Prognostic Value of Neutrophil Extracellular Trap-Related Molecular Signatures in Immune-Related Genes for Patients with Osteosarcoma

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Abstract: This study investigated the prognostic value of neutrophil extracellular trap (NET)-related molecular signatures in immune-related genes for osteosarcoma patients, as well as their potential to provide molecular targets for individualized therapeutic strategies. Osteosarcoma patients' gene expression profiles and clinical follow-up data were retrieved from the TARGET database as the training set and the GEO database (GSE21257) as the external validation set. Consensus clustering was performed for molecular subtyping based on NET-related gene (NRG) expression patterns, and bioinformatic analyses were applied to compare biological functions, tumor microenvironment and immune infiltration characteristics across subtypes. LASSO regression was used to screen core prognostic NRGs and construct a prognostic signature, whose predictive efficacy was validated via multiple statistical methods, and a clinical nomogram was further constructed by integrating the signature's risk score with clinical characteristics. The results showed that patients could be stratified into two molecular subtypes with distinct prognostic and immune-related features, and the constructed NRG-based signature effectively divided patients into high- and low-risk groups with different prognosis, acting as an independent risk factor for poor prognosis in osteosarcoma and outperforming previously published prognostic signatures. The combined nomogram improved prognosis prediction accuracy, and high risk scores were associated with osteosarcoma distant metastasis. In conclusion, NRG expression characteristics are closely correlated with osteosarcoma prognosis and tumor immune microenvironment, and the core NRG-based prognostic signature can serve as a novel molecular biomarker, providing new insights for osteosarcoma risk stratification and precise immunotherapy.

Keywords: Osteosarcoma; Neutrophil Extracellular Traps; Immune-Related Genes; Prognostic Signature; Tumor Immune Microenvironment; Risk Stratification

1. Introduction

Osteosarcoma is the most prevalent primary malignant bone tumor in children and adolescents, predominantly arising in the metaphyses of long bones [1]. It is characterized by high malignancy, strong invasiveness, and a high propensity for distant metastasis, with its high mortality and disability rates posing a severe threat to the life and health of adolescents [2]. Although multidisciplinary comprehensive treatment regimens combining chemotherapy, radiotherapy, and surgery have been widely adopted in clinical practice, and emerging immunotherapeutic strategies have brought new prospects for osteosarcoma treatment [3-5], the therapeutic response and prognosis of osteosarcoma patients exhibit significant individual heterogeneity due to the high genomic diversity of tumors. Notably, the 5-year OS rate of patients with metastatic osteosarcoma remains below 20% [6]. Therefore, screening molecular biomarkers closely associated with osteosarcoma prognosis, constructing efficient prognostic prediction models, and achieving precise risk stratification and individualized treatment for

patients are key research priorities in the basic and clinical study of osteosarcoma.

Abnormal expression of immune-related genes is intricately linked to tumorigenesis, progression, and prognosis. Prognostic signatures constructed based on immune-related genes have been validated in various malignant tumors and have become a research hotspot in tumor immunology [7]. Neutrophil extracellular traps (NETs) are reticular structures released by activated neutrophils, composed of decondensed chromatin, histones, and granule enzymes [8]. Initially recognized as an innate immune defense mechanism against pathogen invasion, accumulating evidence has revealed the dual role of NETs in the TME—they can inhibit tumor growth by activating the host anti-tumor immune response, and can also be induced by tumor cells to promote tumor invasion, metastasis, and immune escape [9,10]. The expression characteristics of NET-related genes (NRGs) have been confirmed to correlate with the prognosis and immune infiltration of multiple tumors [11], but their expression patterns, biological functions, and prognostic value in osteosarcoma remain largely elusive.

With the rapid development of high-throughput sequencing technology and the improvement of public gene databases, mining tumor prognostic molecular biomarkers through bioinformatics analysis has become an efficient and reliable research method [12]. In this study, we took the TARGET and GEO public databases as data sources to systematically analyze the expression characteristics and prognostic value of NRGs in osteosarcoma. We performed molecular subtyping of osteosarcoma via consensus clustering and explored the differences in the immune microenvironment among different subtypes. We further constructed and validated an NRG-based prognostic signature, aiming to provide new experimental evidence for the prognosis evaluation of osteosarcoma and the screening of potential immunotherapeutic targets

2. Materials and Methods

2.1 Data Collection and Preprocessing

RNA sequencing data (FPKM format) and corresponding clinical information (including gender, age, metastatic status, OS time, and survival status) of 89 osteosarcoma patients were downloaded from the Genomic Data Commons (GDC, http://portal.gdc.cancer.gov) of the TARGET database as the training set. Gene expression data and clinical follow-up data of 53 osteosarcoma patients from the GSE21257 dataset were retrieved from the GEO database (https://www.ncbi.nlm.nih.gov/geo/) as the external validation set. Both datasets were standardized using the log2 transformation method. Samples with incomplete clinical information and OS time less than 1 month were excluded to ensure the reliability and accuracy of subsequent bioinformatics analyses.

2.2 Identification of NET-Related Molecular Subtypes of Osteosarcoma

The ESTIMATE algorithm was employed to calculate tumor microenvironment (TME) scores—including the total ESTIMATE score, stromal score and immune score—for each osteosarcoma patient [13]. Single-sample GSEA (ssGSEA) was further used to quantify the activation levels of 13 immune-related pathways and the infiltration enrichment scores of 16 immune cell subsets in all osteosarcoma cases. All these aforementioned scores were subjected to intergroup comparison between the two NETs-related molecular subtypes. Additionally, the expression patterns of immune checkpoint genes and human leukocyte antigen (HLA) family genes were detected to predict the potential response of osteosarcoma patients to immunotherapeutic interventions.

2.3 Analysis of Biological Functions and Immune Characteristics of Different Molecular Subtypes

Differentially expressed genes (DEGs) among different molecular subtypes were screened using the R software with the thresholds of $P<0.05$ and $|\log2 \text{ fold change}|>1$. Gene Ontology (GO) functional enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis were performed to explore the core biological processes and signaling pathways involved in DEGs. GSEA 4.0.1 software was further used to analyze the pathway enrichment characteristics of different subtypes, with $P<0.05$ as the statistical significance criterion.

The ESTIMATE algorithm was used to calculate the TME scores (including immune score, stromal score, and total ESTIMATE score) for each patient, and the score differences among different subtypes were compared. The ssGSEA algorithm was applied to quantitatively analyze

the infiltration levels of 16 types of immune cells and the activation status of 13 immune-related pathways, to explore the differences in immune infiltration characteristics among different subtypes. Meanwhile, the expression levels of immune checkpoint genes and human leukocyte antigen (HLA) family genes in different subtypes were detected to predict the potential response of patients in different subtypes to immunotherapy.

2.4 Immune Characteristics of Clusters 1 and 2

We quantified the tumor microenvironment (TME) profiles of individual osteosarcoma patients via the ESTIMATE algorithm, which generated total ESTIMATE, stromal and immune scores for each case. Single-sample gene set enrichment analysis (ssGSEA) was subsequently performed to assess the activation status of 13 immune-related signaling pathways and the infiltration enrichment levels of 16 distinct immune cell populations in all osteosarcoma samples. All TME and immune-related scores were compared between the two NETs-associated molecular clusters. Furthermore, we detected the expression levels of immune checkpoint genes and human leukocyte antigen (HLA) family genes to predict the potential responsiveness of osteosarcoma patients to immunotherapy.

2.5 Construction and Verification of a Prognostic NRG Signature

For prognostic model development, the TARGET dataset was designated as the training cohort. Survival-related NRGs identified by univariate Cox regression were further screened via LASSO regression using the glmnet R package, which served to select optimal variables for constructing the final signature and prevent overfitting [14]. Additionally, multivariate Cox analysis with the Survminer R package was conducted to generate the risk score equation. The risk score for each osteosarcoma patient was calculated as follows:

Risk score = $\sum_{i=1}^{n}$ coefi × NRG expression

Where Coefi denotes the regression coefficient of each NRG in the signature. Osteosarcoma patients were stratified into high-risk and low-risk groups based on the median value of their risk scores. The GSE21257 dataset, including survival data of 53 osteosarcoma patients, was used as the validation set to evaluate the established NRG signature. Data from both datasets were adjusted using the sva module in R software. The predictive accuracy and reliability of the NRG signature were determined through time-dependent receiver operating characteristic (ROC) curves for 1-, 3-, and 5-year survival, univariate and multivariate Cox analyses, and Kaplan-Meier (K-M) plots with log-rank tests. The predictive ability of the NRG signature was compared with four published prognostic signatures associated with glycolysis [15], DNA Damage Response [16], immune-related genes [17], and Transcription co-factors [18].

Furthermore, a prognostic nomogram for osteosarcoma that integrates the signature-derived risk score and clinical parameters was constructed. The nomogram's prognostic predictive accuracy was validated using time-dependent 1-, 3-, and 5-year ROC curves and calibration curves. Finally, the chi-square test was applied to assess the association between risk groups and the clinicopathological characteristics of osteosarcoma patients [19].

2.6 Statistical Analysis

All statistical analyses were performed using R software (version 4.1.0). The Wilcoxon signed-rank test was used for the comparison of measurement data between groups, and the chi-square test was used for the comparison of count data between groups. Survival analysis was performed using the K-M method with the log-rank test. Univariate and multivariate Cox regression analyses were used to analyze prognostic influencing factors. ROC curves were plotted and AUC values were calculated to evaluate predictive efficacy. A two-sided $P<0.05$ was considered statistically significant [20,21].

3. Results

3.1 Classification and Validation of NET-Related Molecular Subtypes of Osteosarcoma

Univariate Cox regression analysis showed that all 69 included NRGs were significantly associated with the overall survival rate of osteosarcoma patients in the TARGET database (all $P<0.05$), and all were included in subsequent consensus clustering analysis. When the number of clusters k=2, the intra-group correlation of patients was the highest and the inter-group difference was the most significant; therefore, 89 osteosarcoma patients were divided into two molecular subtypes: subtype 1 (n=47) and

subtype 2 (n=42). PCA and tSNE dimensionality reduction analyses showed that the gene expression profiles of the two subtypes could be clearly distinguished with no obvious overlap, confirming the reliability of the clustering and subtyping results. The heatmap indicated the differential expression patterns of 69 NRGs and the distribution of clinicopathological characteristics between the two subtypes, with significant differences in metastatic status between the two subtypes. K-M survival analysis showed that the overall survival rate of patients in subtype 2 was significantly higher than that in subtype 1, with a statistically significant difference (P<0.001).

3.2 GO and KEGG enrichment analyses

With P<0.05 and |log2 fold change|>1 as thresholds, a total of 389 DEGs were screened between the two molecular subtypes [22]. GO and KEGG enrichment analyses were performed on these DEGs (Figure 1A). GO functional enrichment analysis revealed that DEGs were mainly enriched in immune-related biological processes, including T cell activation, immune receptor activity, neutrophil activation, and regulation of monocyte proliferation (Figure 1B). KEGG pathway enrichment analysis showed that DEGs were mainly involved in the hematopoietic cell lineage, cytokine-cytokine receptor interaction, cell adhesion molecules, and osteoclast differentiation signaling pathways (Figure 1C). GSEA results demonstrated that multiple signaling pathways related to anti-tumor immunity were significantly enriched in subtype 2, including antigen presentation and recognition, B cell receptor signaling pathway, chemokine signaling pathway, and allograft rejection (Figure 1D), suggesting that the prognostic difference between the two subtypes may be closely related to immune-related biological functions.

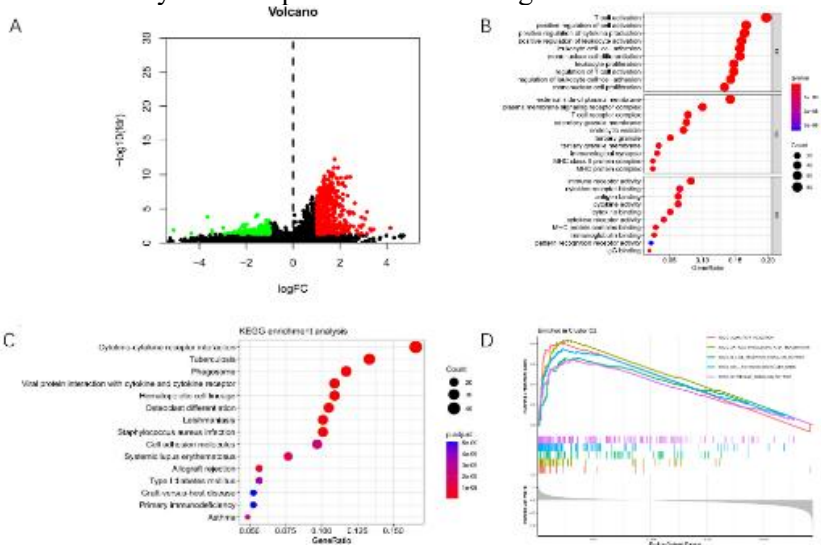

Figure 1. Functional Enrichment Analysis of Differentially Expressed Genes (DEGs) in Different Molecular Subtypes of Osteosarcoma

(A) Volcano plot of DEGs between the two subtypes;
(B) GO functional enrichment analysis results of DEGs;
(C) KEGG pathway enrichment analysis results of DEGs;
(D) GSEA pathway enrichment analysis results of subtype 2.

3.3 Tumor Microenvironment and Immune Infiltration Characteristics of Different Molecular Subtypes

ESTIMATE algorithm analysis showed that the immune score, stromal score, and total ESTIMATE score of patients in subtype 2 were significantly higher than those in subtype 1 (all P<0.05). ssGSEA analysis revealed that the infiltration levels of anti-tumor immune cells including dendritic cells, CD8+ T cells, helper T cells, B cells, natural killer (NK) cells, and macrophages in subtype 2 were significantly higher than those in subtype 1, and the activation levels of immune-related functions and immune signaling pathways were also significantly elevated in subtype 2 (P<0.05), indicating that subtype 2 has a more favorable tumor immune microenvironment.

Detection of the expression levels of immune checkpoint genes and HLA family genes showed that the expression levels of HLA family genes

(HLA-A, HLA-B, HLA-C, HLA-DMA) and immune checkpoint genes (PDCD1, CTLA4, LAG3, TIGIT) in patients with subtype 2 were significantly higher than those in subtype 1 (all P<0.05), suggesting that patients with subtype 2 may have a more positive response to immune checkpoint inhibitor therapy.

3.4 Construction of the NRG-Based Prognostic Signature and Verification of Its Predictive Efficacy

LASSO regression analysis with 10-fold cross-validation screened four core prognostic NRGs (ITGAM, MAPK1, SELPLG, TLR2) to construct an NRG-based prognostic signature for osteosarcoma, and a risk score calculation formula was established by multivariate Cox regression analysis. According to the median risk score, patients in the TARGET training set and GSE21257 validation set were all divided into high- and low-risk groups. The distribution of risk scores and patient survival status showed that the high-risk group had a higher proportion of deaths and shorter OS time. K-M survival curve results indicated that the OS rate of the high-risk group was significantly lower than that of the low-risk group in both the training set and validation set (both P=0.005).

Time-dependent ROC curve results showed that the AUC values for 1-, 3-, and 5-year OS in the training set were 0.772, 0.771, and 0.757, respectively, and those in the validation set were 0.622, 0.719, and 0.744, suggesting that the prognostic signature had good prognostic predictive efficacy in both the training set and validation set. Univariate and multivariate Cox regression analyses confirmed that the risk score was an independent risk factor for the prognosis of osteosarcoma patients (P=0.002, HR=3.375, 95%CI: 1.570-7.240). The heatmap results indicated significant differences in the expression levels of the four core NRGs between the high- and low-risk groups (P<0.05).

Comparison of the NRG-based signature constructed in this study with four previously published osteosarcoma prognostic signatures showed that the AUC values for 1-, 3-, and 5-year OS of the signature in this study were all higher than those of the other four signatures, with a C-index of 0.72, which was significantly higher than that of other models. The 10-year restricted mean survival time curve also showed that the prediction model in this study had better predictive stability, suggesting that the NRG-based prognostic signature constructed in this study had superior predictive accuracy and stability.

3.5 Construction and Validation of a Prognostic Nomogram for Osteosarcoma

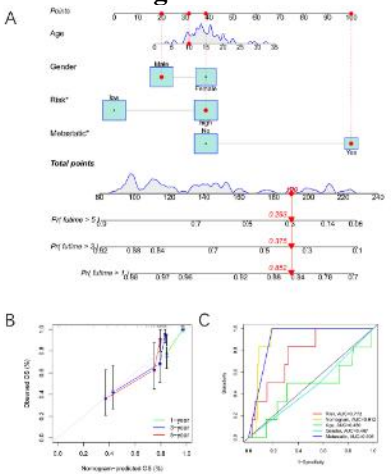

Figure 2. Construction and Validation of a Prognostic Nomogram for Osteosarcoma.

A nomogram for predicting the 1-, 3-, and 5-year OS of osteosarcoma patients was constructed by integrating the risk scores and clinicopathological characteristics (gender, age, metastatic status) of patients in the training set (Figure 2A). Each index was assigned a corresponding score, with a higher total score indicating a poorer prognosis for the patient. Calibration curve results showed that the 1-, 3-, and 5-year OS rates predicted by the nomogram were highly consistent with the actual OS rates, closely approaching the ideal 45° line (Figure2B), suggesting good predictive accuracy of the nomogram. Time-dependent ROC curve results showed that the AUC value of the nomogram was significantly higher than that of a single risk score and other clinicopathological indicators (Figure 2C), indicating that the nomogram could effectively improve the accuracy of prognosis prediction for osteosarcoma patients.

(A) 1-, 3-, and 5-year prognostic nomogram combining risk scores and clinical characteristics; (B) Calibration curves for the nomogram predicting 1-, 3-, and 5-year overall survival rates; (C) ROC curve comparison between the nomogram and single indicators.

4. Discussion

Osteosarcoma is the most common primary malignant bone tumor in adolescents, and improving its prognosis has become a major

research challenge in the field of orthopedic oncology [1]. The high genomic heterogeneity of tumors is the core reason for the significant differences in patient prognosis and poor therapeutic response [1,6]. Identifying molecular biomarkers closely related to osteosarcoma prognosis and constructing efficient prognostic prediction models are of great clinical significance for achieving precise risk stratification of patients and formulating individualized treatment strategies. This study is the first to systematically explore the expression characteristics and prognostic value of NRGs in osteosarcoma. We achieved molecular subtyping of osteosarcoma via consensus clustering and found that NET-related molecular characteristics are closely associated with the prognosis and tumor immune microenvironment of osteosarcoma patients. A prognostic signature with high predictive efficacy was constructed based on four core NRGs, providing new insights for the prognosis evaluation of osteosarcoma and the screening of immunotherapeutic targets.

As an important component of innate immunity, the dual role of NETs in the TME has received extensive attention in recent years [8]. The correlation between abnormal expression of NRGs and tumor prognosis has also been confirmed in pan-cancer studies [14], but there are no relevant reports on the research of NRGs in osteosarcoma to date. In this study, 69 NRGs related to the prognosis of osteosarcoma patients were screened by univariate Cox regression, and osteosarcoma patients were divided into two molecular subtypes based on their expression characteristics. Patients in subtype 2 showed significantly better prognosis than those in subtype 1, which was the first discovery that NRG expression patterns can stratify osteosarcoma patients into different prognostic subtypes. Functional enrichment analysis results showed that DEGs between the two subtypes were mainly enriched in immune-related biological processes and signaling pathways, and multiple anti-tumor immune pathways were significantly enriched in subtype 2. This result suggests that NRGs may affect the prognosis of osteosarcoma patients by regulating the tumor immune microenvironment, which is consistent with the conclusion of previous studies that NETs are involved in tumor immune regulation [8,10], and also provides a direction for subsequent exploration of the regulatory mechanism of NETs in osteosarcoma[23,24].

The tumor immune microenvironment is a key factor affecting tumor progression, prognosis, and immune therapy response, and the infiltration level of anti-tumor immune cells is closely associated with patient prognosis [25,26]. This study found that subtype 2 with a favorable prognosis had higher immune scores, stromal scores, and total ESTIMATE scores, and the infiltration levels of anti-tumor immune cells including CD8+ T cells, B cells, NK cells, and dendritic cells were significantly higher than those in subtype 1. CD8+ T cells are the core effector cells of anti-tumor adaptive immunity, which can specifically recognize and kill tumor cells, and their high infiltration is a well-recognized marker of favorable prognosis in various malignant tumors [27,28]. B cells participate in anti-tumor immunity by secreting specific antibodies and presenting tumor antigens to T cells [29]. As an important component of innate immunity, NK cells can directly kill tumor cells in a major histocompatibility complex-unrestricted manner, and their functional abnormalities are closely associated with tumor immune escape [30,31]. The high infiltration of the above anti-tumor immune cells forms an immune-activated tumor microenvironment in subtype 2, which is an important molecular basis for the favorable prognosis of patients in this subtype.

Meanwhile, the expression levels of HLA family genes and immune checkpoint genes were significantly increased in subtype 2. The high expression of HLA genes is a prerequisite for effective presentation of tumor neoantigens and the initiation of anti-tumor immune responses, which is essential for the efficacy of immunotherapy [32]. The high expression of immune checkpoint genes indicates that tumor cells are under strong immune surveillance pressure, and such patients are more likely to benefit from immune checkpoint inhibitor therapy [33,34]. These results suggest that NET-related molecular subtypes can serve as potential biomarkers for screening osteosarcoma patients who may benefit from immunotherapy, providing a theoretical basis for the precise immunotherapy of osteosarcoma.

Based on the molecular subtype analysis, this study screened four core NRGs (ITGAM, MAPK1, SELPLG, TLR2) by LASSO regression and constructed a prognostic signature, which could effectively stratify

osteosarcoma patients into high- and low-risk groups. Multivariate Cox regression analysis confirmed that the risk score was an independent risk factor for prognosis. This signature showed good predictive efficacy in both the training set and external validation set, and its predictive accuracy was superior to four previously published osteosarcoma prognostic signatures, suggesting its high clinical application value. All four core genes are key regulatory genes for NET formation and release: CD11b encoded by ITGAM is an important adhesion molecule that mediates neutrophil recruitment and NET formation [11]; MAPK1, as a core molecule of the MAPK signaling pathway, can mediate NET formation and also regulate the proliferation, invasion, and metastasis of osteosarcoma cells [35]; SELPLG is involved in the adhesion of neutrophils to vascular endothelial cells and the subsequent release of NETs [14]; TLR2 can activate neutrophils through pattern recognition and induce the formation of NETs [8]. Abnormal expression of the above genes can affect the tumor immune microenvironment and biological behavior of tumor cells by regulating NET formation and release, thereby affecting the prognosis of osteosarcoma patients. This molecular mechanism explains the good predictive efficacy of the NRG-based signature constructed in this study.

This study also found that high risk scores were significantly associated with distant metastasis of osteosarcoma, and the metastasis rate of the high-risk group was three times that of the low-risk group. Distant metastasis is the main cause of treatment failure and death in osteosarcoma patients, suggesting that this NRG-based signature can also serve as a potential biomarker for evaluating the metastatic risk of osteosarcoma. In addition, previous studies by our research team found that icariin can inhibit the proliferation and metastasis of osteosarcoma cells by regulating the mTOR signaling pathway [36], and recent studies have confirmed that the mTOR signaling pathway is a key regulatory pathway for NET formation [10]. Targeting the mTOR signaling pathway can inhibit NET release and further suppress tumor invasion and metastasis, suggesting that NETs may become a new target for targeted therapy of osteosarcoma. The NRG-based signature constructed in this study can serve as a basis for screening patients who may benefit from NET-targeted therapy, providing a new direction for the development of targeted therapeutic drugs for osteosarcoma.

This study also has certain limitations that need to be acknowledged. First, all research data were obtained from public databases with a relatively small sample size, and the study was a retrospective analysis, which may have selection bias. Subsequent large-sample, multicenter, prospective clinical studies are needed to further verify the research results. Second, this study only explored the expression characteristics and prognostic value of NRGs through bioinformatics analysis, and did not verify the biological functions and regulatory mechanisms of core genes through in vitro and in vivo experiments, which need to be further investigated through cytological experiments and animal models. Third, this study did not verify the correlation between the expression characteristics of NRGs and prognosis using clinical tissue samples, and the clinical translation of the research results still needs further verification. Finally, the predictive value of NRG subtypes and signatures for immune therapy response in this study is only a bioinformatics speculation and has not been verified by clinical trials, and subsequent relevant clinical studies are needed to confirm it.

5. Conclusions

NET-related genes are abnormally expressed in osteosarcoma, and their expression characteristics are closely correlated with the prognosis and tumor immune microenvironment of osteosarcoma patients. The prognostic signature constructed based on four core NRGs (ITGAM, MAPK1, SELPLG, TLR2) can effectively predict the prognosis of osteosarcoma patients, and the risk score is an independent risk factor for poor prognosis. Meanwhile, this signature can be used to evaluate the metastatic risk of osteosarcoma patients. The nomogram constructed by combining the risk score with clinical characteristics can significantly improve the accuracy of prognosis prediction for osteosarcoma patients. This study provides a new molecular biomarker for the prognosis evaluation and risk stratification of osteosarcoma, and also provides new insights for precise immunotherapy and the screening of targeted therapy targets for osteosarcoma.

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