# Signature based on immune-related LncRNA can predict overall survival of osteosarcoma patients

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# Abstract

**Background:** Osteosarcoma is a malignant bone tumor common in children and adolescents. Metastatic status remains the most important guideline for classifying patients and making clinical decisions. Despite many efforts, newly diagnosed patients receive the same therapy that patients have received over the last 4 decades. With the development of high-throughput sequencing technology and the rise of immunotherapy, it is necessary to deeply explore the immune molecular mechanism of osteosarcoma.

**Methods:** We obtained RNA-seq data and clinical information of osteosarcoma patients from TCGA database and TARGET database. With the help of co-expression analysis we identified immune-related lncRNA and then by means of univariate Cox regression analysis prognostic-related lncRNA was screened out. And also by using least absolute shrinkage and selection operator regression method a model based on immune-related lncRNA was constructed. The differences in overall survival, immune infiltration, immune checkpoint gene expression, and tumor microenvironmental immunity type between the two groups were evaluated.

**Results:** We constructed a signature consisting of 13 lncRNA. Our results show that signatures can reliably predict the overall survival of patients with osteosarcoma and can bring net clinical benefits. Further more, the signatures can be used for further risk stratification of the metastasis patients. Patients in the low-risk group had higher immune cell infiltration and immune checkpoint gene expression. The results from gene set variation analysis show that patients in low-risk group are closely related to immune-related pathways when compared with patients in high-risk group. Finally, patients in the low-risk group are more likely to be classified as TMIT I and hence more likely to benefit from immunotherapy.

**Conclusion:** Our signature may be a reliable marker for predicting the overall survival of patients with osteosarcoma. Keywords: Osteosarcoma, TCGA, LncRNA, Tumor immunology, Prognosis.


# 1 Background

Osteosarcoma is a malignant bone tumor that commonly affects children and adolescents[1]. In the 1970s, chemotherapy was introduced and significantly improved patient-survival. Currently, patients with newly diagnosed osteosarcoma routinely receive neoadjuvant chemotherapy, surgical removal of the lesion, and undergo adjuvant chemotherapy[2].Today, clinical characteristics such as metastatic status remain the most important criteria for stratifying patients and making clinical decisions. A number of studies have shown that the 5-year survival rate of metastatic patients is only 20% to 30%, while the 5-year survival rate of local osteosarcoma at the same period is about 65-70%[3-6]. Patients with lung-only metastases at initial presentation should be treated aggressively with surgery and chemotherapy, and pulmonary metastatectomy should be considered. However, the prognosis of patients with bone metastases is not optimistic, and this outcome should be considered when developing individual treatment plans.[7] However, in clinical work, a notable difference in the prognosis of patients with similar clinical characteristics is often observed. In addition, perhaps due to the relatively low incidence of

osteosarcoma, the formulation of new drugs and new treatment programs has reached a deadlock, and there has been no breakthrough in the past 40 years [8]. Hence, in order to find new biomarkers and therapeutic targets we need an even deeper understanding of the molecular mechanism of osteosarcoma, which will help us.

Nowadays, immunotherapy has been used as a new type of anti-tumor method, which has shown reliable efficacy in a variety of tumors including melanoma and hepatocellular carcinoma[9-11]. However, due to many factors, the effectiveness of these new therapies in osteosarcoma-patients is still unclear. Therefore, there is an urgent need to understand the immune molecular mechanisms of osteosarcoma. LncRNA is defined as a kind of non-coding RNA longer than 200 nucleotides[12]. Over a decade ago, little was known about this RNA. However, with the rapid development of technology, more and more evidence indicated that this non-coding RNA has a vital role in the development of many tumors, including osteosarcoma[13-16]. Recent studies have shown that LncRNAs such as DANCR, AFAP1-AS1, RP11-361F15.2, KCNQ1OT1 are related to the occurrence, proliferation, metastasis and chemotherapy resistance of osteosarcoma[17-20].

Therefore, with the help of co-expression analysis, this study identified the immune-related lncRNA in osteosarcoma and developed reliable prognostic signatures based on the lncRNA to stratify the patients more precisely. In addition, the differences in immune characteristics between high-risk and low-risk patients were identified. Finally, the relationship between our signature and the efficacy of immunotherapy was also explored based on marker or typing developed in the previous literature.

# 2 Methods

## 2.1 Data collection

Normalized RNA-Seq (FPKM format) data for 88 osteosarcoma patients was downloaded from the TCGA database (https://cancergenome.nih.gov/). At the same time, we downloaded the clinical data related to these patients from the TARGET database (https://ocg.cancer.gov/programs/target). Table 1 lists the clinical characteristics of these patients. All these datas were obtained from a public database, so no additional informed consent was required.

## 2.2 Identification of immune-related lncRNA

A list of immune-related genes was downloaded from the immunology database and the analysis portal (ImmPort) database[21]. The list consisted of a total of 2498 unique immune-related genes. The lncRNA profile from RNA-seq data was extracted using R software. Correlation between lncRNA and immune-related genes was then calculated. LncRNA with a correlation coefficient of 0.4 and $P < 0.05$ are considered as immune-related lncRNA and were used for subsequent analysis.

## 2.3 Construction and evaluation of lncRNA model

Univariate Cox regression analysis was used to identify survival-related lncRNA from the above-mentioned immune-related lncRNA. The lncRNA with a P value from the univariate Cox proportional hazards analysis (Wald test for predictive potential) of less than 0.5 is considered as a prognostic-related lncRNA. The prognosis-related lncRNA expression data set was used as an input to the survival model. Subsequently, 1000 iterations were performed using the least absolute shrinkage and selection operator (LASSO), and the retained lncRNA in more than 50 iterations was considered as an important lncRNA. These important lncRNAs are incorporated into the proportional hazards model in turn, and the area under the receiver's operating characteristic curve (AUROC) was calculated. The model when AUROC reaches the peak is the optimal model. Subsequently, each patient's risk score based on the best model was calculated, and time-dependent receiver operating characteristic (ROC) curve analysis was used to determine the optimal cut-off value for the risk score[22]. Risk score = βlncRNA(1) × expr lncRNA(1) + βlncRNA(2) × exprlncRNA(2) +···+βlncRNA(n)× exprlncRNA(n)[23]. The patients were then divided into high-risk group and low-risk group based on the best cut-off value. The log-rank test was used to achieve the overall survival difference between the two groups, and the KM survival curve was drawn. Using the receiver operating characteristic (ROC) curve analysis the specificity and sensitivity of the risk score was assessed. Finally, multivariate Cox regression analysis was used to explore the independent prognostic value of risk scores.

## 2.4 Construction and evaluation of lncRNA model

The relationship between risk scores and clinical characteristics was further investigated. We divided patients into 6 subgroups based on metastatic status, gender and other clinical characteristics, and explored the prognostic value of risk scores in each subgroup. The forest plot of subgroup analysis was then drawn by means of R software. In addition, based on the metastatic status and risk status, the patients were divided into four groups, and the overall difference in the survival rate among patients in each group was calculated and a KM survival curve was drawn.

## 2.5 Estimation of immune infiltration

Using the transcriptome data of osteosarcoma patients in the TCGA database as input files, the immune infiltration was evaluated with the help of the "microenvironmental cell counter (MCP-counter)" method. This method can reliably quantify the absolute abundance of 8 immune and 2 stromal cell population[24]. Then by means of the ‘ESTIMATE’ R package the stromal score, immune score and estimate score were estimated (https://sourceforge.net/projects/ estimateproject /)[25]. Finally, the single sample GSEA was used to evaluate the enrichment of 29 immune-related gene sets in each sample[26]. The difference in immune infiltration between the two groups of patients was evaluated. ‘Tumor microenvironment immune type (TMIT)’ was used to speculate the efficacy of anti-PD-1 / PD-L1

treatment. TMIT divides patients into four types based on PD-L1 and CD8A mRNA expression, which has been shown to predict patients' response to immune checkpoint inhibitors in pan-cancer analysis[27]. Using SubMap analysis (Gene Pattern) to compare gene expression profiles of osteosarcoma patients with melanoma patients treated with immunotherapy to indirectly predict the efficacy of immunotherapy in osteosarcoma patients[28, 29].

## 2.6 Gene set variation analysis

Genome Variation Analysis (GSVA) is an unsupervised gene set enrichment method that can estimate the scores of certain pathways or markers over a sample population[30]. We downloaded the 'c2.cp.kegg.v7.1.symbols' and 'c5.all.v7.1.symbols' gene sets from the 'Molecular Signatures Database' for GSVA. Subsequently, the differential analysis of these gene sets was performed using the LIMMA package of R software, and the gene set with adjusted P <0.05 was regarded as the differentially expressed gene set.

## 2.7 Construction of the nomogram

The rms package of R software was used to build a nomogram based on clinical factors and immune-related lncRNA risk scores. We then assessed the predictive capability using the concordance index (C-index). In addition, the calibration plots were drawn to verify the accuracy of the nomogram. Finally, by means of decision curve analysis, the clinical utility of the nomogram was assessed.

## 2.8 Statistical analysis

All analyses in this study were performed with R software (version 3.6.3) and P < 0.05 was considered statistically significant.

# 3 Results

## 3.1 Identification of prognostic-related immune-related lncRNA in osteosarcoma

Firstly, we isolated lncRNA expression data from the RNA-SEQ data of 88 patients downloaded from the TCGA database. Subsequently, a total of 2498 immune-related genes were extracted from the ImmPort database. Supplementary Table 1 provides detailed information on these immune-related genes. As shown in Supplementary Table 2, 1986 immune-associated lncRNA were identified by constructing immune-lncRNA co-expression networks. We analyzed the relationship between these immune-related lncRNA and the survival of 85 patients using univariate Cox regression analysis (3 patients lack valid clinical data). Finally, 240 lncRNAs were identified as survival-related immune-related lncRNA and used

for further analysis. Supplementary Figure 1 and Supplementary Table 3 shows the results of univariate cox analysis of these lncRNAs.

## 3.2 Establishing a risk score and testing its prognostic value in osteosarcoma

As mentioned above, these lncRNAs related to prognosis were subjected to 1000 iterations using LASSO analysis. Subsequently, the 29 lncRNA retained in more than 50 iterations were considered important lncRNA for further analysis. Supplementary Table 1 shows the details of these lncRNA. Incorporating these important lncRNA into the COX model, the optimal model consisting of 13 lncRNA was determined based on the 5-year survival AUROC. The risk score of each patient was calculated, and the cut-off value of the risk score was determined to be 0.186 using the time-dependent receiver operating characteristic (ROC) curve analysis. The results of the survival analysis showed that patients in the low-risk group had longer survival rate than patients in the high-risk group (Figure 1D, P<0.001). Figure 1 shows the process of building this model. Supplementary Figure 2 shows the relationship between 13 lncRNA and immune genes.

To verify the independent prognostic value of the risk score, we performed a multivariate regression analysis. As shown in Figure 2B, after adjusting for other variables (including age, gender, and metastatic information), we found that the risk score may be an independent predictor. The results of the forest plot show that the higher the risk score, the shorter the overall survival of the patient (hazard ratios: 2.974, 95% of confidence intervals: 2.164-4.088, P= 1.89e−11). The results of the time-dependent ROC analysis show that the risk score has good discriminative ability. As shown in Figure 2A, no matter how the patient's survival time changes, the risk score always has an excellent discriminating ability. On the contrary, as the patient's survival time prolongs, the discriminating ability of metastatic status continues to decline.

## 3.3 Relationship between risk score and clinical characteristics

To further explore the strength of the risk score, we divided patients into 6 subgroups based on their age, gender and metastatic status, and explored the prognostic value of risk scores in each subgroup. Among them, 48 were in the male group, 37 were in the female group, 66 were in the Age<18 years old group, 19 were in the Age>=18 years old group, 64 were in the non-metastasis group, and 21 were in the metastasis group. As shown in Figure 3A, the risk score shows a good prognostic value in each subgroup. Metastatic status is an important basis for formulating treatment plans for patients with osteosarcoma. Therefore, we further divided patients into 4 groups according to their metastatic status and risk status. Among them, group 1 has 45 patients, group 2 has 8 patients, group 3 has 19 patients, and group 4 has 13 patients. As shown in Figure 3B, there was no significant difference in overall survival rate between

metastatic patients and non-metastatic patients in the low-risk group. Among patients in the metastasis group, patients in the low-risk group had a longer overall survival than those in the high-risk group. Since the prognosis of metastatic patients is affected by some clinical factors such as the metastatic location and treatment methods. We further analyzed whether the prognostic value of the risk score for metastatic patients is affected by the location of metastasis. According to the patient's clinical information, 6 out of 21 metastatic patients with complete survival data had bone metastases. Our results show that after adjusting the metastatic site, the risk score is an independent prognostic factor for metastatic patients.

## 3.4 Evaluation of differences in immune infiltration between high- and low-risk groups

We further evaluated the differences in immunological characteristics between the two groups of patients. As mentioned above, the abundance of 10 immune-related cells was calculated using the MCP-counter method. As shown in Figure 4A, a significant difference was observed between the two groups of patients. Compared with patients in the high-risk group, the abundance of the 8 cell populations in the patients in the low-risk group was higher (B-cell lineage, CD8+T cells, Cytotoxic lymphocytes, Endothelial cells, Monocytic lineage cells, Neutrophils, NK cells, T cells). Then, the estimate, immune and stromal scores were calculated using the ESTIMATE algorithm. Similarly, patients in the low-risk group had higher estimate scores, immune scores, and stromal scores than those in the high-risk group ($p < 0.001$, Figure 4B). We further used ssGSEA to evaluate the difference of 29 immune-related gene sets or immune cells between the two groups of patients as a supplement. The results of ssGSEA have reached similar conclusions. Most immune-related gene sets or immune cells are significantly enriched in the low-risk group (Figure 4C). Finally, we explored the relationship between abundance of 10 immune-related cells and risk score. As shown in Figure 5, with the increasing risk score, the abundance of immune cells kept decreasing, especially CD8 + T cells.

The differences in gene expression of some immune-related pathways were further evaluated and heatmaps were drawn to show the results. We found that genes related to activated T effector and IFNγ pathway such as STAT1, CCL4, CXCL9, CXCL10 were significantly up-regulated in the low-risk group (Figure 6B).

## 3.5 The relationship between signature and the expression of immune checkpoint gene, tumor microenvironment type

As mentioned above, the association between the expression of 7 potentially targetable immune checkpoint genes between the two groups was assessed. As shown in the figure, all immune checkpoint genes in the low-risk group were more highly expressed, although PDCD1 did not show statistical significance (Figure 6A). Further analysis showed that the CD8A gene is also highly expressed in the low-risk group. Similarly, tumor lymphocyte infiltration was higher in the low-risk group. However, the optimal

cut-off values for PD-L1 and CD8A mRNA expression have not been determined. Therefore, we analyzed the relationship between the risk score value and PD-L1 gene expression, CD8A gene expression, and TIL. The results showe that as the risk score increased, the expression of PD-L1 gene and CD8A gene decreased (Figure 6C-D). Unfortunately, although TIL also showed a similar trend, it did not reach statistical significance (Figure 6E). However, the results of the box plot show that the TIL of the low-risk group is higher than that of the high-risk group (Figure 4C).

In addition, we used SubMap analysis to further study the relationship between MRGP signature and immunotherapy efficiency. Using subclass mapping, the expression profiles of the two groups of patients (high-risk group and low-risk group) were compared with a published immunotherapy data set. This data set records the expression data of 47 melanoma patients treated with programmed cell death protein 1 (PD-1) immune checkpoint inhibitors or cytotoxic T lymphocyte associated protein 4 (CTLA-4) immune checkpoint inhibitors. The results showed that the expression profiles of patients in the low-risk group were correlated with those in the PD-L1 response group (Figure 6F, Bonferroni P value=0.016). This indicates that patients in the low-risk group are more likely to benefit from PD-L1 therapy. We further evaluated the characteristics of patients in the lncRNA signature group. As shown in Figure 6G, there is a higher proportion of metastatic patients in the high-risk group.

## 3.6 Identifying the differences in biological pathways and processes between the two groups of patients

We used GSVA to study the differences in biological pathways between the two groups of patients. As shown in the figure 7, significant differences were observed in the biological pathways between the two groups of patients. We found that in the low-risk group, most immunization-related pathways had higher GSVA scores. It is worth noting that patients in the high-risk group had higher GSVA scores for certain metabolic-related pathways. Supplemental Table 4 shows detailed information on GSVA results.

## 3.7 Construction of Nomogram Based on Risk Score

We developed a nomogram that combined risk scores with traditional clinical factors (Figure 8), and tested the accuracy of the nomogram using a calibration curve. As shown in Figure 8B, the 3-year and 5-year overall survival predictions show that the nomograms have good accuracy. The C index of the nomogram is 0.924, which indicates that our nomogram has a good degree of discrimination. These results suggest that the new nomogram shows reliable performance in predicting patient prognosis. Finally, the results of the DCA analysis show that the model combined with the risk score can bring clinical net benefits.

# 4 Discussion

This study identified immune-related lncRNA in osteosarcoma patients based on IRG in the Immport database. These lncRNA were then used to construct a signature consisting of 13 lncRNA. Our signature can reliably identify high-risk patients. The results of subgroup analysis and nomogram further support our conclusion. Considering that our signature is based on immune-related lncRNA, we used three methods to study the differences in immune characteristics between the two groups of patients. The results showed that the low-risk group was closely related to immune cell infiltration and immune-related gene expression. In addition, compared with the high-risk group, the immune checkpoint gene expression was higher in the low-risk group. These results indicate that our signature can effectively identify high-risk patients and help to understand the immune microenvironment of osteosarcoma. In summary, we believe that our signature has certain clinical value. Several recent studies have provided some potential directions for the application of signatures. Zhang et al used qRT-PCR to detect the expression of signature genes in frozen tissue samples of patients and further calculated the risk scores of these patients. Consistent with the results of the public database, there were significant differences in RFS between the two groups[31]. Yao et al evaluated the protein expression of signature genes through immunohistochemistry (IHC) stained tissue microarrays to verify the application of signatures at the protein level. Similar to the transcriptome analysis results of public databases, the signature shows great potential in predicting the prognosis of HNSCC patients[32]. Similarly, signature LncRNA needs to be measured in an independent cohort with complete clinical information to verify our conclusions.

Presently, the presence or absence of metastases at diagnosis is still a key indicator for identifying patients with high-risk osteosarcoma[33]. However, some patients have a good prognosis, despite the diagnosis of metastasis. Therefore, effective identification of this part of patients helps to formulate more accurate treatment plans. By means of further analysis of the patient's risk score and metastatic status, we found that the risk score can effectively screen this part of the patient. Compared with metastatic patients in the high-risk group, metastatic patients in the low-risk group had significantly higher overall survival. Further analysis showed that after adjusting the factor of metastasis site, risk score is an independent prognostic factor for metastatic patients. Unfortunately, the TCGA database is unable to obtain relevant information on the treatment of patients with metastases. Therefore, our conclusions may be potentially biased and further research is needed to verify our conclusions.

Today, immune checkpoint inhibitor therapy has been shown to produce durable clinical responses to patients with various advanced cancers, such as melanoma, non-small cell lung cancer, bladder cancer, renal cell carcinoma, and Hodgkin’s lymphoma[34-37]. Unfortunately, only some patients can benefit from this treatment strategy. Even in melanoma, a cancer that is considered to be highly immunogenic, 24% of patients treated with the combination of nivolumab plus ipilimumab still showed progressive disease[38]. According to the results of published clinical trials, in osteosarcoma, the therapeutic effect of this treatment is even more pessimistic. The results of SARC028 showed that 1 out of 22 patients with osteosarcoma achieved partial response and 6 patients had stable disease[39]. The results of the PEMBROSARC study showed that of 17 patients with advanced osteosarcoma, 15 patients can be assessed for the primary efficacy endpoint. 4 cases had tumor shrinkage, 1 case had partial response, and 5 cases had stable disease[40]. This new treatment method has evoked questions about how to

identify patients responding to this therapy. Previous studies have shown that the use of immunohistochemistry to detect the expression of PD-L1 on the surface of tumor cells can be used as a factor in predicting patient response to treatment[41]. Unfortunately, the predictive power of this method is limited, because not all PD-L1 positive patients respond well[42, 43]. In addition, recent studies have shown that tumor mutation burden (TMB) is also expected to be a marker for identifying patients who can benefit from immunotherapy. TMB, as a marker for predicting immunotherapy response, has shown encouraging results in non-small cell lung cancer and melanoma[44, 45]. However, in renal clear cell carcinoma, the relationship between TMB and immunotherapy efficacy remains controversial[46]. Moreover, studies have shown that the tumor microenvironment has a certain relationship with the response of patients receiving immunotherapy. Combining the abundance of tumor invasive lymphocytes with the expression of PD-L1 gene to divide patients into four subtypes has been preliminarily proved to be able to predict the response to immunotherapy in melanoma patients[47]. Another study based on pan-cancer data proposed a TMIT classification based on the expression of PD-L1 gene in CD8A gene (TMIT I (PDL1 (+), CD8A (+)), TMIT II (PDL1 (- ), CD8A (-)), TMIT III (PDL1 (+), CD8A (-)), TMIT IV (PDL1 (-), CD8A (+)). Among them, TMIT type I is associated with high PD-L1 expression, high mutation load/neoantigen, high MSI, PD-L1 amplification, and the presence of oncogenic viruses[27]. In addition, Yu-Pei Chen et al further proved that in some solid tumors, patients with TMIT type I are most likely to benefit from immunotherapy and calculate the optimal cut-off value[48]. In general, many efforts have recently been made to develop markers that can identify patients who will benefit from immunotherapy. However, there is no reliable marker that can be widely used in clinical practice. Based on these markers, our research explored the relationship between lncRNA signatures and the immune efficacy of patients with osteosarcoma. First, we explored the expression differences of 7 immune checkpoint genes including PD-L1 gene between the two groups. The results showed that although the PDCD1 gene did not reach statistical significance, all immune checkpoint genes tended to be highly expressed in the low-risk group. Subsequently, we evaluated the relationship between Tumor microenvironment type and lncRNA signatures. In our study, compared with patients in the high-risk group, patients in the low-risk group were more likely to be classified as type I because of the high expression of the PD-L1 gene and TIL. Similarly, our results show that since patients with lower risk scores tend to have higher expression of PD-L1 and CD8A genes, patients in the low-risk group are more likely to be classified as TMIT I patients. Finally, we compared the expression profiles of osteosarcoma patients with melanoma patients treated with immune checkpoint inhibitors by submap analysis. There is a certain correlation between the expression profiles of the low-risk group and the PD-L1 response group. In summary, our results indicate that patients in the low-risk group are more likely to benefit from immunotherapy. Conversely, patients in the high-risk group may not respond well to immunotherapy. We further analyzed the clinical characteristics of the two groups of patients. Interestingly, most patients in the high-risk group are metastatic patients. Consistent with our conclusions, the conclusions of the SARC028 and PEMBROSARC trials that only recruited patients with metastatic osteosarcoma are not optimistic. However, due to the lack of data on immunotherapy for patients with osteosarcoma, our results need to be interpreted with caution. At the same time, RNA-seq data from osteosarcoma patients receiving immunotherapy are needed to verify this conclusion.

Compared with the uncertainty of the efficacy of immune checkpoints, Mifamurtide, as an immune adjuvant therapy, is an important progress in the treatment of osteosarcoma in recent years[49-51]. Mifamurtide is a synthetic analogue of muramyl dipeptide, which is a component of the cell wall of Bacille Calmette-Guerin and can activate the innate immune system[52]. Monocytes and macrophages activated by Mifamurtide are related to the upregulation of tumoricidal activity and the secretion of pro-inflammatory cytokines, including tumor necrosis factor (TNF)-a, IL-1α, IL-1β, IL-6, and IL-8[53-55].Tumor-associated macrophages, as the most abundant infiltrating immune cells in the tumor microenvironment, may promote tumorigenesis through immunosuppression, matrix degradation protein expression and support for angiogenesis[56]. In contrast to many other tumors, recent studies have shown that high numbers of TAM are associated with a lower risk of metastasis and better prognosis in patients with osteosarcoma[57]. Further studies have shown that in the presence of IFN-γ, liposomal muramyl tripeptide can significantly induce the anti-tumor activity of M1-like macrophages. At the same time, the inhibitory effect of activated M1-like macrophages on the growth of osteosarcoma cells is mediated by soluble factors[58]. The latest research shows that Mifamurtide regulates the function of macrophages by converting the polarization of macrophages to a TAM-like intermediate M1/M2 phenotype, thereby inhibiting the proliferation of osteosarcoma cells[59]. Consistent with previous results, our results show that patients in the low-risk group have a higher abundance of macrophage infiltration.

It must be admitted that our research has some limitations. Firstly, the sample size is relatively small. Although osteosarcoma is a rare tumor with a low incidence, we believe that only a larger sample size will make the conclusion more convincing. Secondly, due to the scarcity of lncRNA research in osteosarcoma, we were unable to find other lncRNA data with clinical information, so we failed to set up external validation. Finally, due to the lack of immunotherapy data of osteosarcoma patients, we could only combine the conclusions of previous studies to speculate the value of risk scores in predicting patients' response to immunotherapy. Further research is needed to prove our conclusion.

# 5 Conclusion

In conclusion, our signature can effectively predict the overall survival of osteosarcoma patients and can be used as a reliable supplement to the metastatic state to formulate more accurate treatment plans. Finally, our signature is expected to provide some guidance for identifying patients who may benefit from immunotherapy.

# Abbreviations

**TCGA**: The Cancer Genome Atlas; **TARGET**: Therapeutically Applicable Research To Generate Effective Treatments; **LASSO**: Least Absolute Shrinkage and Selection Operator; **K-M**: Kaplan-Meier; **ROC**: Receiver Operating Characteristic; **AUC**: area under the ROC curve; **AUROC**: area under the receiver's operating characteristic curve; **MCP-counter**: microenvironmental cell counter; **TMIT**: Tumor microenvironment immune type; **GSVA**: Genome Variation Analysis; **ssGSEA**: single sample Gene Set Enrichment Analysis; **OS**: Overall survival

## Declarations

# Ethics approval and consent to participate

Not applicable.

# Consent for publication

Not applicable

# Availability of data and materials

The datasets analysed during the current study are available in the TCGA repository and TARGET repository, https://portal.gdc.cancer.gov; https://ocg.cancer.gov/programs/target.

# Competing interests

The authors declare that they have no competing interests

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# Author Contributions

L-LQ collected and analyzed the data and wrote the paper. Z-LH and Manhas assisted in collecting the data and participated in the writing. Z-Y, L-XC, Z-Y, W-J, L-T, Y-YB and L-YK assisted in the design of this study. L-JZ is responsible for all the integrity of data and the accuracy of data analysis. All authors have thoroughly revised the manuscript.

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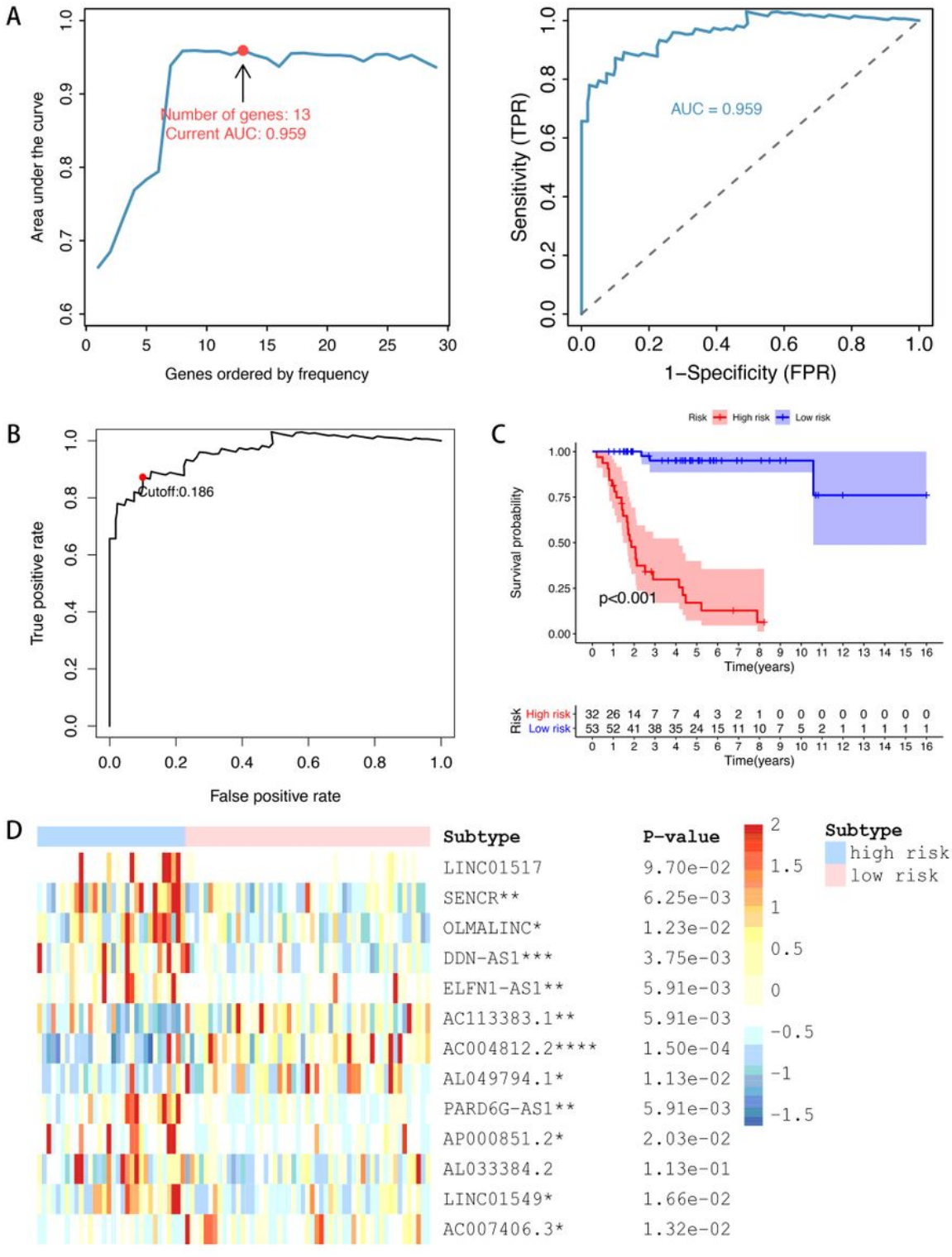
# Tables

Table 1. Summary of clinical characteristics of Osteosarcoma patient data sets in the study.

| Characteristic | | TCGA dataset N=88 |
|---|---|---|
| Vital status, n (%) | Alive | 57(64.8) |
| | Dead | 29(33.0) |
| | Unknown | 2(0.2) |
| Age, n (%) | > = 18 | 19(21.6) |
| | <18 | 69(78.4) |
| Gender, n (%) | Male | 51(58.0) |
| | Female | 37(42.0) |
| Metastasis, n (%) | M0 | 66(75) |
| | M1 | 22(25) |

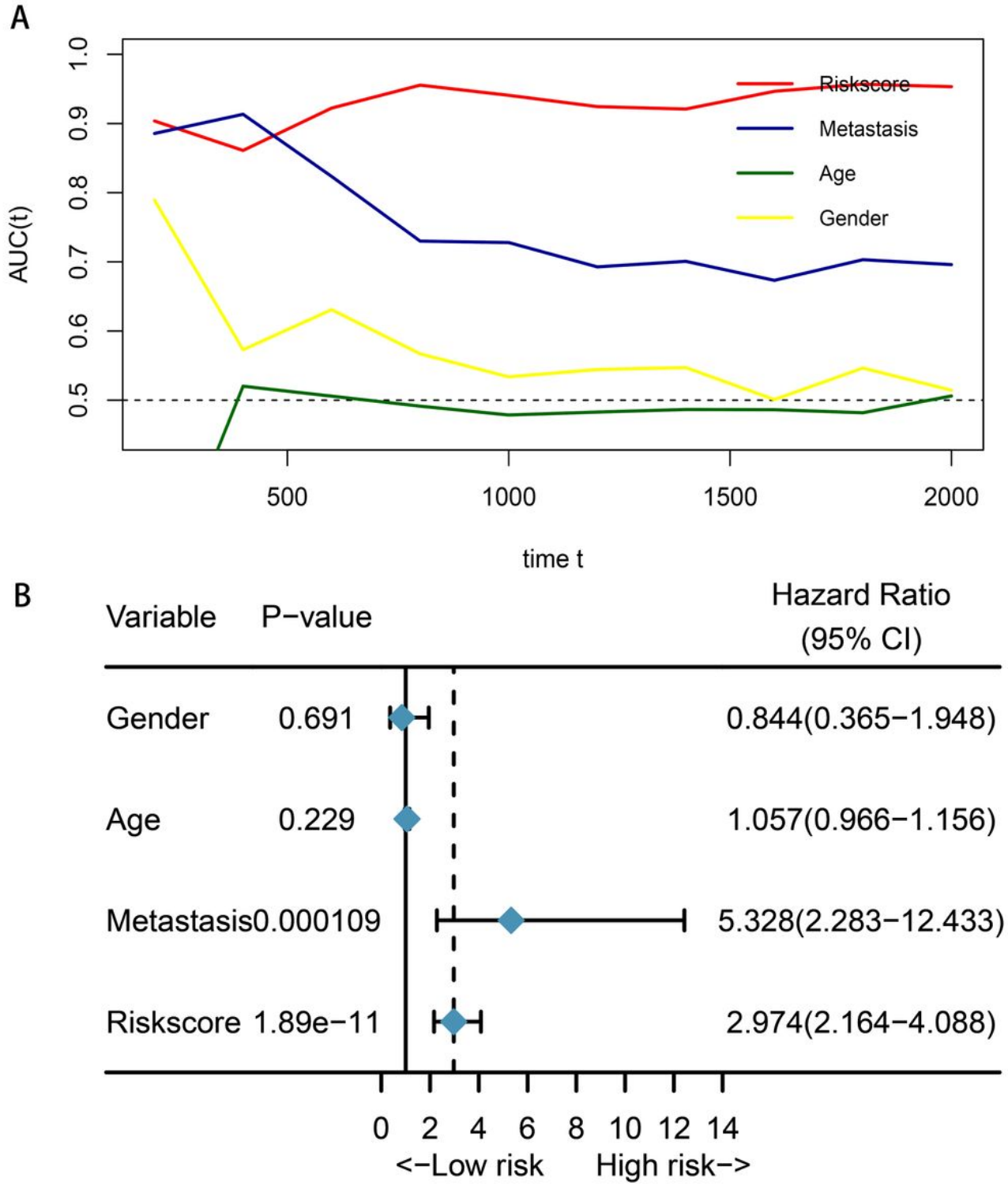
# Figures




## Figure 1

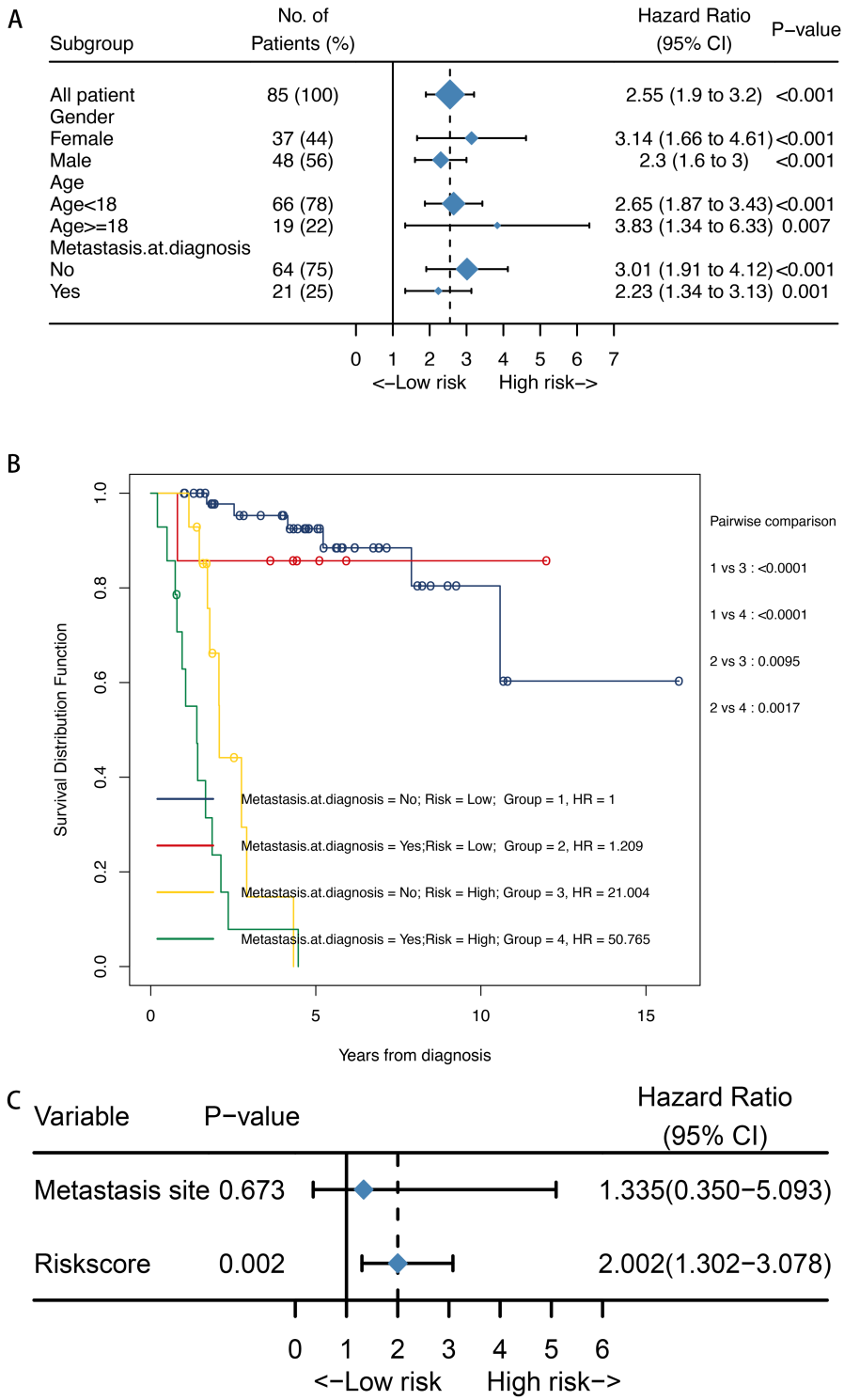
Construction of lncRNA-based signature. (A) Calculate the area under the receiver operating characteristic curve (AUROC) and identify its peak value. (B) Time-dependent ROC curve of LncRNA signature. The optimal cut-off value of LncRNA signature is 0.186, and patients are divided into high-risk group and low-risk group according to the cut-off value (C) Kaplan–Meier curves of overall survival according to LncRNA

signature groups in the TCGA cohort. (D) Heat map of the expression of lncRNA constituting the signature in two groups of patients.



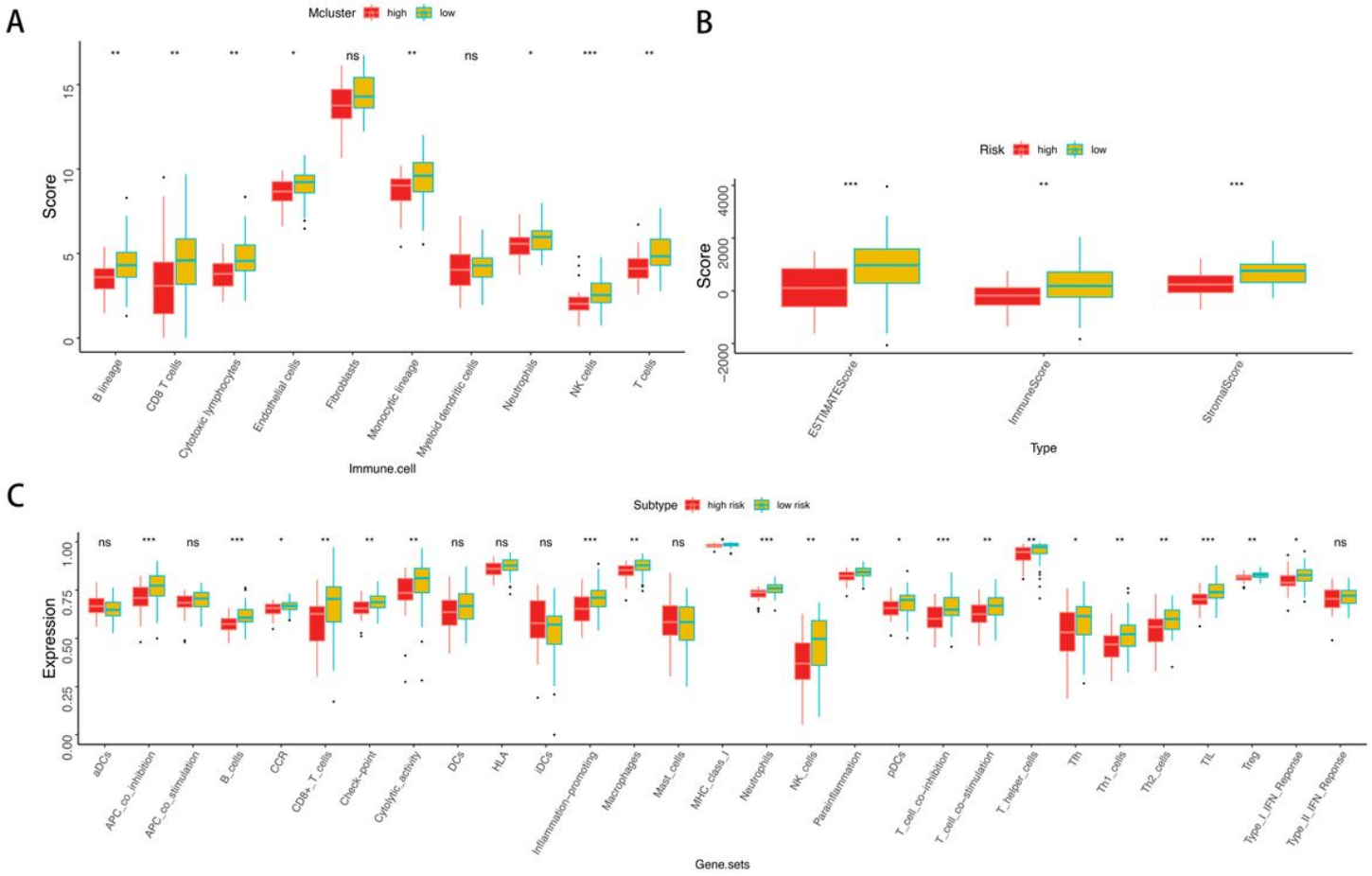

## Figure 2

Evaluate whether lncRNA signature is an independent prognostic factor. (A) ROC curve of clinical characteristics and lncRNA signature. (B) Forest plot of multivariate Cox regression results of lncRNA signature and clinical characteristics.



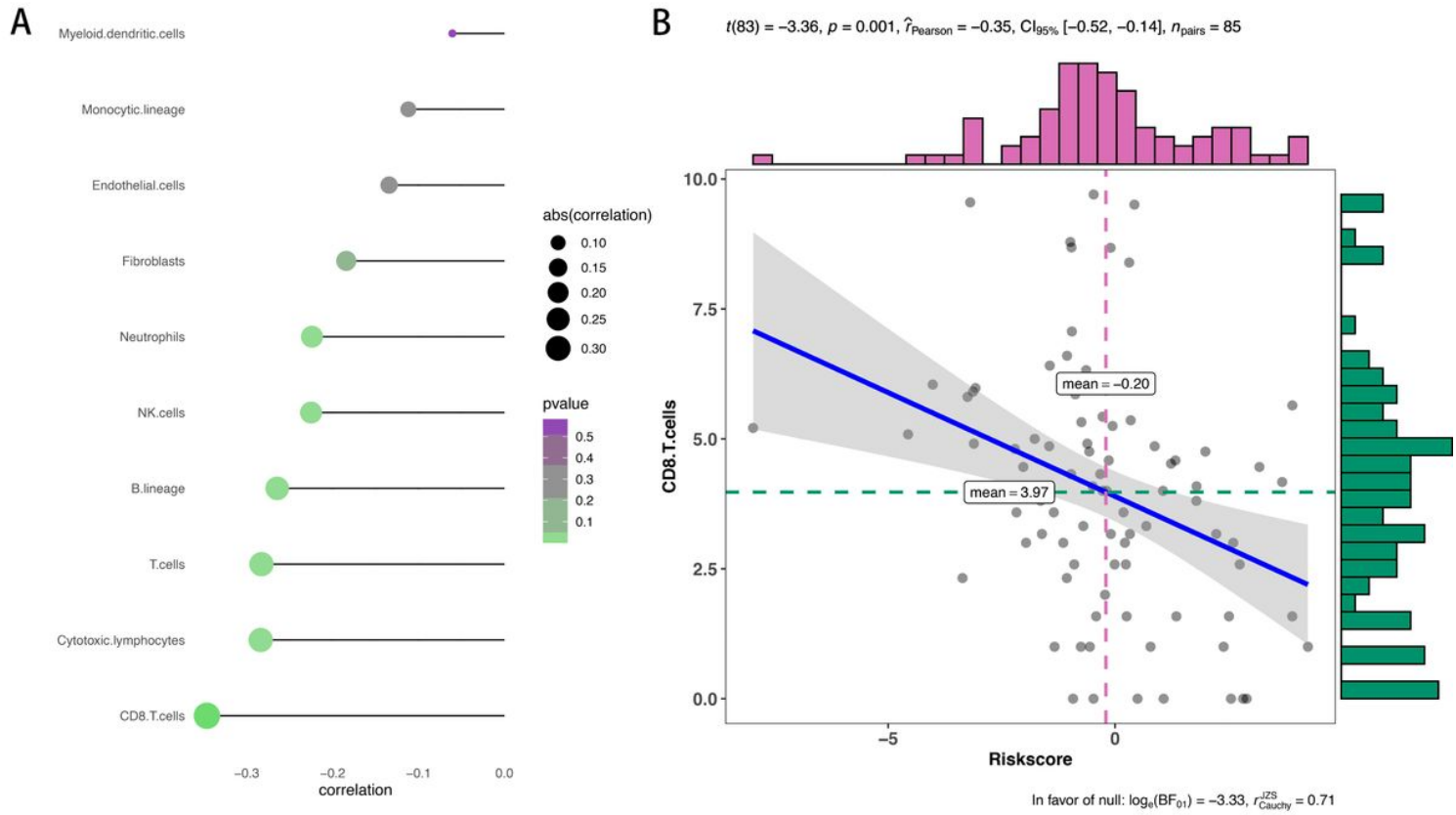

## Figure 3

Subgroup analysis based on clinical characteristics. (A) Forest plot to assess the prognostic value of lncRNA signature in subgroups. (B) KM survival curve of four groups of patients classified by signature and metastatic status. (C) Forest plot of multivariate Cox regression results of lncRNA signature and metastasis site.



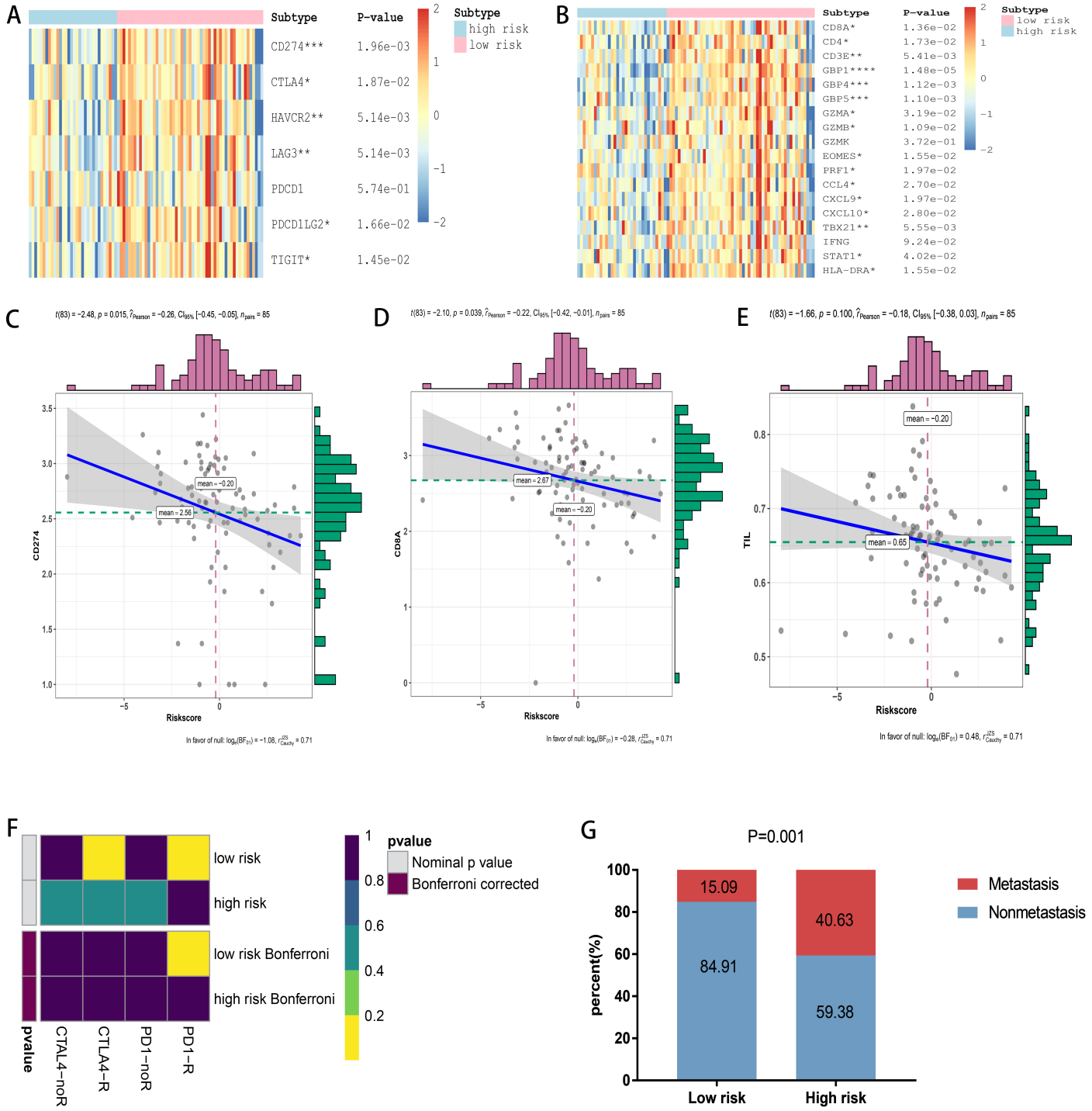

## Figure 4

Assess the difference in immune infiltration between the two groups. (A) Differences of 10 cell abundances calculated by MCP-counter method between two groups of patients. (B) Box plot of stromal score, immune score and estimate score calculated by ESTIMATE method. (C) Box plot of 29 gene sets calculated by ssGSEA. *P<0.05, **P<0.01, ***P<0.001




## Figure 5

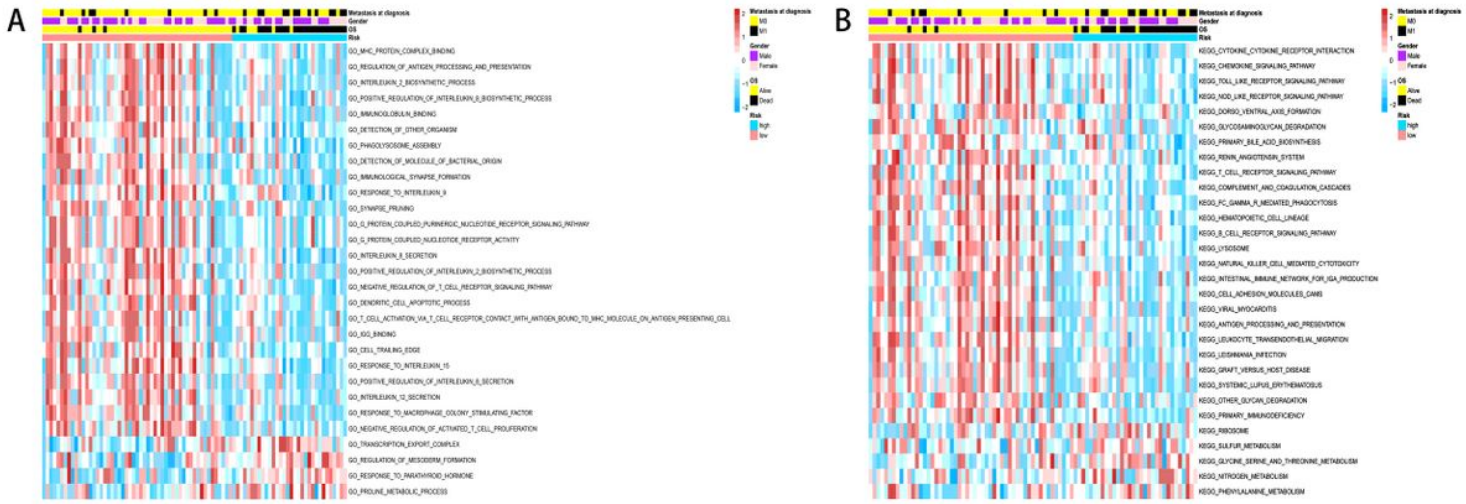
Assess the relationship between immune infiltration and risk score. (A) Assess the relationship between abundance of 10 immune-related cells and risk score. (B) Assess the relationship between CD8.T cell abundance and risk score.




## Figure 6

Assess the patient's Tumor microenvironment immune type. (A) Heat map of 7 immune checkpoint gene expressions in two groups of patients. (B) Heat map of expression of immune-related genes in two groups of patients. (C) Relationship between CD247 expression and risk score. (D) Relationship between CD8A expression and risk score. (E) Relationship between TIL and risk score. (F) Heatmap of correlation between expression profiles of patients in the LncRNA signature group and patients receiving

immunotherapy. (G) Histogram to assess the metastatic status of the two groups of patients. *P<0.05, **P<0.01, ***P<0.001




## Figure 7

Biological function of two groups of patients. (A) Heat map of KEGG pathway score calculated by GSVA for two groups of patients. (B) Heat map of GO score calculated by GSVA for two groups of patients.

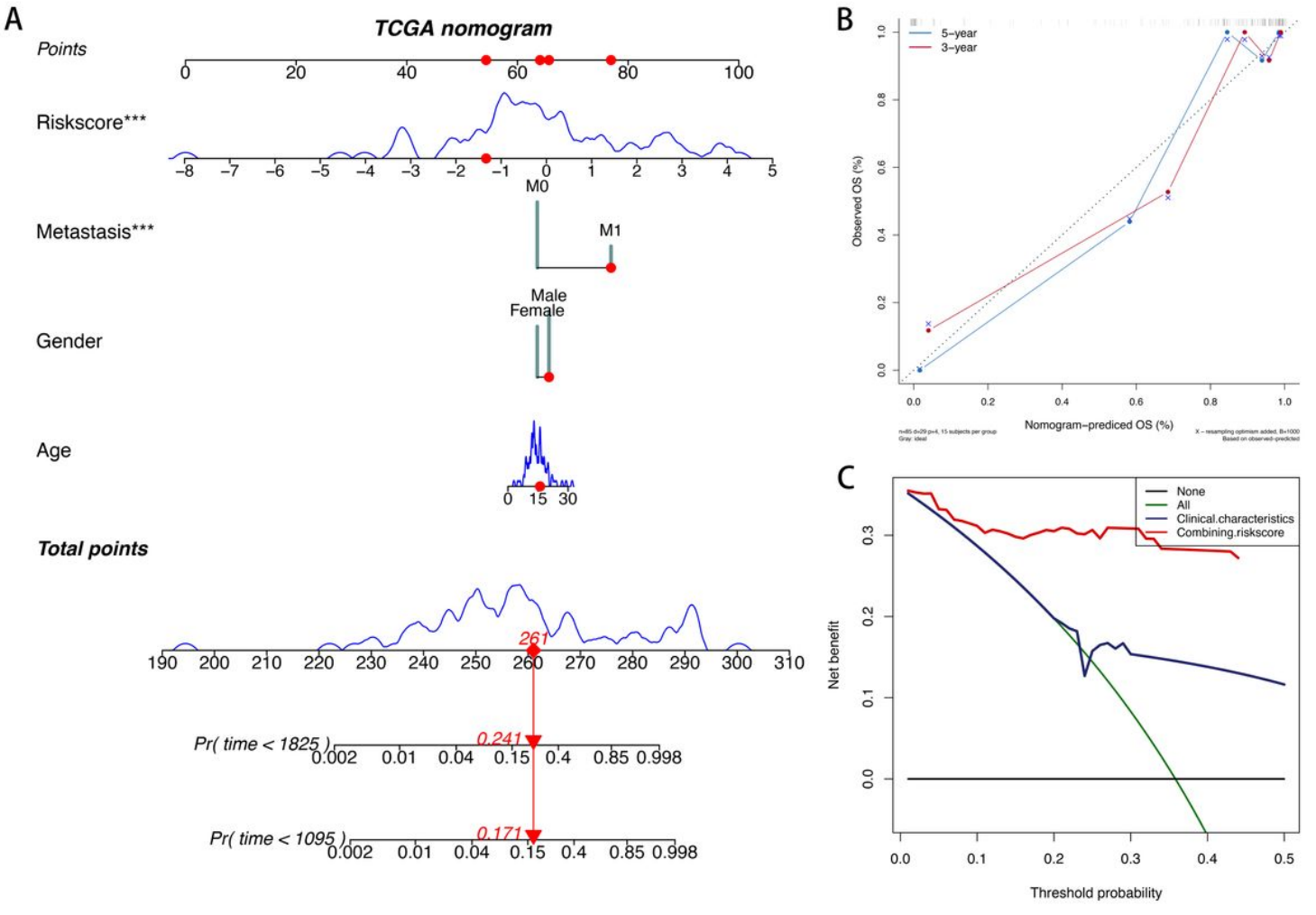


**Figure 8**

Construct and evaluate nomogram. (A) Nomograms for predicting the probability of patient mortality based on lncRNA signature and clinical variables. (B) The calibration plot for internal validation of the nomogram. (C) Decision curve analyses of the nomograms based on lncRNA signature for 3-year overall survival. *P<0.05, **P<0.01, ***P<0.001

# Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementarytable.xlsx
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