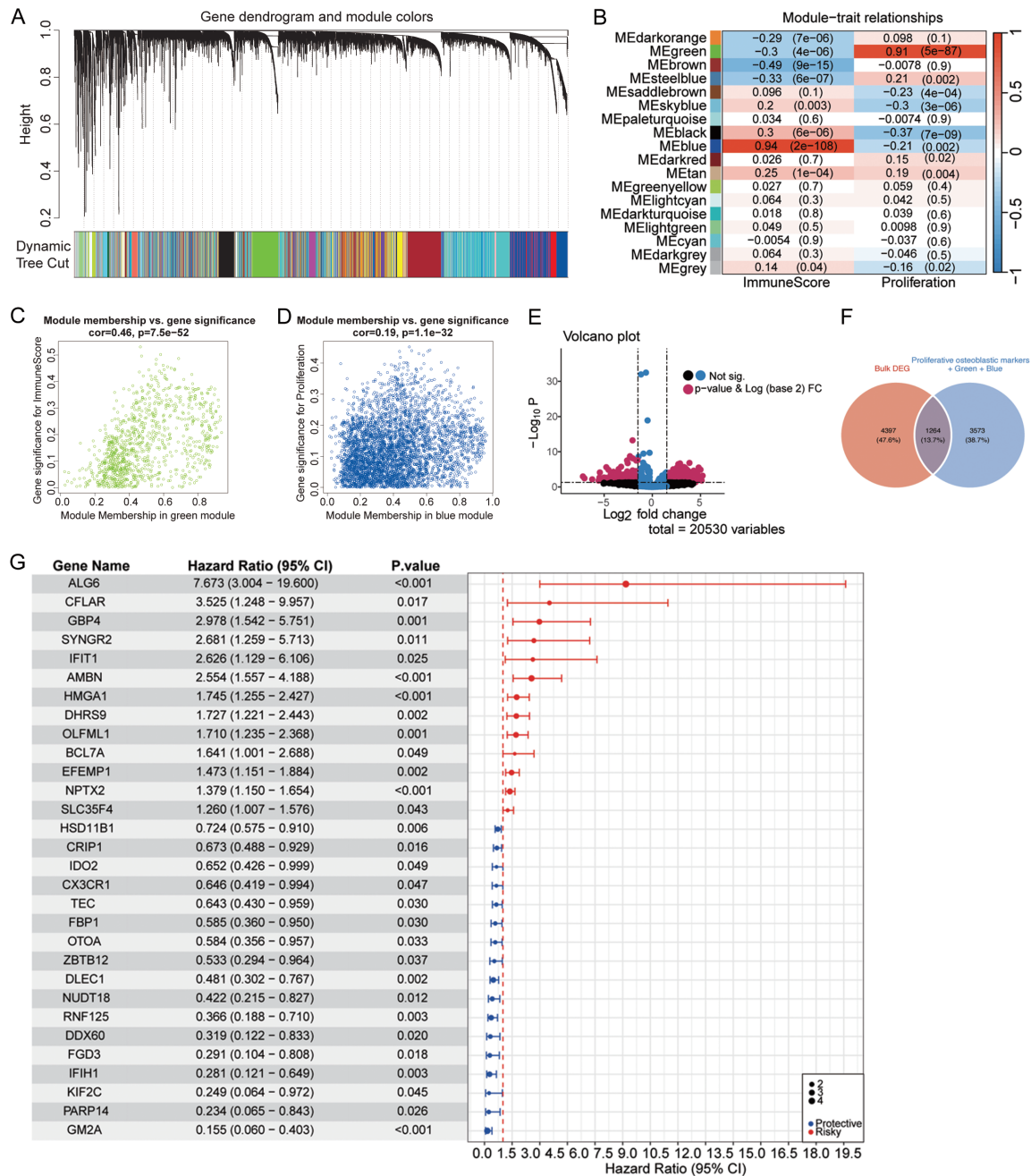


Figure 2. Identification of immune-proliferation-related signatures (IPRS) by weighted gene co-expression network analysis (WGCNA) and prognostic screening. A. Gene dendrogram and module colors generated by WGCNA. B. Heatmap showing correlations between gene modules and immune or proliferation-related traits. C, D. Scatter plots showing the relationship between module membership and gene significance in key modules. E. Volcano plot showing differentially expressed genes in the bulk transcriptomic cohort. F. Venn diagram showing the overlap between bulk differentially expressed genes and the union of proliferative osteoblastic marker genes and WGCNA blue and green module genes. The union contained 3,573 genes, of which 1,264 overlapped with the bulk differentially expressed genes. G. Forest plot of univariate Cox regression analysis.

age and IPRS risk score (Figure 4C). Calibration curves showed good agreement between predicted and observed survival probabilities

(Figure 4D). Time-dependent ROC analysis yielded AUC values of 0.701, 0.711, 0.721 and 0.721 for 1-, 3-, 5- and 10-year overall

Immune-proliferation signatures in osteosarcoma

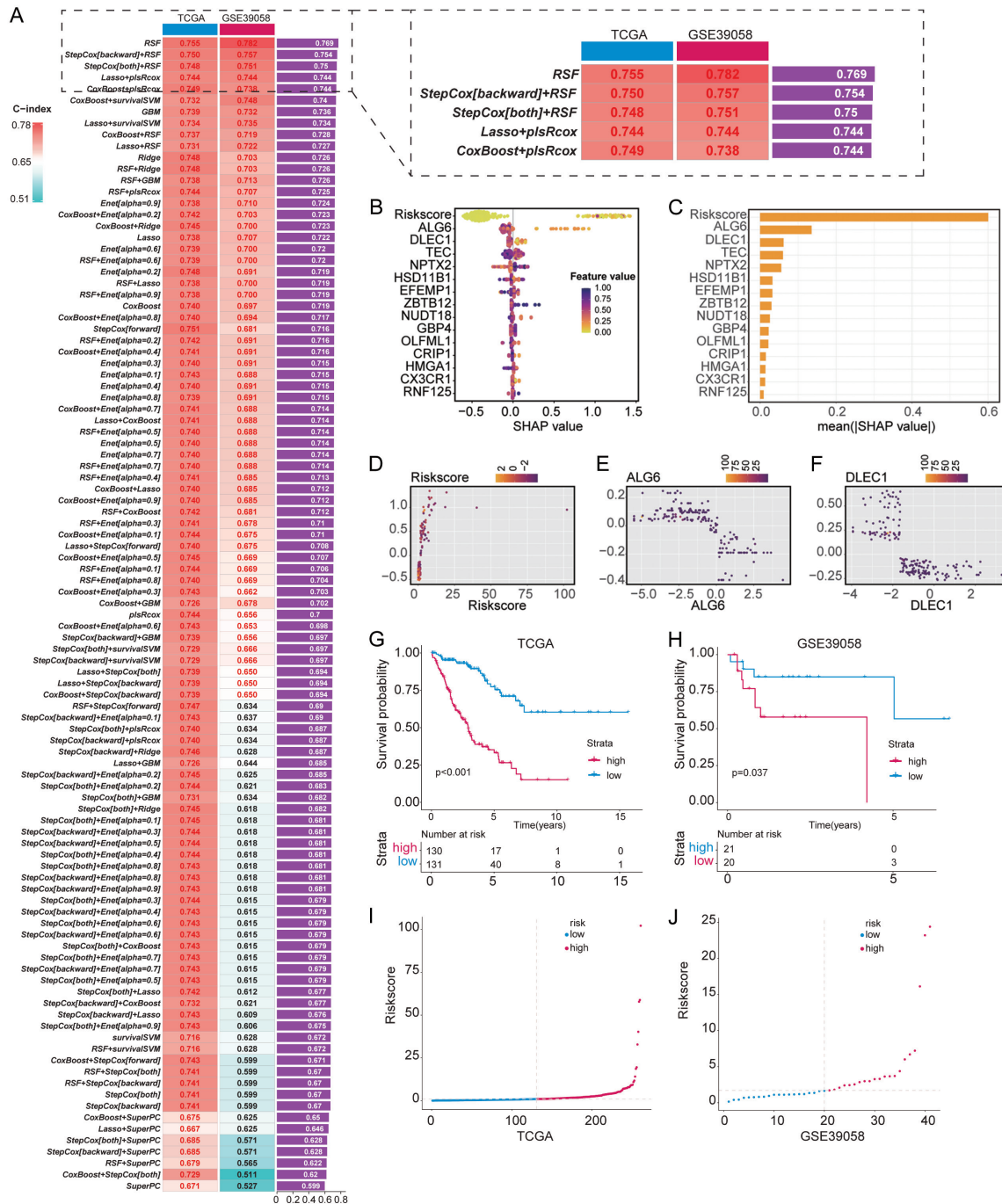


Figure 3. Construction and validation of the IPRS-based machine-learning prognostic model. **A.** Heatmap comparing the concordance index (C-index) values of 117 machine-learning models in the TCGA-SARC training cohort and GSE39058 validation cohort. The TCGA value (0.755) represents the 10-fold cross-validated C-index, whereas the GSE39058 value (0.782) represents the external validation C-index. **B.** Shapley additive explanations (SHAP) summary plot showing the contribution of IPRS score and model genes to prognostic prediction. **C.** Feature-importance plot based on mean absolute SHAP values. **D-F.** SHAP dependence plots showing the relationship between risk score or representative genes and model output. **G, H.** Kaplan-Meier survival curves comparing overall survival between high- and low-IPRS score groups in the TCGA-SARC cohort and GSE39058 cohort. **I, J.** Distribution of IPRS scores in the TCGA and GSE39058 cohorts.

survival, respectively (Figure 4E). The model therefore maintained relatively stable pre-

dictive performance across the evaluated time points.

Immune-proliferation signatures in osteosarcoma

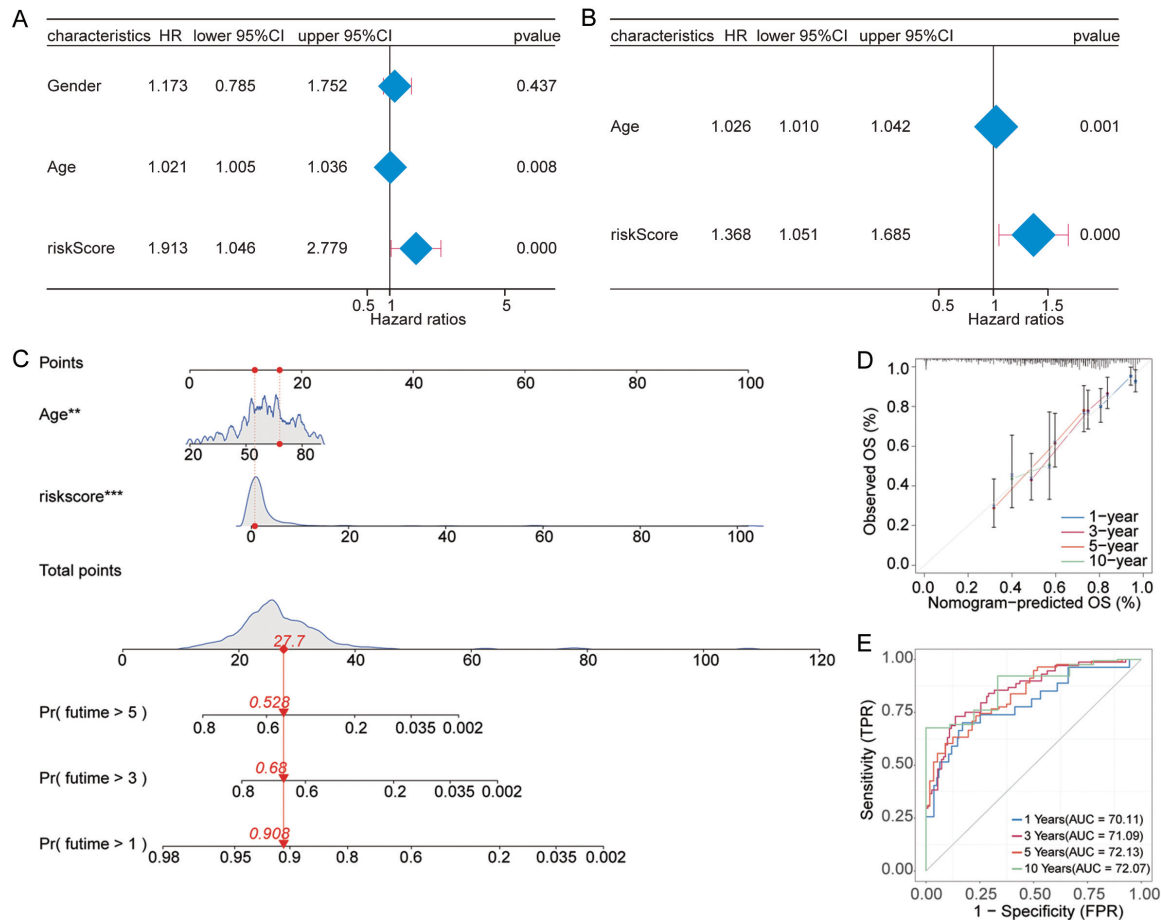


Figure 4. Independent prognostic value of the IPRS score and construction of a prognostic nomogram. A. Univariate Cox regression analysis evaluating the prognostic value of sex, age and IPRS score. B. Multivariate Cox regression analysis. C. Nomogram integrating age and IPRS score to predict 1-, 3-, 5- and 10-year overall survival probabilities. D. Calibration curves assessing the agreement between nomogram-predicted and observed overall survival probabilities. E. Time-dependent ROC curves evaluating the predictive performance of the nomogram at 1, 3, 5 and 10 years.

The IPRS score was associated with immune microenvironment features in osteosarcoma

We next explored the relationship between the IPRS score and the osteosarcoma immune microenvironment. CIBERSORT was used to estimate the relative proportions of 22 immune-cell types. Several immune-cell populations differed between the high- and low-IPRS score groups, including naive B cells, plasma cells, CD8 T cells, resting CD4 memory T cells, follicular helper T cells, resting NK cells, activated NK cells, monocytes, M0/M1/M2 macrophages, dendritic cells, mast cells, eosinophils and neutrophils (**Figure 5A**). Immune composition also varied substantially among

individual patients, as shown in the stacked bar plots (**Figure 5B**).

The correlation analyses provided a similar picture. The IPRS score was associated with multiple immune-cell infiltration levels (**Figure 5C**). In the ESTIMATE analysis, higher IPRS scores were negatively correlated with ESTIMATEScore, ImmuneScore and StromalScore, with correlation coefficients of -0.29, -0.29 and -0.24, respectively (all $P < 0.001$) (**Figure 5D**). Negative correlations were also observed for M1 macrophages, resting mast cells and CD8 T cells (**Figure 5E**). Overall, tumors with higher IPRS scores tended to show lower immune and stromal components and reduced infiltration of selected immune-cell populations.

Immune-proliferation signatures in osteosarcoma

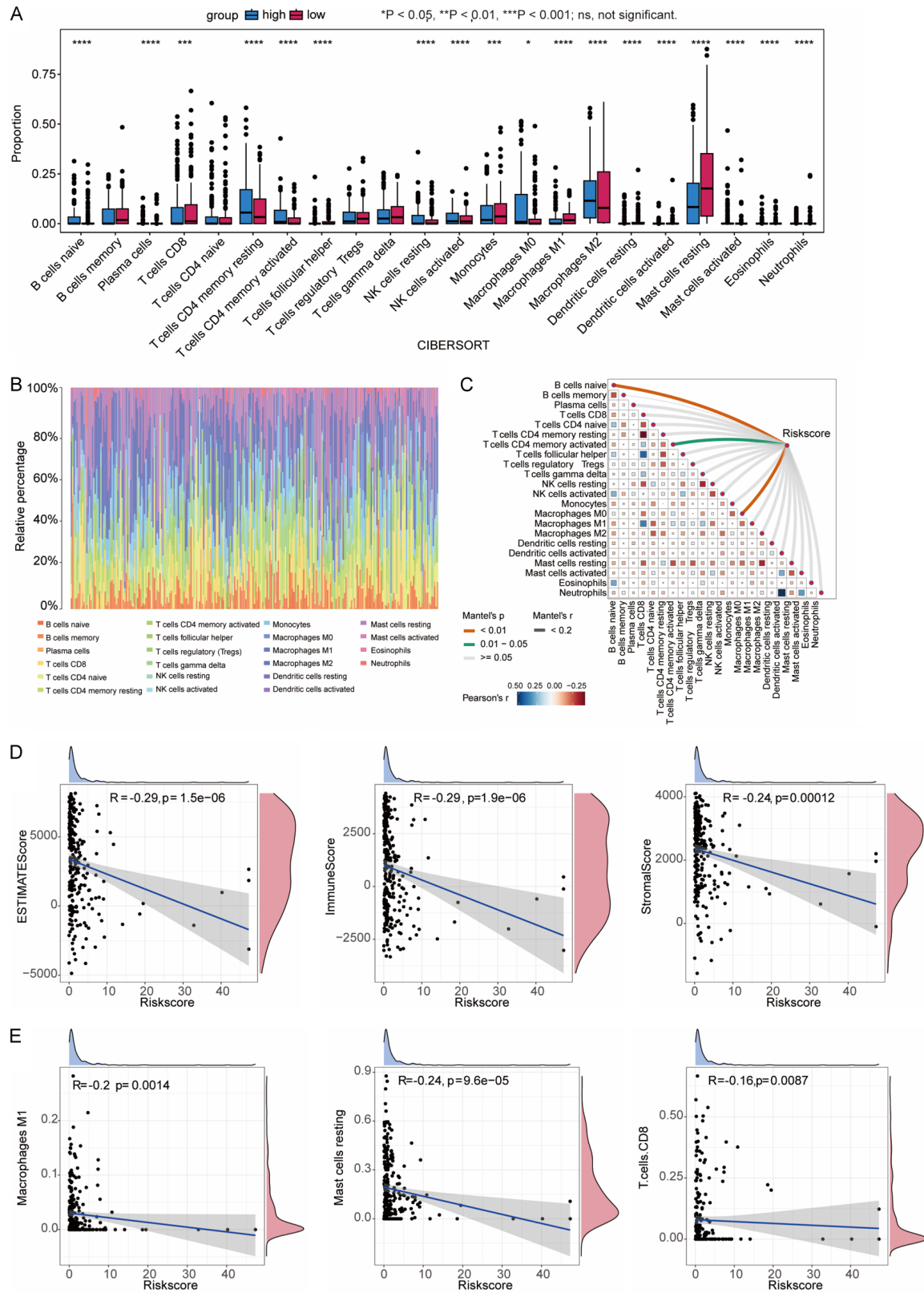


Figure 5. Association between IPRS score and the osteosarcoma immune microenvironment. A. Boxplots comparing the relative proportions of 22 immune-cell types between high- and low-IPRS score groups based on CIBERSORT analysis. B. Stacked bar plot showing the immune-cell composition of individual osteosarcoma samples. C. Correlation matrix and Mantel test showing associations between IPRS score and immune-cell infiltration features. D.

Immune-proliferation signatures in osteosarcoma

Correlation analysis between IPRS score and ESTIMATEScore, ImmuneScore and StromalScore. E. Scatter plots showing correlations between IPRS score and representative immune-cell populations, including M1 macrophages, resting mast cells and CD8 T cells. Significance was determined by Wilcoxon rank-sum test. *P*-values were adjusted using the Benjamini-Hochberg false discovery rate (FDR) method. *FDR-adjusted *P* < 0.05, **FDR-adjusted *P* < 0.01, ***FDR-adjusted *P* < 0.001, ****FDR-adjusted *P* < 0.0001; ns, not significant.

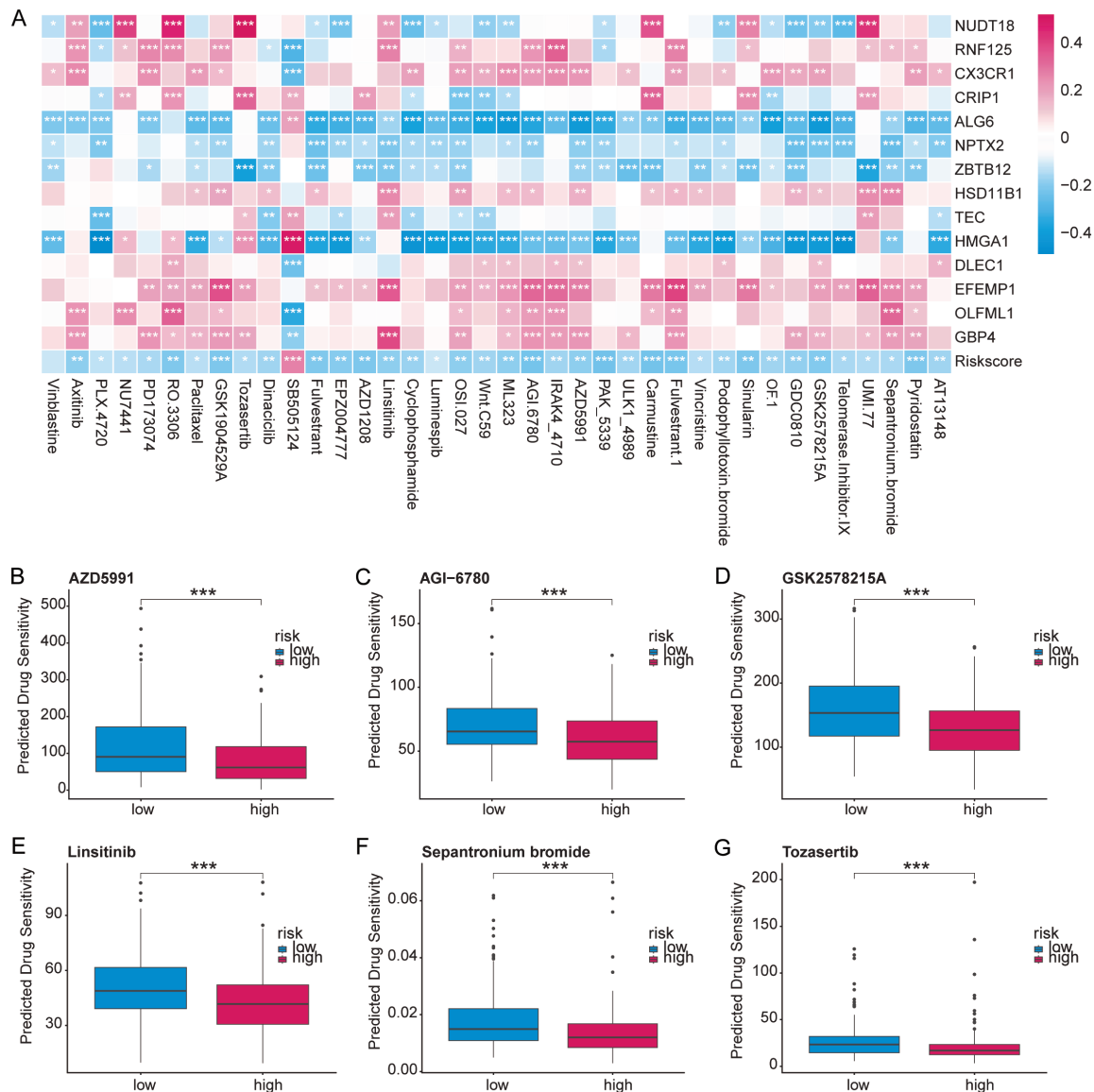


Figure 6. Drug sensitivity analysis based on IPRS-related genes and IPRS score. A. Heatmap showing correlations between IPRS-related genes, IPRS score and predicted sensitivity to multiple antitumor drugs. B-G. Boxplots comparing predicted drug sensitivity between high- and low-IPRS score groups for AZD5991, AGI-6780, GSK2578215A, Linsitinib, Sepantronium bromide and Tozasertib. ****P* < 0.001.

The IPRS score indicated potential differences in drug sensitivity

We also assessed whether the IPRS score was related to predicted treatment response. Correlation heatmaps showed that multiple IPRS-

related genes and the IPRS score were significantly associated with predicted sensitivity to different antitumor agents (Figure 6A). After stratification by IPRS score, several drugs showed significant differences in predicted sensitivity between the high- and low-risk groups.

These included AZD5991, AGI-6780, GSK-2578215A, Linsitinib, Sepantronium bromide and Tozasertib (**Figure 6B-G**).

These findings suggest that IPRS-based risk scoring may capture differences in predicted drug response among patients with different prognostic risks. This result should be interpreted cautiously. The drug-sensitivity analysis was computational and will require further validation in cellular experiments, organoid models or animal models.

ALG6, a core IPRS gene, promoted proliferation and suppressed apoptosis in osteosarcoma cells

Among the IPRS genes, *ALG6* made a substantial contribution to the model predictions and was associated with poor prognosis in Cox analysis. We therefore selected *ALG6* for functional validation. qRT-PCR confirmed efficient knockdown of *ALG6* in both 143B and U2OS cells after si-*ALG6* transfection compared with si-NC (**Figure 7A**). The functional effects were consistent across assays. Immunofluorescence staining also showed markedly reduced *ALG6* fluorescence intensity in si-*ALG6*-transfected 143B and U2OS cells (**Figure 7B, 7C**), confirming successful *ALG6* knockdown. CCK-8 assays demonstrated that *ALG6* knockdown significantly inhibited the proliferation of 143B and U2OS cells (**Figure 7D, 7E**). Colony formation assays further showed that *ALG6* silencing reduced the colony-forming ability of both cell lines (**Figure 7F**). Moreover, Annexin V-FITC/PI flow cytometry showed that *ALG6* knockdown significantly increased the proportion of apoptotic cells (**Figure 7G**). Taken together, these results support a functional role for *ALG6* in maintaining osteosarcoma cell proliferation and limiting apoptosis. *ALG6* may therefore represent a biologically relevant IPRS gene with potential therapeutic value.

Discussion

Patients with osteosarcoma do not follow a uniform clinical course. Even after similar surgical and chemotherapeutic treatment, some patients remain disease-free, whereas others develop relapse, metastasis or drug resistance. Conventional clinicopathological factors cannot fully explain this difference. In this study, we examined this problem from the single-cell

level and identified a proliferative osteoblastic subpopulation with active chromosome segregation, mitotic activity and microtubule-related processes. We then integrated these proliferative features with immune-related information to construct IPRS. The resulting IPRS score separated patients with different survival risks and was also associated with immune microenvironmental features and predicted drug sensitivity.

The single-cell analysis revealed marked heterogeneity within the osteoblastic compartment. One notable finding was the presence of a proliferative osteoblastic subpopulation enriched for genes involved in chromosome segregation, nuclear division and microtubule binding. This pattern suggests an actively cycling and potentially chromosomally unstable cell state, rather than a simple difference in osteoblastic differentiation. This is biologically meaningful. Osteosarcoma is characterized by rapid growth and genomic instability, and such a highly proliferative cell state may contribute to tumor expansion, clonal evolution and the regrowth of residual tumor cells after treatment. In line with this observation, the IPRS-based RSF model showed stable prognostic performance in both the training and external validation cohorts.

The association between IPRS and the immune microenvironment provides another important layer of information. In our analysis, higher IPRS scores were negatively correlated with ImmuneScore, StromalScore and ESTIMATEScore. They were also related to the infiltration levels of CD8⁺ T cells, M1 macrophages and resting mast cells. In practical terms, high-risk tumors appeared to have fewer immune and stromal components and a weaker antitumor immune contexture. This observation fits with the single-cell findings. A rapidly proliferating osteoblastic tumor-cell state may reshape the surrounding immune environment through nutrient competition, metabolite accumulation and paracrine signaling [2]. The IPRS score may therefore reflect not only survival risk, but also the interaction between tumor-cell proliferation and immune remodeling.

Immune suppression in osteosarcoma is unlikely to result from a single pathway. Several mechanisms may act in parallel. Dense stro-

Immune-proliferation signatures in osteosarcoma

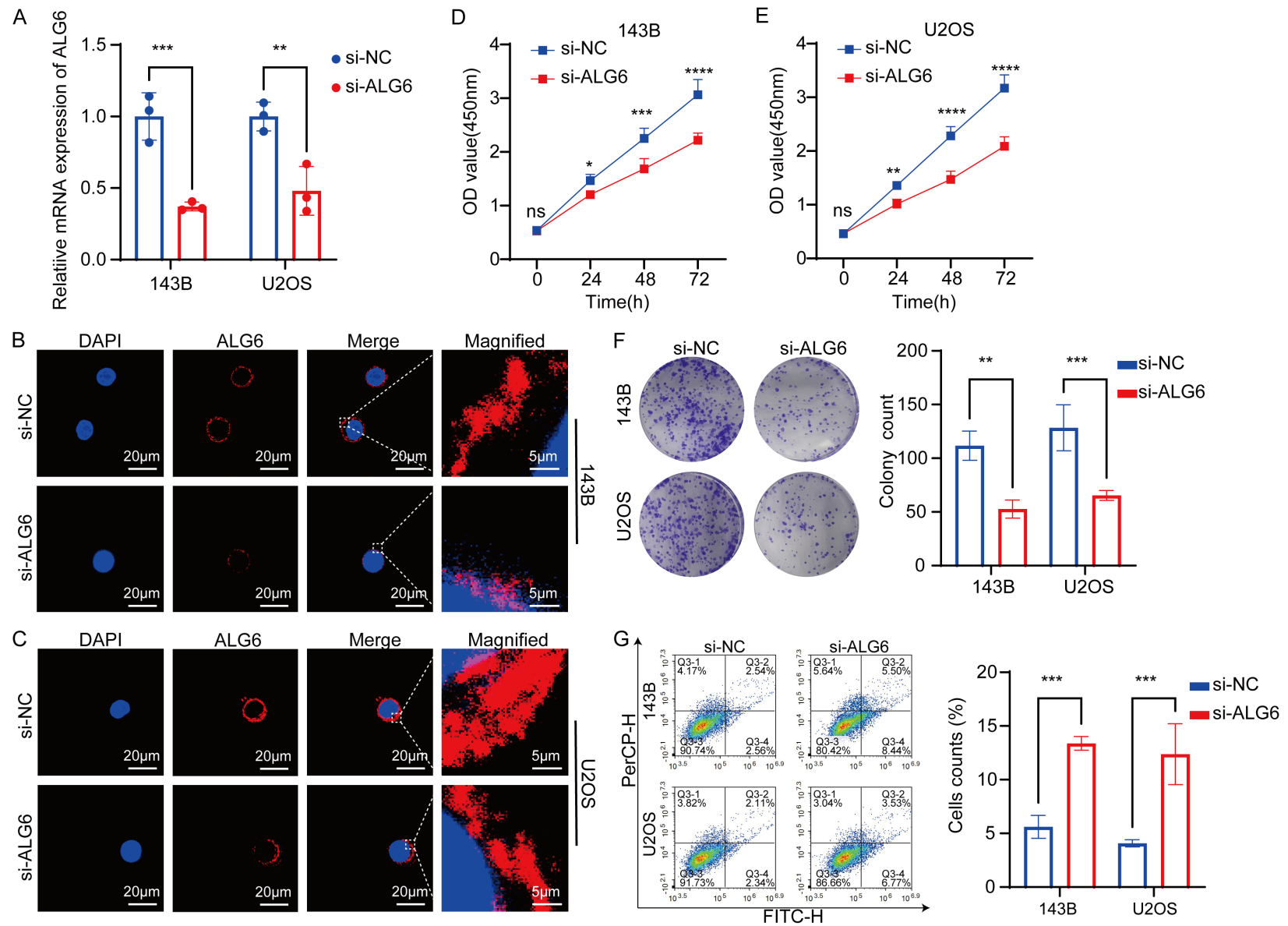


Figure 7. Functional validation of ALG6 in osteosarcoma cells. A. Quantitative real-time PCR (qRT-PCR) analysis of ALG6 mRNA expression in 143B and U2OS cells transfected with si-NC or si-ALG6. B. Representative immunofluorescence images in 143B cells. C. Representative immunofluorescence images in U2OS cells. D, E.

CCK-8 assays showing the proliferation of 143B and U2OS cells following *ALG6* knockdown. F. Representative images and quantitative analysis of colony formation in 143B and U2OS cells. G. Representative flow-cytometry plots and quantitative analysis of apoptosis in 143B and U2OS cells using Annexin V-FITC/PI staining. ns, not significant; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$.

mal components, abnormal vasculature and hypoxia can restrict the entry of T cells into tumor tissue [20-22]. Tumor-associated macrophages, myeloid-derived suppressor cells and regulatory T cells may further impair CD8+ T-cell and NK-cell function through mediators such as IL-10 and TGF- β . Metabolism may also be involved. Enhanced glycolysis, lactate accumulation, and lipid and amino acid metabolic reprogramming can favor macrophage polarization toward tumor-promoting phenotypes [11, 23]. In some tumors, reduced MHC-I or B2M expression may limit immune recognition even when immune cells are present [24]. These processes together may help explain the cold-tumor state seen in a subset of osteosarcoma patients, with immune exclusion, myeloid suppression and defective antigen presentation.

These findings also have therapeutic implications. For many patients with osteosarcoma, immune checkpoint inhibition alone may be insufficient. The tumor immune microenvironment may need to be modified before effector-cell killing can be effectively enhanced. Potential strategies include targeting CSF-1R- or CCR2-related pathways, reprogramming tumor-associated macrophages, reducing metabolic competition, and improving vascular or stromal architecture [25, 26]. These approaches may create a more permissive immune contexture. They could then be combined with PD-1/PD-L1 inhibitors, CAR-T cells, CAR-NK cells, chemotherapy, or targeted therapy according to the biological features of individual tumors [27-29]. In this setting, IPRS may help identify patients with different proliferative and immune states and may support more individualized treatment stratification.

Several limitations should be considered. First, this study was based mainly on public transcriptomic datasets. Differences in sample processing, sequencing platforms and clinical annotation may have introduced heterogeneity. In addition, metastatic status, tumor necrosis rate, and surgical margin status were unavailable or incompletely annotated in the public cohorts and therefore could not be incorporat-

ed into the nomogram. Consequently, the current nomogram should be regarded as a preliminary molecular prognostic model rather than a comprehensive clinicopathological prediction tool. Second, although an external validation cohort was included, the validation sample size was relatively small. Larger independent osteosarcoma cohorts are needed to confirm the robustness of the IPRS model. Third, immune-cell infiltration and drug sensitivity were inferred using computational methods. These results should therefore be interpreted as predictive evidence rather than direct measurements of immune status or treatment response. Fourth, although our in vitro experiments showed that *ALG6* knockdown inhibited osteosarcoma cell proliferation and promoted apoptosis, these experiments did not establish the downstream molecular mechanisms. *ALG6* encodes an alpha-1,3-glucosyltransferase involved in the early steps of endoplasmic reticulum-associated N-linked glycosylation [30, 31]. N-linked glycosylation is an important post-translational modification that regulates protein folding, stability, trafficking and cell-surface expression [31, 32]. Therefore, *ALG6* may influence osteosarcoma progression by affecting the maturation, stability or cell-surface expression of glycoproteins. Altered glycosylation-related enzymes have also been linked to osteosarcoma cell survival and proliferation [33, 34]. Aberrant glycosylation of membrane-associated proteins, including growth factor receptors such as IGF-1R and EGFR [32, 35], integrins [36] and immune checkpoint molecules such as PD-L1 [37], may affect tumor-cell proliferation, survival and immune escape. However, these potential mechanisms remain hypothetical in osteosarcoma and require further validation using glycoproteomic and functional experiments. Fifth, the single-cell and bulk transcriptomic datasets used in this study were derived from independent patient cohorts rather than matched samples, which may limit the direct translational inference of the proliferative subpopulation identified at single-cell resolution to the bulk-level prognostic model. Finally, whether IPRS can predict benefit from immunotherapy requires further validation in

clinical cohorts receiving immunotherapeutic regimens and in patient-derived experimental models.

Overall, this study suggests that IPRS captures part of the link between proliferative tumor states and immune contexture in osteosarcoma. The model showed prognostic value, but its clinical utility still needs further confirmation. Future studies using spatial transcriptomics, single-cell multi-omics or patient-derived models may help determine whether IPRS reflects spatial immune exclusion, myeloid suppression or other clinically relevant immune states. Such work may also clarify how proliferative tumor cells interact with the immune microenvironment and how this interaction could be used to guide combination therapy in osteosarcoma.

Conclusion

In conclusion, this study integrated single-cell transcriptomic analysis, bulk transcriptomic profiling, machine-learning modeling, and experimental validation to characterize immune- and proliferation-related prognostic features in osteosarcoma. We identified a proliferative osteoblastic subpopulation marked by active chromosome segregation and mitotic activity, supporting proliferative heterogeneity as a relevant feature of osteosarcoma biology. By combining proliferation-related signals with immune-associated gene modules, we established an immune-proliferation-related signature (IPRS) model that showed stable prognostic performance in the training and external validation cohorts. The IPRS score was independently associated with overall survival and was also linked to immune microenvironmental features and predicted drug sensitivity. In vitro assays further showed that knockdown of *ALG6*, a key IPRS gene, inhibited osteosarcoma cell proliferation and promoted apoptosis. Overall, these findings support the prognostic value of IPRS in osteosarcoma and suggest that *ALG6* warrants further mechanistic investigation.

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Disclosure of conflict of interest

None.

Address correspondence to: Haoling Zhang, Department of Biomedical Sciences, Advanced Medical and Dental Institute, Universiti Sains Malaysia, Jalan Tun Hamdan Sheikh Tahir, 13200 Kepala Batas, Pulau Pinang, Malaysia. E-mail: zhanghaolingedu@163.com; Wenjun Meng, Department of Pain Management, West China Hospital, Sichuan University, No. 37 Guoxue Alley, Wuhou District, Chengdu 610041, Sichuan, China. E-mail: mwj1995@scu.edu.cn; Tao Deng, The Fourth Stationed OPD, The General Hospital of Western Theater Command, Chengdu 610036, Sichuan, China. E-mail: 13628018180@163.com

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Immune-proliferation signatures in osteosarcoma

Supplementary Table 2. Proliferation-associated gene set

	Type	Sequence (5'-3')
β-actin	Forward	CATGTACGTTGCTATCCAGGC
	Reverse	CTCCTTAATGTCACGCACGAT
ALG6	Forward	GGCCTCAAAGGAAAGGGGTT
	Reverse	CACGATCAACCGGGAAGAGT
si-ALG6	Sense	AATTCTTATTCAGGTGCTGGTAA
	Antisense	TTACCAGCACCTGAATAAGAATT
si-NC	Sense	TTCTCCGAACGTGTCACGT
	Antisense	ACGTGACACGTTCGGAGAA

Abbreviations: siRNA, small interfering RNA; si-ALG6, ALG6-targeting siRNA.