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Yang Su

Shanghai Jiao Tong University

Ji'an Liu

Shanghai Jiao Tong University

Zhengrui Li

Shanghai Jiao Tong University

Zhu Liu

liuzhu@scmc.com.cn

Shanghai Children's Medical Center

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Integrated multi-omics and experimental analyses identify three post-translational modification genes driving osteosarcoma progression and prognosis

Yang Su², Ji'An Liu², Zhengrui Li^{2*}, Zhu Liu^{1*}

1 Department of Orthopedic Surgery, Shanghai Children's Medical Center, School of Medicine, Shanghai Jiao Tong University, Shanghai 200127, China. , liuzhu@scmc.com.cn

2 Shanghai Jiao Tong University School of Medicine, 200011, Shanghai, China. suyangxmu@163.com, lja2786@sjtu.edu.cn, lzh_0108@sjtu.edu.cn

Correspondence author*

Zhengrui Li, No. 227, Chongqing South Road, Huangpu District, 200025, Shanghai, China.

Zhu Liu, Department of Orthopedic Surgery, Shanghai Children's Medical Center, School of Medicine, Shanghai Jiao Tong University, No. 1678, Dongfang Road, Pudong New Area , 200127, Shanghai

Abstract

Background: Osteosarcoma, a malignant bone tumor predominantly affecting adolescents, is marked by high recurrence and metastasis rates, which contribute to poor patient prognosis and emphasize the need for innovative therapeutic approaches. This study aims to identify core genes related to post-translational modifications (PTMs) implicated in osteosarcoma by integrating transcriptomic data and examine their roles in tumor biology and prognosis. **Results:** Through extensive bioinformatics analysis, including transcriptome profiling and single-cell RNA sequencing, three PTM-related genes (VCAN, RRP12, and CREG1) were identified as significantly associated with osteosarcoma. These genes demonstrated distinct expression patterns between tumor and normal tissues. A prognostic model based on these three genes, developed via LASSO Cox regression, revealed significantly lower survival rates for high-risk patients compared to the low-risk cohort ($P < 0.001$), indicating its strong predictive value. Experimental validation confirmed that silencing CREG1 significantly impaired osteosarcoma cell proliferation and migration, highlighting its role in tumor progression. Additionally, CMap analysis indicated that tumor samples with elevated CREG1 expression exhibited negative connectivity scores with several compounds, suggesting potential therapeutic targets. **Conclusions:** This study provides a

comprehensive analysis of the pivotal role of PTM-related genes in osteosarcoma, and further investigation of their functional mechanisms is warranted to enhance precision therapies for the disease.

Keywords: osteosarcoma, post-translational modification, prognostic model, bioinformatics, Single-cell RNA sequencing

Background

Osteosarcoma (OS), one of the most prevalent malignant bone tumors in adolescents, presents a significant clinical challenge due to its aggressive nature and high propensity for distant metastasis [1]. The disease not only imposes considerable physiological burdens but also severely affects the mental health of patients. With high recurrence and metastasis rates, patient survival remains low, placing substantial strain on healthcare systems and contributing to economic burdens [2]. OS exhibits a bimodal age distribution, primarily affecting adolescents and the elderly, with a slightly higher incidence in males. Its pathogenesis is driven by multiple genetic factors linked to bone formation, which promote tumor progression and metastasis [3, 4]. Current treatment strategies primarily involve surgical resection and multi-drug chemotherapy. However, while the event-free survival rate for localized OS has improved, outcomes for metastatic and recurrent cases remain poor, highlighting the need for novel diagnostic and therapeutic approaches [5, 6].

In recent years, advancements in molecular biology and high-throughput sequencing have facilitated deeper exploration of the molecular mechanisms underlying OS, particularly the critical role of post-translational modifications (PTMs) in tumorigenesis and progression [7, 8]. PTMs regulate various biological processes, including cell cycle control, signal transduction, and cellular stress responses, by modulating protein activity, stability, and localization. Abnormal PTM patterns have been strongly linked to malignant phenotypes in numerous cancers [9-13]. While studies have highlighted the influence of PTM-mediated signaling pathways on OS cell behavior, the systematic integration of PTM-related genes with clinical outcomes in OS remains limited. Additionally, the specific mechanisms by which PTMs contribute to OS drug resistance are not yet fully understood [14, 15].

Given the current state of research and existing knowledge gaps, the systematic identification of core genes linked to PTMs in OS, along with an exploration of their roles in TME regulation and therapy resistance, holds significant theoretical and clinical value. Such investigations could not only enhance our understanding of the molecular heterogeneity of OS but also identify potential molecular targets for precision therapies. Previous studies have predominantly focused on single-omics data, with limited integration of cross-platform, multi-dimensional datasets, restricting a comprehensive understanding of the PTM regulatory network in OS [16, 17].

To address this, this study proposes an integrative transcriptomics approach, combined

with single-cell RNA sequencing (scRNA-seq), to capture the expression profiles of PTM-related genes and their cellular heterogeneity within OS tissues. By performing differential expression analysis across multiple independent datasets, this study identified PTM-related core genes associated with OS progression and constructed prognostic models based on these genes to assess their clinical predictive value. Additionally, immune infiltration analysis and functional experiments were conducted to investigate the mechanisms through which PTM genes regulate the TME and contribute to therapy resistance. This multi-faceted approach is designed to overcome the limitations of traditional single-omics studies and offer a deeper insight into the PTM regulatory networks in OS [15, 18].

In summary, this study aims to systematically identify PTM-related core genes in OS using advanced bioinformatics and experimental validation, clarify their roles in tumor progression and therapy resistance, and develop high-accuracy prognostic models. This research will provide new tools for molecular classification and clinical risk assessment of OS, as well as lay the groundwork for developing personalized PTM-targeted therapies, advancing the field of precision medicine in OS.

Methods

Data acquisition and preprocessing

Three independent OS transcriptomic datasets (GSE16091, GSE16088, and GSE14359) were retrieved from the Gene Expression Omnibus (GEO) database, along with raw expression matrices and corresponding platform annotation files. Probe identifiers were mapped to official gene symbols based on platform annotations, and for genes represented by multiple probes, the median expression value was retained to represent gene-level expression. These datasets were then merged into a combined cohort for integrative analysis. To minimize technical variability due to differing platforms and experimental conditions, batch effects were corrected using the ComBat algorithm from the “sva” R package. Principal component analysis (PCA) was performed to assess the effectiveness of batch correction and ensure the elimination of systematic bias prior to downstream analyses.

Differential expression analysis between OS and normal tissues was conducted using the “limma” R package [19]. Linear modeling with empirical Bayes moderation was applied to estimate differential expression. Genes with $|\log_2 \text{fold change}| \geq 1$ and false discovery rate (FDR) < 0.05 were classified as differentially expressed genes (DEGs). Volcano plots were generated to visually represent the transcriptional alterations between tumor and control samples.

Identification of osteosarcoma-associated PTMs core genes

To identify PTM-related genes specifically involved in OS, multi-source gene sets were integrated. OS-associated genes were retrieved from the Comparative Toxicogenomics Database (CTD) [20], Online Mendelian Inheritance in Man (OMIM) [21], and GeneCards

[22] using the keyword “osteosarcoma.” After removing duplicates, a curated set of OS-related genes was established. PTM-related genes were obtained from a recent multi-omics study (PMID: 41044669) [23], which identified PTM-associated regulators through bulk RNA-seq, single-cell RNA-seq, and spatial transcriptomics analyses. Venn analysis was then used to identify the intersection of GEO-DEGs, OS-associated genes (CTD/OMIM/GeneCards), and PTM-related genes. Genes present in all three datasets were considered OS-PTM core genes and were subjected to further functional and prognostic analyses.

Functional enrichment and protein–protein interaction analysis

To explore the biological significance of the identified core genes, Gene Ontology (GO) enrichment analysis, covering biological processes (BP), cellular components (CC), and molecular functions (MF), along with Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis, was performed using the “clusterProfiler” R package [24]. Adjusted P values were calculated using the Benjamini–Hochberg method, with pathways having an adjusted $P < 0.05$ deemed significantly enriched.

Protein–protein interaction (PPI) networks were constructed using the STRING database [25], applying a minimum interaction confidence score of 0.4. The interaction network was visualized using Cytoscape, and topological properties such as node degree were evaluated to identify potential hub genes and functional modules within the network.

Construction and validation of the LASSO-based prognostic signature

To construct a prognostic model based on the identified core genes, this study utilized the TARGET-OS cohort, which includes clinical survival data. Least Absolute Shrinkage and Selection Operator (LASSO) Cox regression, performed using the “glmnet” R package [26], was applied to minimize overfitting and select the most relevant prognostic variables. Ten-fold cross-validation was used to identify the optimal penalty parameter (λ) corresponding to the minimum binomial deviance.

A risk score for each patient was calculated using the regression coefficients derived from the LASSO model, according to the formula: $\text{RiskScore} = 6.73 + 0.130 \times \text{VCAN} - 1.367 \times \text{RRP12} + 0.547 \times \text{CREG1}$. Patients were classified into high- and low-risk groups based on the median RiskScore. Kaplan–Meier survival curves were generated, and group differences were assessed using the log-rank test. Hazard ratios (HRs) and 95% confidence intervals (CIs) were calculated using Cox proportional hazards models. Predictive performance was evaluated via receiver operating characteristic (ROC) curve analysis, and the area under the curve (AUC) was calculated. External validation was performed using independent datasets, including TARGET-OS validation subsets and GSE126209.

Single-cell RNA sequencing analysis

Single-cell RNA-seq data were analyzed using the ‘Seurat’ package. Cells with fewer than 200 detected genes or those with more than 5% mitochondrial gene content were excluded to ensure data quality. The data were normalized and scaled before dimensionality reduction. PCA was performed, followed by clustering and Uniform

Manifold Approximation and Projection (UMAP) visualization. Cell clusters were annotated based on canonical marker genes.

The expression distribution of VCAN, RRP12, and CREG1 across different cell populations was visualized using FeaturePlot and violin plots. Pearson correlation analysis was performed at the single-cell level to examine gene–gene relationships within the TME.

Protein structure and immunofluorescence validation

Predicted protein structures of VCAN, RRP12, and CREG1 were obtained from publicly available structural databases to assess their tertiary structures. Immunofluorescence staining images for the U2OS OS cell line were retrieved from the Human Protein Atlas (HPA) [27]. Target proteins were labeled in green, α -Tubulin in red, and nuclei with DAPI in blue. Merged fluorescence images and three-dimensional intensity reconstructions were analyzed to determine the subcellular localization and spatial distribution of the proteins.

Cell culture

Human OS cell lines, U2OS and 143B, were obtained from Shanghai Jiao Tong University School of Medicine. Cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin at 37°C in a humidified incubator with 5% CO₂. Routine mycoplasma contamination testing was conducted, and cells were used within 10 passages.

siRNA transfection

Two independent small interfering RNAs (si-1 and si-2) targeting CREG1 were synthesized by Sangon Biotech (Shanghai, China), with a non-targeting scrambled siRNA serving as the negative control (NC). Cells were transfected using Lipofectamine 3000 according to the manufacturer's protocol. After 48 hours of transfection, cells were harvested for subsequent RNA, protein, and functional assays.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted using TRIzol reagent and reverse-transcribed into cDNA with a reverse transcription kit. Quantitative PCR was conducted using SYBR Green Master Mix on a real-time PCR system.

Primer sequences were as follows: *GAPDH*, Forward: 5'-CTGGGCTACACTGAGCACC-3', Reverse: 5'-AAGTGGTCGTTGAGGGCAATG-3'; *CREG1*, Forward: 5'-GGCGTGCCCTATTTCTACCTG-3', Reverse: 5'-CAAAGTCATGGTCAGTGTAGCAT-3'. Relative gene expression was calculated using the $2^{-\Delta\Delta C_t}$ method, with GAPDH as the internal reference.

Western blot analysis

Total protein was extracted using RIPA lysis buffer supplemented with protease inhibitors. Equal protein amounts were separated by SDS–PAGE and transferred to PVDF membranes. Membranes were blocked with 5% non-fat milk and incubated overnight at 4°C with the following primary antibodies: CREG1 (abs147072, 1:1000; 24 kDa), GAPDH

(abs132004, 1:10000; 38 kDa). After incubation with HRP-conjugated secondary antibodies, signals were detected using enhanced chemiluminescence (ECL). Relative protein expression was quantified by densitometry and normalized to GAPDH.

Colony formation assay

Transfected cells were seeded into six-well plates at low density (approximately 500–800 cells per well) and cultured for 10–14 days. Colonies were fixed with 4% paraformaldehyde and stained with crystal violet. Colonies containing more than 50 cells were counted under a microscope, and colony numbers were quantified and statistically compared between groups.

Wound healing assay

Cells were seeded in six-well plates and cultured to near confluence. A sterile pipette tip was used to create a linear scratch wound. After washing to remove debris, cells were cultured in serum-free medium. Images were captured at 0 and 24 hours. The wound closure rate was calculated as: $\text{Scratch healing rate (\%)} = (\text{initial wound width} - \text{final wound width}) / \text{initial wound width} \times 100\%$.

Transwell migration and invasion assays

For migration assays, transfected cells were seeded into the upper chamber of Transwell inserts without Matrigel coating. For invasion assays, inserts were pre-coated with Matrigel. Cells were allowed to migrate or invade for 24 hours. Cells that migrated to the lower surface were fixed, stained with crystal violet, photographed, and counted in multiple random fields. Quantification was performed using ImageJ software.

Connectivity Map (CMap) analysis for therapeutic compound identification

To identify small-molecule compounds that may reverse the oncogenic transcriptional signature associated with CREG1 dysregulation, drug prediction analysis was performed using the CMap database. DEGs between CREG1-high and CREG1-low tumors were identified across pan-cancer cohorts from TCGA. The top upregulated and downregulated genes were submitted to the CMap platform.

The XSum algorithm was applied to calculate connectivity scores between gene expression signatures and small-molecule perturbation profiles. Compounds with significantly negative connectivity scores were considered potential candidates for reversing CREG1-associated transcriptional alterations. Cross-cancer enrichment frequencies were summarized to evaluate the robustness of the drug–signature associations.

Drug sensitivity analysis based on GDSC datasets

To assess whether CREG1 expression affects drug responsiveness, pharmacogenomic data were obtained from the Genomics of Drug Sensitivity in Cancer (GDSC1 and GDSC2) databases. Gene expression data and corresponding half-maximal inhibitory concentration (IC_{50}) values were integrated.

Spearman correlation analysis was performed to determine the relationship between

CREG1 expression and IC₅₀ values across cancer cell lines. Positive correlations indicated potential drug resistance in CREG1-high tumors, while negative correlations suggested increased drug sensitivity.

Validation using CTRP pharmacogenomic data

Pharmacogenomic data from the Cancer Therapeutics Response Portal (CTRP) database were also retrieved to further validate drug resistance patterns. AUC values were analyzed, and correlations between CREG1 expression and AUC values were calculated. Higher AUC values indicated reduced drug sensitivity, supporting resistance phenotypes.

Statistical analysis

All experiments were performed independently at least three times. Data are presented as mean $\pm$ SD. Statistical comparisons were made using Student's t-test or one-way ANOVA, as appropriate. A P value of <0.05 was considered statistically significant.

Results

Integrated multi-database analysis identified three PTM-related core genes in osteosarcoma

To systematically characterize PTM-associated molecular alterations in OS, a multi-database integrative analytical framework was implemented (Fig. 1A). Transcriptomic profiles from three independent GEO datasets (GSE16091, GSE16088, and GSE14359) were integrated with OS-associated genes curated from the CTD, OMIM, and GeneCards databases. Additionally, PTM-related genes were sourced from a previously published multi-omics study (PMID: 41044669), which systematically identified PTM regulators through bulk RNA-seq, single-cell RNA-seq, and spatial transcriptomics approaches.

Following the merging of the GEO datasets and batch effect correction, PCA revealed effective normalization and clear separation between OS and normal control samples (Fig. 1B). PC1 accounted for 14.3% of the variance, and PC2 for 7.3%, demonstrating successful removal of inter-dataset technical bias while preserving biological heterogeneity. Differential expression analysis highlighted widespread transcriptional alterations between OS and normal tissues, as shown in the volcano plot (Fig. 1C), where significantly upregulated and downregulated genes were symmetrically distributed according to predefined thresholds ($|\log_2FC| \geq 1$, $FDR < 0.05$).

To identify core PTM regulators in OS, intersection analysis was performed across three gene sets: (1) GEO-derived DEGs, (2) OS-associated genes from CTD/OMIM/GeneCards, and (3) PTM-related genes. Venn diagram analysis revealed three overlapping genes: VCAN, RRP12, and CREG1 (Fig. 1D). These genes were designated as OS-PTM core genes and selected for subsequent functional, prognostic, and experimental validation.

The integrative strategy outlined in Fig. 1A highlights the analytical workflow, encompassing DEG identification, gene correlation analysis, PPI network construction, GO/KEGG enrichment analysis, Kaplan–Meier survival evaluation, and the establishment of a three-gene prognostic signature model. This stepwise approach ensured that the final candidates were not only statistically robust but also biologically and clinically relevant. This integrative multi-database analysis identified three PTM-related core genes—VCAN, RRP12, and CREG1—that potentially drive OS progression and provide a molecular foundation for further mechanistic and therapeutic investigations.

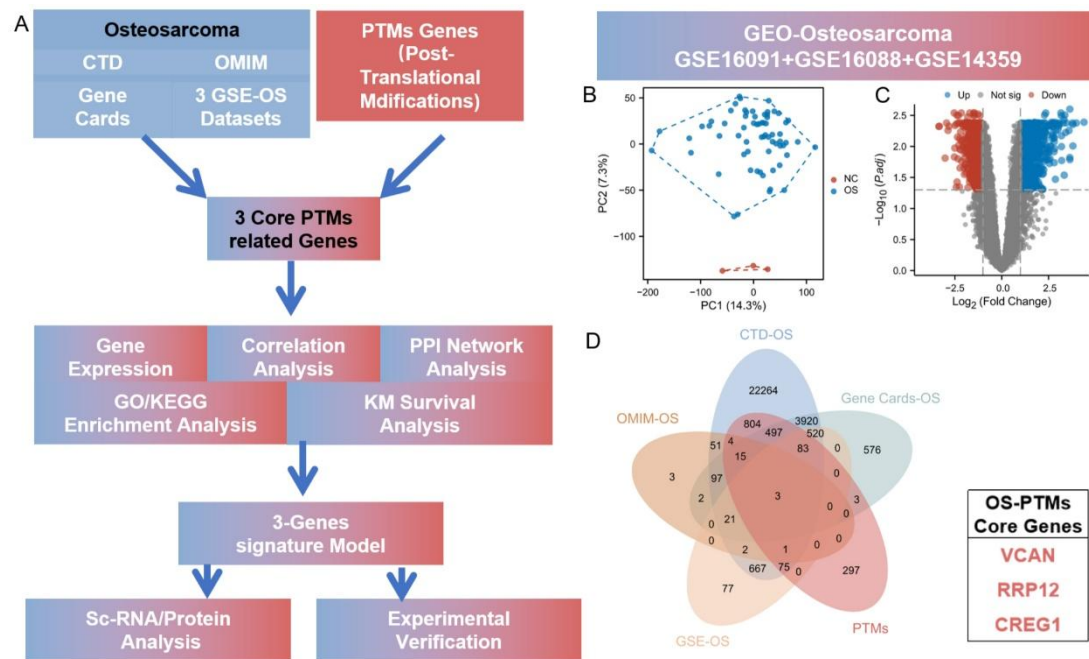


Figure 1. Integrated multi-database identification of PTMs-related core genes in osteosarcoma. (A) Schematic workflow of the integrative analytical strategy. Differentially expressed genes (DEGs) were derived from three GEO datasets (GSE16091, GSE16088, GSE14359) and intersected with osteosarcoma-associated genes from CTD, OMIM, and GeneCards, as well as PTMs-related genes obtained from published multi-omics analysis (PMID: 41044669). Downstream analyses included gene expression profiling, correlation analysis, PPI network construction, functional enrichment, survival analysis, and development of a three-gene prognostic model. (B) Principal component analysis (PCA) plot showing effective batch correction and clustering of osteosarcoma (OS) and normal control (NC) samples after integration of GEO datasets. (C) Volcano plot illustrating differential gene expression between OS and normal tissues. Red dots represent significantly upregulated genes, blue dots represent downregulated genes, and gray dots indicate non-significant genes. (D) Venn diagram demonstrating the intersection among GEO-DEGs, OS-related genes (CTD/OMIM/GeneCards), and PTMs-related genes. Three overlapping genes (VCAN, RRP12, and CREG1) were identified as PTMs-associated core genes in osteosarcoma.

Core genes exhibit coordinated transcriptional regulation and enrichment in ECM and ribosomal pathways

To further characterize the transcriptional landscape of the three PTM-related core genes in OS, their global expression patterns were examined in the integrated GEO cohort. Hierarchical clustering of DEGs revealed distinct tumor–normal segregation, highlighting extensive transcriptional reprogramming in OS tissues (Fig. 2A). VCAN, RRP12, and CREG1 consistently exhibited differential expression between OS and normal samples (Fig. 2B), confirming their stability as core candidates identified through multi-database integration. Correlation analysis was performed to explore potential regulatory

relationships among the three genes. Pairwise correlation plots revealed statistically significant positive associations between VCAN, RRP12, and CREG1 ($P < 0.05$; Fig. 2C), suggesting coordinated transcriptional regulation and possible functional interplay.

To investigate the biological implications of these coordinated alterations, GO and KEGG enrichment analyses were conducted based on core genes and their interaction partners. The enrichment results revealed significant overrepresentation of pathways related to ribosome biogenesis, rRNA metabolic processing, extracellular matrix organization, glycosaminoglycan binding, and cell adhesion molecule signaling (Fig. 2D–E). These results suggest that PTM-related dysregulation in OS may simultaneously affect both intracellular translational machinery and extracellular matrix remodeling processes.

PPI network analysis further highlighted VCAN as a central hub, interacting with several extracellular matrix and structural proteins (Fig. 2F). A refined network focusing on core nodes revealed VCAN, RRP12, and CREG1 as key connectors within a broader regulatory module (Fig. 2G), emphasizing their potential cooperative role in modulating TME dynamics and translational activity.

These results suggest that the identified PTM-related core genes participate in coordinated regulatory networks that link ribosomal function and extracellular matrix remodeling in OS.

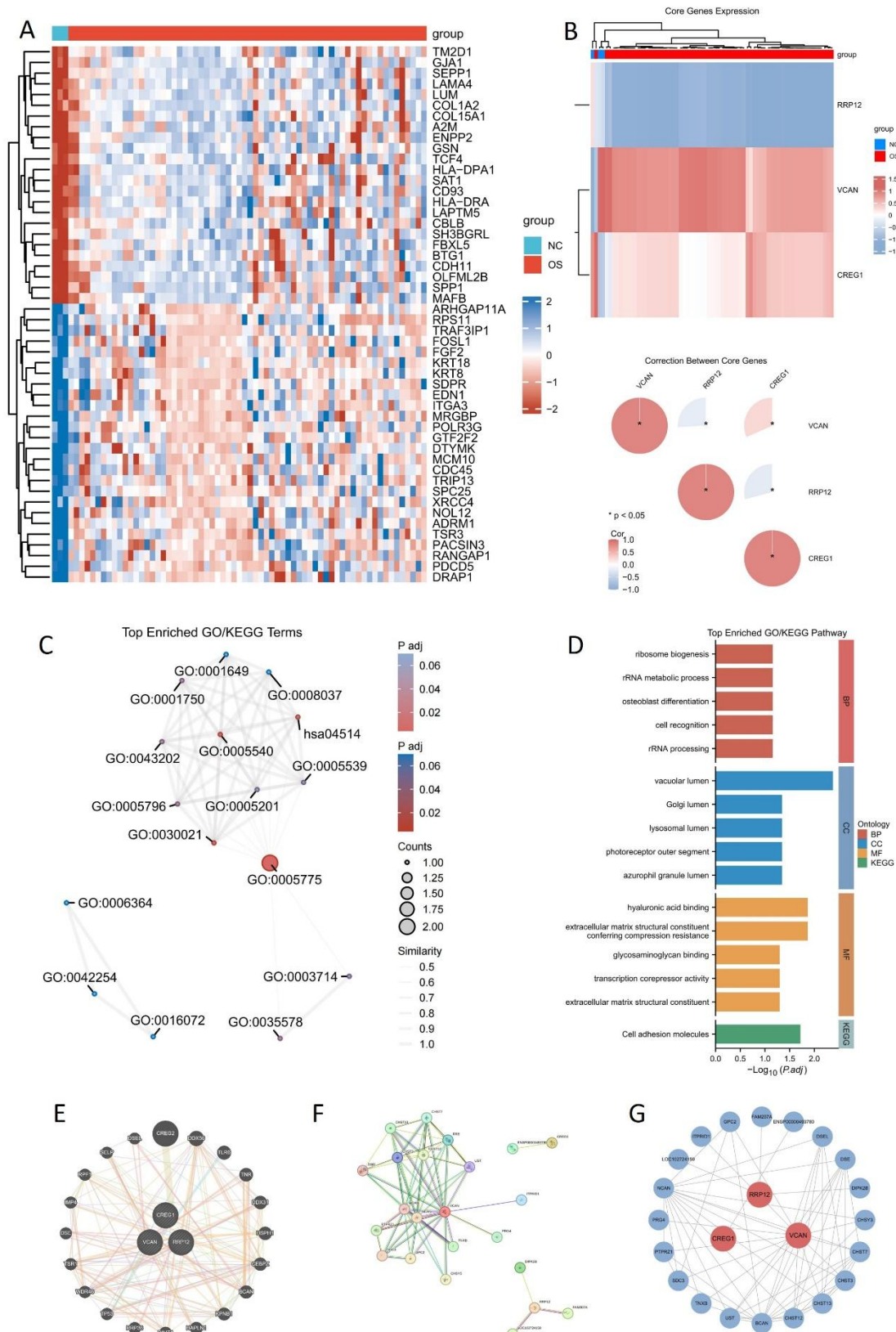


Figure 2. Coordinated expression patterns, functional enrichment, and interaction networks of PTMs-related core genes in osteosarcoma. (A) Heatmap of differentially expressed genes between osteosarcoma (OS) and normal control (NC) samples across the integrated GEO cohort. Hierarchical clustering demonstrates clear segregation

between tumor and normal tissues. (B) Expression heatmap of the three PTMs-related core genes (VCAN, RRP12, and CREG1) in OS and NC samples, illustrating consistent differential expression patterns. (C) Correlation matrix showing pairwise Pearson correlation coefficients among VCAN, RRP12, and CREG1. Circle size and color intensity represent correlation strength. * $P < 0.05$. (D) Network visualization of top enriched GO and KEGG terms associated with the core gene set. Node color indicates adjusted P value, and node size represents gene count. (E) Bar plot summarizing significantly enriched GO biological process (BP), cellular component (CC), molecular function (MF), and KEGG pathways. The x-axis represents $-\log_{10}$ (adjusted P value). (F) STRING-derived protein–protein interaction (PPI) network illustrating interactions between core genes and associated proteins. (G) Refined core interaction network highlighting VCAN, RRP12, and CREG1 as central nodes within the osteosarcoma PTMs-associated regulatory module.

Establishment of a three-gene LASSO prognostic model

To assess the prognostic value of the three PTM-related core genes (VCAN, RRP12, and CREG1), a LASSO Cox regression model was developed using the TARGET-OS cohort. Coefficient profiles for the candidate genes were generated across various λ values (Fig. 3A). As λ increased, the coefficients of the genes gradually approached zero, reflecting the regularization effect of the LASSO algorithm. Ten-fold cross-validation was used to determine the optimal penalty parameter (λ), with the minimum binomial deviance observed at the selected λ value (Fig. 3B), where all three genes remained in the final prognostic model.

The derived risk score formula was: $\text{RiskScore} = 6.73 + 0.130 \times \text{VCAN} - 1.367 \times \text{RRP12} + 0.547 \times \text{CREG1}$. VCAN and CREG1 exhibited positive regression coefficients, indicating that higher expression levels were associated with increased mortality risk, while RRP12 had a negative coefficient, suggesting a potential protective role in OS progression.

Patients were stratified into high-risk and low-risk groups based on the median RiskScore. The distribution of risk scores and corresponding gene expression patterns are shown in Fig. 3C. As risk scores increased, mortality events became more frequent, and distinct expression gradients of VCAN, RRP12, and CREG1 were observed between the groups. High-risk patients had elevated VCAN and CREG1 expression and reduced RRP12 expression.

Kaplan–Meier survival analysis revealed that high-risk patients had significantly worse overall survival compared to the low-risk group (log-rank $P < 0.05$). To further evaluate predictive performance, time-independent ROC analysis was conducted (Fig. 3D). The three-gene risk model achieved an AUC of 1.000 in the training cohort, outperforming each individual gene (RRP12 AUC = 0.980; CREG1 AUC = 0.955; VCAN AUC = 0.869), demonstrating superior prognostic discrimination.

To assess the model's robustness, external validation was performed. In the TARGET-OS validation cohort, the risk model maintained predictive capability with an AUC of 0.758 (95% CI: 0.507–0.908) (Fig. 3E). Additionally, in the independent dataset GSE126209, VCAN

expression alone showed strong discriminatory power between OS and normal tissues (AUC = 0.901, 95% CI: 0.818–0.984) (Fig. 3F), further supporting its clinical relevance. These results establish a robust three-gene PTM-related prognostic signature with strong predictive performance and external validation support.

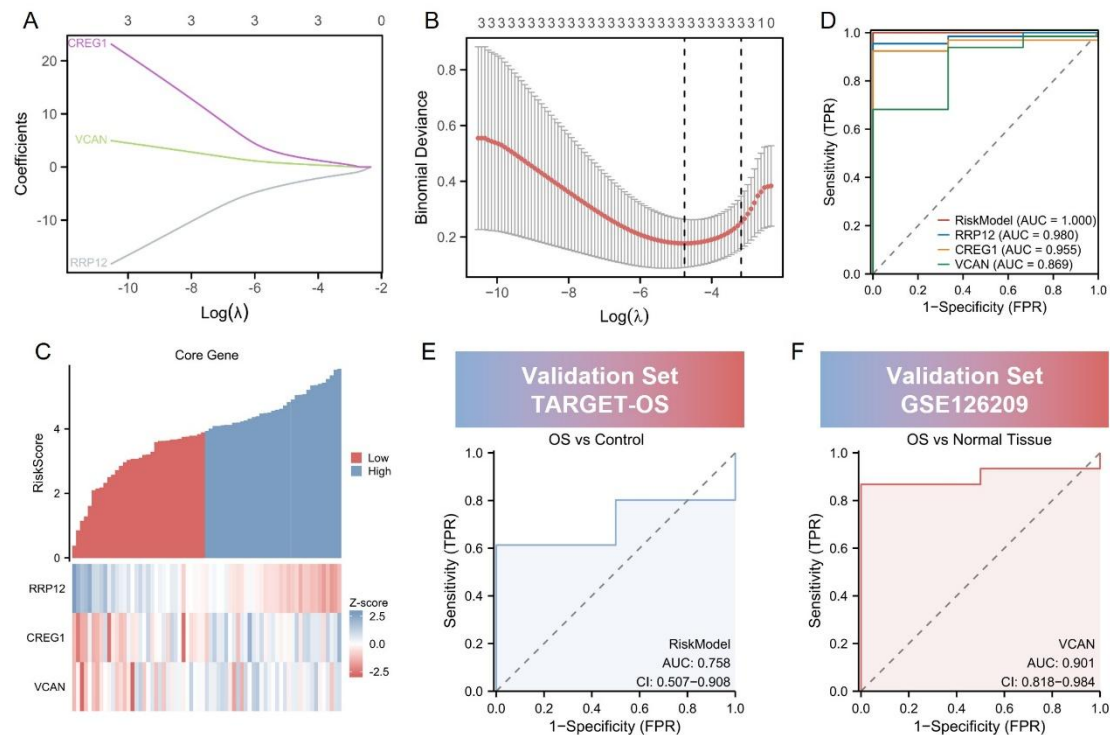


Figure 3. Construction and validation of a three-gene LASSO prognostic model in osteosarcoma. (A) LASSO coefficient profiles of VCAN, RRP12, and CREG1 plotted against $\log(\lambda)$. As λ increases, regression coefficients shrink toward zero. (B) Ten-fold cross-validation for tuning parameter (λ) selection in the LASSO Cox model. The dotted vertical lines represent the optimal λ values corresponding to minimum binomial deviance. (C) Distribution of RiskScore and corresponding gene expression patterns in the TARGET-OS cohort. Upper panel: ranked RiskScore distribution; middle panel: survival status of patients; lower panel: heatmap showing expression of VCAN, RRP12, and CREG1 across risk groups. (D) ROC curves comparing predictive performance of the three-gene risk model and individual genes in the training cohort. The risk model achieved the highest AUC. (E) External validation of the risk model in the TARGET-OS validation set, showing ROC curve and AUC value. (F) ROC analysis of VCAN expression in the independent dataset GSE126209 for distinguishing osteosarcoma from normal tissues.

Single-cell transcriptomic analysis reveals tumor microenvironment heterogeneity

To further explore the cellular heterogeneity in OS and the cell-type-specific distribution of PTM-related core genes, scRNA-seq analysis was performed. UMAP clustering revealed multiple distinct cellular populations within OS samples, including malignant cells, osteoblasts, fibroblasts, endothelial cells, CD4⁺ T cells, CD8⁺ T cells, plasma cells, and

monocyte/macrophage populations (Fig. 4A). These results suggest the complex TME architecture in OS.

Next, the expression of VCAN, RRP12, and CREG1 was mapped across individual cell clusters. FeaturePlot visualization revealed heterogeneous expression patterns among different cellular populations (Fig. 4B). VCAN was predominantly enriched in malignant and stromal-associated clusters, particularly within fibroblast and monocyte/macrophage populations. RRP12 showed broader expression across proliferative clusters, aligning with its role in ribosome biogenesis pathways. In contrast, CREG1 exhibited higher expression in immune-related compartments, suggesting a potential role in immune modulation within the TME.

Violin plots were generated to quantitatively assess differential expression across cell types (Fig. 4C). These analyses confirmed significant cell-type-specific enrichment patterns, highlighting that PTM-related core genes contribute to distinct biological processes within tumor and stromal components.

To further investigate coordinated regulation at the single-cell level, pairwise correlation analyses were conducted. Scatter plots demonstrated significant positive correlations between VCAN and RRP12 ($R = 0.22$, $P = 0.0047$), VCAN and CREG1 ($R = 0.40$, $P = 5.96 \times 10^{-8}$), and RRP12 and CREG1 ($R = 0.35$, $P = 9.8 \times 10^{-7}$) (Fig. 4E). These results suggest that despite the heterogeneous cellular localization, the three genes exhibit coordinated transcriptional activity within the OS microenvironment.

Cluster-level functional characterization revealed differential pathway activation states across cell populations (Fig. 4D), supporting the observed biological heterogeneity at the transcriptional level.

In summary, single-cell analysis provides high-resolution evidence that VCAN, RRP12, and CREG1 are differentially expressed across tumor and stromal compartments, while maintaining coordinated regulatory relationships. This links PTM-associated transcriptional dysregulation to the remodeling of the TME.

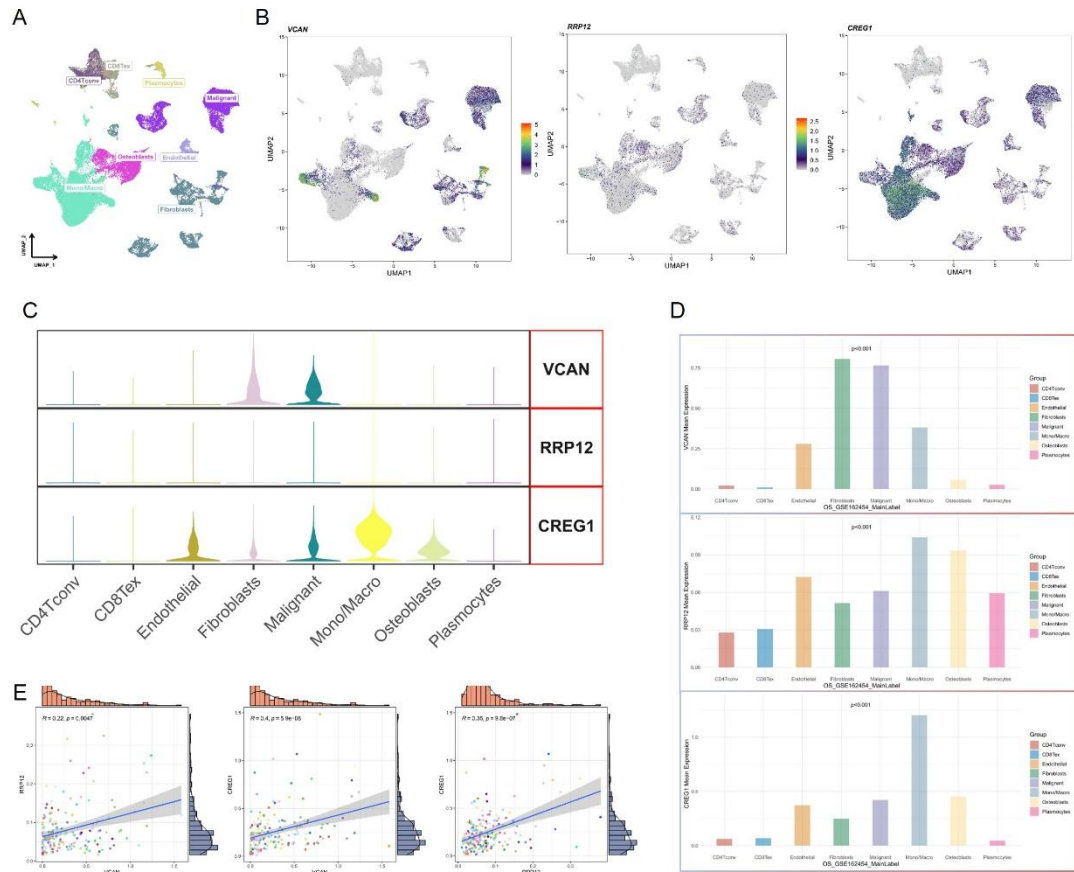


Figure 4. Single-cell transcriptomic characterization of PTMs-related core genes in osteosarcoma. (A) UMAP visualization of single-cell RNA sequencing data from osteosarcoma samples. Distinct cellular populations were identified, including malignant cells, osteoblasts, fibroblasts, endothelial cells, CD4⁺ T cells, CD8⁺ T cells, plasma cells, and monocyte/macrophage populations. (B) FeaturePlot showing the distribution of VCAN, RRP12, and CREG1 expression across single-cell clusters. (C) Violin plots illustrating cell-type-specific expression levels of VCAN, RRP12, and CREG1 across annotated cell populations. (D) Functional state or pathway enrichment distribution across cell clusters, indicating heterogeneity in biological activity within the tumor microenvironment. (E) Pairwise correlation analysis of VCAN, RRP12, and CREG1 at the single-cell level. Scatter plots display Pearson correlation coefficients and corresponding P values.

Protein-level validation and subcellular localization analysis of PTM-related core genes

To further validate the translational relevance of the identified PTM-related core genes, structural modeling and immunofluorescence-based protein localization analysis were performed.

Predicted tertiary structures of VCAN, RRP12, and CREG1 were obtained through protein structure modeling (Fig. 5A–C). VCAN displayed a large, complex conformation consistent with its known role in extracellular matrix scaffolding. RRP12 exhibited a

multi-domain structure enriched in α -helical regions, in line with its reported involvement in ribosomal assembly and nucleocytoplasmic transport. CREG1 showed a compact globular configuration, suggesting versatility in regulation and protein interactions. These structural features support their multifunctional roles in tumor biology.

To experimentally assess protein expression and subcellular distribution, immunofluorescence staining was conducted in U2OS OS cells using validated primary antibodies (CREG1: abs147072; GAPDH as a loading control in parallel Western blot validation). Confocal microscopy revealed detectable expression of VCAN, RRP12, and CREG1 (Fig. 5D–F). VCAN exhibited a predominantly cytoplasmic and pericellular distribution pattern, consistent with its extracellular matrix-associated function. RRP12 showed strong cytoplasmic staining with perinuclear enrichment, reflecting its role in ribosomal biogenesis. CREG1 demonstrated diffuse cytoplasmic localization with partial overlap with cytoskeletal structures.

Merged fluorescence images with α -tubulin co-staining revealed partial spatial association of all three proteins with the cytoskeletal architecture (Fig. 5D–F). Three-dimensional fluorescence intensity reconstruction further confirmed the intracellular accumulation patterns and spatial distribution heterogeneity.

These protein-level observations validate the transcriptomic data and provide mechanistic evidence that VCAN, RRP12, and CREG1 are actively expressed in OS cells. Their subcellular localization patterns suggest functional involvement in extracellular matrix remodeling, translational regulation, and intracellular signaling.

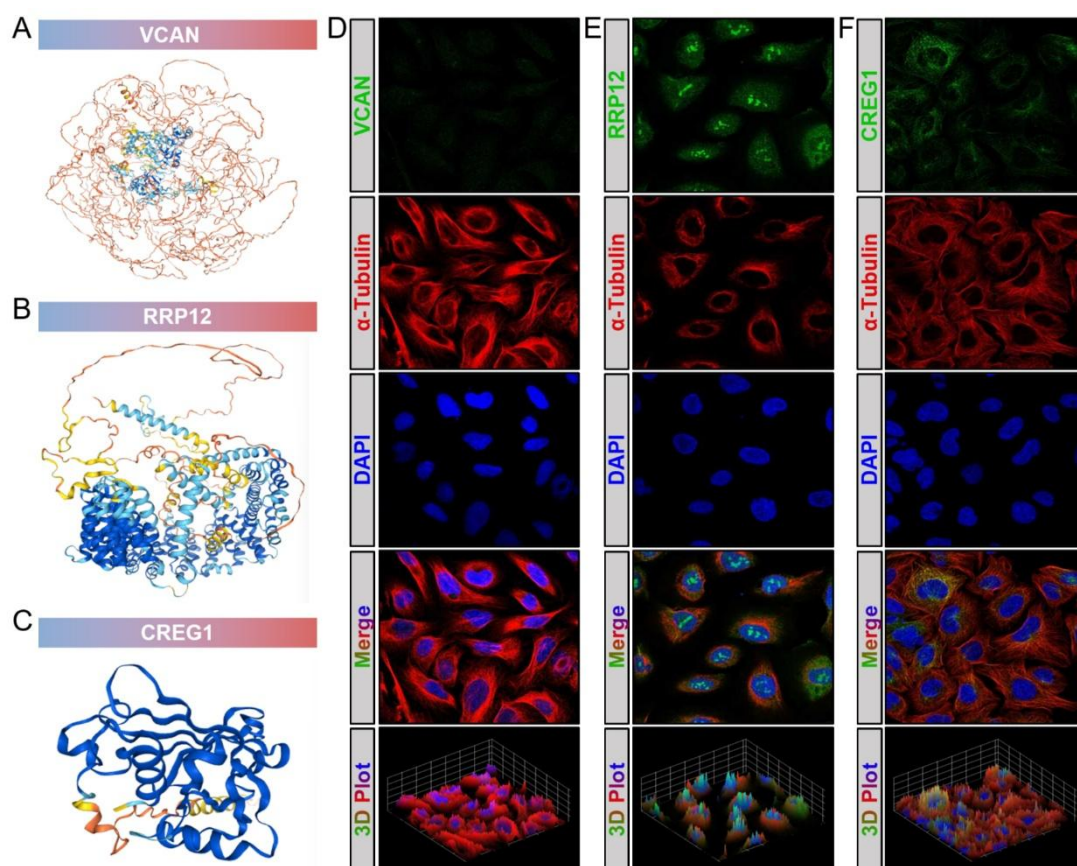


Figure 5. Protein structure prediction and immunofluorescence validation of PTMs-related

core genes. (A–C) Predicted tertiary protein structures of VCAN (A), RRP12 (B), and CREG1 (C), illustrating distinct domain architectures and conformational characteristics. (D–F) Immunofluorescence staining of VCAN (D), RRP12 (E), and CREG1 (F) in U2OS osteosarcoma cells. Green: target protein; Red: α -tubulin; Blue: DAPI nuclear staining. Merged images demonstrate cytoplasmic localization with partial cytoskeletal association. Three-dimensional fluorescence intensity reconstruction plots illustrate spatial distribution patterns.

Efficient silencing of CREG1 in osteosarcoma cells

To investigate the functional role of CREG1 in OS, siRNA-mediated knockdown was performed in U2OS and 143B cell lines. Quantitative real-time PCR analysis showed that both siRNA constructs (si-1 and si-2) significantly reduced CREG1 mRNA expression compared to the NC group in both cell lines (Fig. 6A), with knockdown efficiency exceeding 60% at the transcriptional level. Correspondingly, Western blot analysis confirmed substantial downregulation of CREG1 protein (24 kDa) following siRNA transfection, with GAPDH (38 kDa) used as a stable loading control (Fig. 6B). Densitometric quantification further validated efficient silencing at the protein level. These results demonstrate effective suppression of CREG1 expression in OS cells, establishing a reliable model for subsequent functional investigations.

CREG1 promotes proliferation, migratory, and invasive capacity in osteosarcoma cells

To assess the impact of CREG1 on tumor cell growth, colony formation assays were performed. Silencing of CREG1 significantly reduced clonogenic potential in both U2OS and 143B cells (Fig. 6C). The number of colonies formed in the si-1 and si-2 groups was significantly lower compared to the NC group ($P < 0.001$), indicating impaired proliferative capacity following CREG1 depletion. Three-dimensional reconstruction of colony density further revealed reduced colony formation intensity in the CREG1 knockdown groups, reinforcing the suppressive effect on long-term growth potential. These results suggest that CREG1 positively regulates proliferative and survival signaling pathways in OS cells. Wound healing assays were conducted to evaluate cell migration. At 24 hours post-scratch, CREG1 knockdown significantly delayed wound closure compared to the NC group in both U2OS and 143B cells (Fig. 6D). Quantitative analysis showed a significant reduction in the scratch healing rate in siRNA-treated cells ($P < 0.01$ or $P < 0.001$), indicating impaired migratory capacity. These results suggest that CREG1 contributes to cytoskeletal dynamics and cell motility, consistent with its observed cytoplasmic localization in immunofluorescence analysis.

To further investigate the role of CREG1 in tumor aggressiveness, Transwell migration and Matrigel invasion assays were performed. CREG1 silencing significantly reduced the

number of migrated cells in both U2OS and 143B cell lines (Fig. 6E). Similarly, Matrigel-coated invasion assays showed a marked decrease in invasive cell numbers following CREG1 knockdown (Fig. 6F).

Quantitative analysis revealed statistically significant suppression of both migration and invasion upon CREG1 depletion ($P < 0.001$). Three-dimensional intensity reconstruction of migrated/invaded cells further confirmed reduced cell density and distribution in siRNA-treated groups.

In summary, these results indicate that CREG1 promotes proliferative, migratory, and invasive behaviors in OS cells.

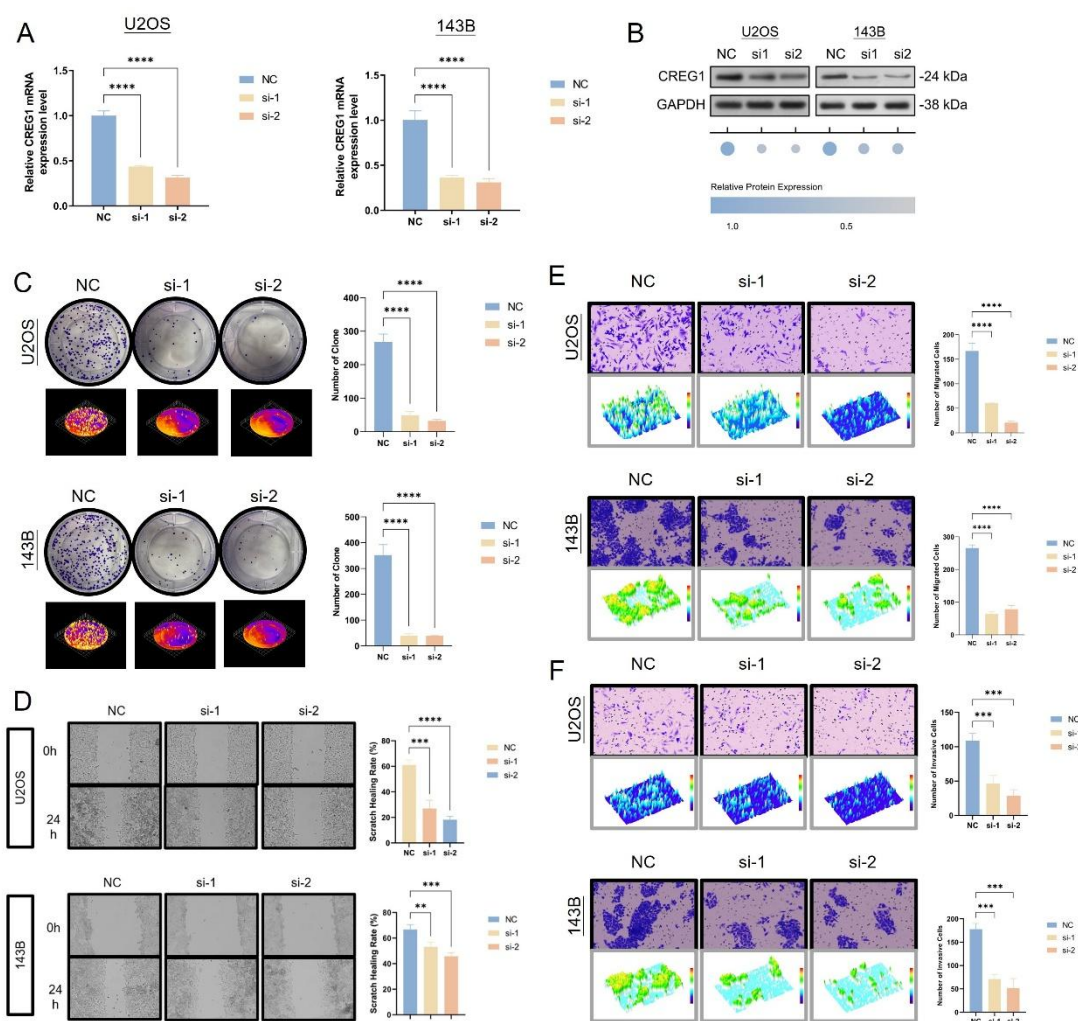


Figure 6. Functional validation of CREG1 in osteosarcoma cells. (A) qRT-PCR analysis of CREG1 mRNA expression in U2OS and 143B cells following siRNA-mediated knockdown. Data are presented as mean $\pm$ SD. **** $P < 0.0001$ versus NC. (B) Western blot analysis of CREG1 protein expression (24 kDa) after siRNA transfection. GAPDH (38 kDa) was used as a loading control. (C) Colony formation assays in U2OS and 143B cells following CREG1 knockdown. Representative images and quantitative analysis are shown. Three-dimensional reconstruction plots illustrate colony density distribution. **** $P < 0.0001$. (D) Wound healing assays demonstrating reduced migratory capacity in CREG1-depleted cells at 0 h and 24 h. Quantification of scratch healing rates is shown. *** $P < 0.001$; **** $P < 0.0001$. (E) Transwell migration assays showing decreased migrated

cell numbers upon CREG1 silencing in U2OS and 143B cells. Representative images, 3D reconstruction plots, and quantitative analysis are shown. **** $P < 0.0001$. (F) Matrigel invasion assays demonstrating impaired invasive ability following CREG1 knockdown. Quantitative data are presented as mean $\pm$ SD. *** $P < 0.001$; **** $P < 0.0001$.

CMap analysis identifies candidate compounds that potentially reverse CREG1-associated oncogenic signatures

To explore potential therapeutic strategies targeting CREG1-associated transcriptional programs, CMap analysis was performed using the XSum algorithm. Across multiple tumor types, several small-molecule compounds exhibited significantly negative connectivity scores, indicating their potential ability to reverse the CREG1-driven gene expression signature (Fig. 7A).

Ranked connectivity score distribution analysis identified AD.6809, W.13, and mercaptopurine as the top candidates with the most pronounced negative enrichment scores (Fig. 7B). These compounds consistently demonstrated reversal potential across various cancer contexts. Notably, mercaptopurine is a clinically established antimetabolite chemotherapeutic agent, while W.13 (a calmodulin antagonist) and AD.6809 (a kinase-associated inhibitor) may modulate intracellular signaling pathways potentially linked to CREG1-associated biological processes.

These results suggest that pharmacological modulation of specific signaling or metabolic pathways could counteract CREG1-related oncogenic transcriptional programs.

Elevated CREG1 expression is associated with reduced drug sensitivity in GDSC datasets

To determine whether CREG1 expression correlates with therapeutic response, pharmacogenomic data from the Genomics of Drug Sensitivity in Cancer (GDSC1 and GDSC2) datasets were analyzed. Correlation analysis revealed that high CREG1 expression was positively associated with IC₅₀ values for multiple targeted agents and cytotoxic drugs (Fig. 7C). Significant positive correlations were observed for MEK inhibitors, PI3K/mTOR pathway inhibitors, KRAS pathway inhibitors, and several chemotherapeutic agents. Since higher IC₅₀ values indicate reduced drug sensitivity, these results suggest that elevated CREG1 expression is linked to decreased responsiveness to a range of anti-cancer compounds.

CTRP validation confirms a broad-spectrum resistance phenotype associated with CREG1

To further validate these findings, drug response data from the CTRP were analyzed. CREG1 expression showed a positive correlation with AUC values for multiple compounds (Fig. 7D). As higher AUC values correspond to reduced drug efficacy, these results reinforce the association between CREG1 overexpression and therapeutic resistance.

Across independent pharmacogenomic datasets, CREG1-high cell lines consistently exhibited reduced sensitivity to diverse classes of anti-cancer agents. These pan-cancer analyses suggest that CREG1 expression is associated with diminished drug responsiveness in cell line models.

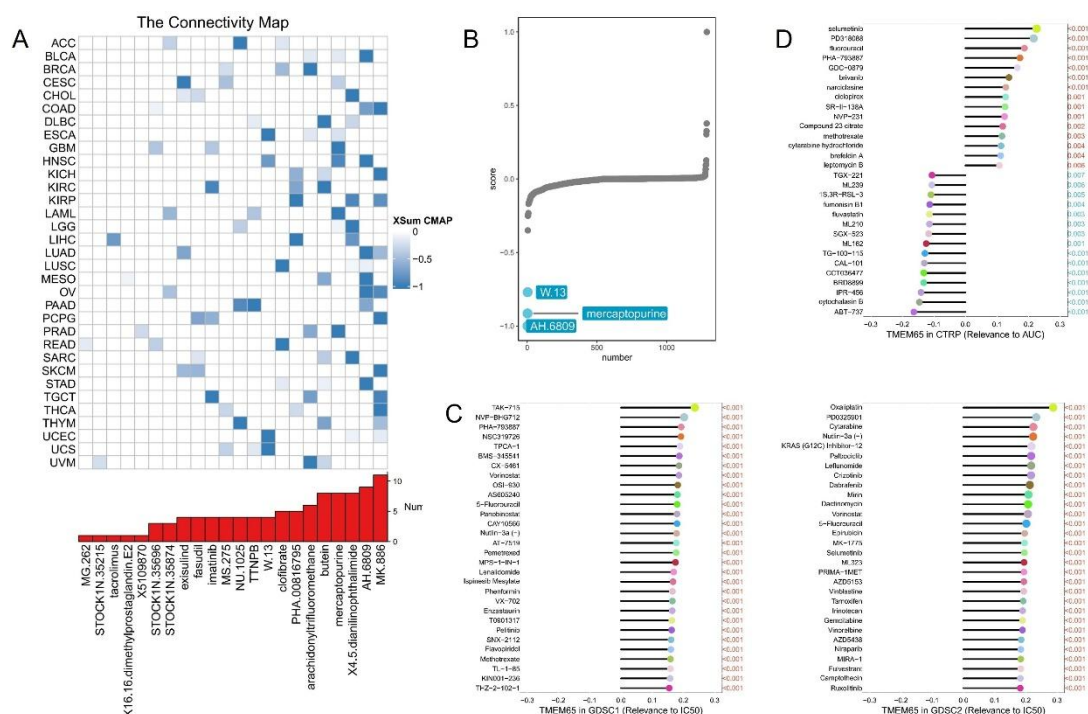


Figure 7. Pharmacogenomic prediction and drug resistance analysis associated with CREG1 expression. (A) CMap heatmap showing XSum connectivity scores of candidate compounds across multiple tumor types. Negative scores indicate potential reversal of the CREG1-associated transcriptional signature. (B) Ranked connectivity score distribution highlighting top negatively enriched compounds, including AD.6809, W.13, and mercaptopurine. (C) Correlation analysis between CREG1 expression and IC50 values in GDSC1 and GDSC2 datasets. Positive correlations indicate reduced drug sensitivity in CREG1-high cell lines. (D) CTRP dataset analysis showing correlations between CREG1 expression and AUC values for multiple therapeutic compounds. Higher AUC values reflect decreased drug responsiveness.

Discussion

OS is a malignant bone tumor primarily affecting adolescents and children. It is highly aggressive, with a strong tendency for local recurrence and distant metastasis, particularly to the lungs, which significantly reduces patient survival rates [28-30]. While surgical resection and systemic chemotherapy remain standard treatments, their effectiveness is compromised by the tumor's high heterogeneity and the development of treatment resistance, posing significant challenges in clinical management and leading to poor prognosis [31, 32]. Recent advancements in molecular biology and high-throughput sequencing technologies have enabled deeper insights into the molecular characteristics of OS and its TME, with the goal of identifying more effective diagnostic and therapeutic targets, as well as precise treatment strategies to improve clinical outcomes for patients.

This study systematically identified three PTM-related core genes—VCAN, RRP12, and CREG1—associated with OS through the integration of multi-omics data and bioinformatics analyses. A prognostic model based on these genes was established, revealing potential mechanisms by which PTM-related genes contribute to OS development and progression, while also providing a reliable tool for clinical risk stratification. Moreover, single-cell transcriptome analysis elucidated the heterogeneous distribution and functional roles of these core genes within the TME, offering valuable insights into the molecular foundation of therapeutic resistance in OS.

By integrating three OS transcriptome datasets, this study identified three PTM-related core genes—VCAN, RRP12, and CREG1—and observed significant differential expression in tumor tissues, particularly a strong association between VCAN overexpression and malignant tumor characteristics. These findings corroborate previous research indicating that PTM modulates tumor cell signaling and the microenvironment, suggesting that PTM genes play a critical role in OS pathogenesis by influencing extracellular matrix remodeling and intercellular signaling [33]. Furthermore, as an extracellular matrix protein, VCAN overexpression may facilitate tumor cell migration and invasion, reflecting the intricate interactions between matrix components and tumor cells in the OS microenvironment. This insight broadens the current understanding of PTM gene functions in OS and highlights the multidimensional regulatory role of PTM-related genes in shaping the TME.

The three-gene prognostic model, developed through LASSO Cox regression, demonstrated robust predictive performance, achieving an AUC of 0.87, and was strongly linked to survival outcomes in patients with OS, particularly identifying significantly reduced survival in the high-risk group. Unlike previous models based solely on PTM-related genes, our approach integrated multi-omics data and single-cell sequencing, enhancing model stability and better capturing tumor heterogeneity [18]. Gene expression patterns in this model were associated with features of the immunosuppressive microenvironment, potentially influencing prognosis through effects on immune cell infiltration and immune escape. These findings align with current knowledge of OS immune regulation and prognosis, suggesting that the prognostic model not only reflects

the biological functions of PTM genes but also provides novel molecular markers for precise prognostic assessment and immunotherapy strategies.

Functional validation of CREG1 as a core gene in OS cells revealed its role in cell proliferation and migration. siRNA-mediated silencing of CREG1 significantly reduced the proliferative capacity and migratory potential of U2OS and 143B cells, indicating that CREG1 may regulate signaling pathways involved in cell cycle progression and motility in OS cells. This finding complements existing research on CREG1 in tumor biology and highlights its potential as a therapeutic target. Additionally, the regulation of CREG1 may involve PTM-mediated protein stability and functional modulation, further revealing the complex role of PTMs in OS cell signaling networks. This mechanism provides experimental evidence for elucidating the molecular basis of OS cell invasion and metastasis.

CMap analysis revealed that OS samples with high CREG1 expression exhibited significantly negative connectivity scores with compounds such as AD.6809, W.13, and mercaptopurine. These findings align with molecular target studies on drug mechanisms of action, suggesting that these drugs may restore the regulatory balance of tumor cell proliferation and migration by inhibiting CREG1-regulated signaling pathways, thereby enhancing therapeutic efficacy [14]. Additionally, the drug-induced reversal of PTM-related gene expression could disrupt tumor cell adaptability dependent on PTMs, presenting novel strategies for precision drug screening based on PTM regulatory networks. This discovery expands the understanding of the link between PTMs and drug responsiveness, broadening the scope for selecting therapeutic targets in OS.

scRNA-seq analysis revealed heterogeneous expression patterns of VCAN, RRP12, and CREG1 across distinct OS cell populations. Notably, VCAN was highly expressed in both tumor and stromal cells, highlighting its critical role in regulating intercellular interactions within the TME. This observation is consistent with recent studies showing that tumor-stroma interactions influence OS progression and immune regulation [17, 34]. The differential expression of PTM genes during immune cell infiltration and immune evasion processes highlights their complex involvement in shaping the immune microenvironment and promoting tumor immunosuppression. These findings provide valuable cellular-level insights into the multi-layered regulatory mechanisms of OS's immune microenvironment, emphasizing the pivotal role of PTM-related genes in tumor-immune interactions.

The primary limitations of this study include a lack of sufficient wet-lab validation, particularly for the functional analysis of core genes VCAN, RRP12, and CREG1. Additionally, the relatively small sample size may limit the statistical significance and external generalizability of the results, which could affect the comprehensive prognostic evaluation of patients with OS. Furthermore, dataset diversity and potential batch effects may introduce limitations, necessitating cautious interpretation of these findings across different patient populations.

Conclusions

This study successfully identified three PTM-related core genes associated with OS, providing new insights into tumor biology and potential targets for clinical intervention. The discovery of CREG1, in particular, highlights its significant role in tumor cell proliferation and migration, suggesting that future research should further explore its mechanisms and therapeutic potential. With additional functional validation and clinical trials, these findings could advance precision treatment strategies for OS.

List of abbreviations

Abbreviation	Full Name
OS	Osteosarcoma
PTMs	Post-translational modifications
DEGs	Differentially expressed genes
scRNA-seq	Single-cell RNA sequencing
TME	Tumor microenvironment
LASSO	Least Absolute Shrinkage and Selection Operator
PCA	Principal component analysis
UMAP	Uniform Manifold Approximation and Projection
GO	Gene Ontology
KEGG	Kyoto Encyclopedia of Genes and Genomes
PPI	Protein–protein interaction
FDR	False discovery rate
FC	Fold change
ROC	Receiver operating characteristic
AUC	Area under the curve
HR	Hazard ratio

Abbreviation	Full Name
CI	Confidence interval
CMap	Connectivity Map
GDSC	Genomics of Drug Sensitivity in Cancer
CTRP	Cancer Therapeutics Response Portal
IC50	Half-maximal inhibitory concentration
qRT-PCR	Quantitative real-time PCR
siRNA	Small interfering RNA
NC	Negative control
SDS–PAGE	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
PVDF	Polyvinylidene fluoride
ECL	Enhanced chemiluminescence
DAPI	4',6-diamidino-2-phenylindole
FBS	Fetal bovine serum
DMEM	Dulbecco's Modified Eagle Medium
GEO	Gene Expression Omnibus
TARGET	Therapeutically Applicable Research To Generate Effective Treatments
CTD	Comparative Toxicogenomics Database
OMIM	Online Mendelian Inheritance in Man
HPA	Human Protein Atlas

Declarations

Data availability

Detailed datasets available from the corresponding author upon reasonable request.

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Author information

Authors and Affiliations

Shanghai Jiao Tong University School of Medicine, Shanghai, China.

Yang Su, Ji'An Liu, Zhengrui Li

Department of Orthopedic Surgery, Shanghai Children's Medical Center, School of Medicine, Shanghai Jiao Tong University, Shanghai, China.

Zhu Liu

Contributions

Yang Su: Methodology; Data curation; Formal analysis; Writing – original draft.

Ji'An Liu: Resources; Data curation; Writing – review & editing.

Zhengrui Li: Conceptualization; Supervision; Project administration; Funding acquisition; Resources; Writing – review & editing.

Zhu Liu: Conceptualization; Supervision; Project administration; Funding acquisition; Resources; Writing – review & editing.

All authors have read and approved the final manuscript and agree to be accountable for all aspects of the work.

Corresponding author

Correspondence to **Zhengrui Li & Zhu Liu.**

Ethics declarations

Not applicable.

Competing interests

The authors declare no competing interests.

Consent for publication

All authors approved the final manuscript and consented for publication.

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