Heliyon 10 (2024) e24990

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

LINC00960 affects osteosarcoma treatment and prognosis by regulating the tumor immune microenvironment

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ARTICLE INFO

Keywords:
Osteosarcoma
Prediction model
Tumor immune microenvironment
Therapy
Prognosis
Single-cell analysis

ABSTRACT

Background: Osteosarcoma (OS), the commonest primary malignant bone tumor, is mainly seen in children and teenagers. LINC00960, a newly discovered long intergenic non-protein coding RNA, has been shown to be important in certain cancers. The objective of this study was to assess LINC00960's prognostic and therapeutic value and analyze its mechanism of action in osteosarcoma.

Methods: With the transcriptome information of 85 osteosarcomas from the TARGET database, the Cox regression analyses, K-M curve, and ROC curve, were conducted for survival and prognostic analysis. The functional analysis was conducted using GO, KEGG, GSEA, and GSVA. The ESTIMATE, ssGSEA, MCP-counter, ImmuCellAI algorithms, and immune checkpoint correlation analysis were performed for immune-related analysis. The single-cell RNA sequencing data of 6 osteosarcoma patients was obtained from the Gene Expression Omnibus database. The Tumor Immune Dysfunction and Exclusion algorithm and the "pRRophetic" R package were performed to predict the response to immunotherapy and chemotherapy.

Results: LINC00960 overexpression is associated with osteosarcoma metastasis and poor prognosis. Based on the LINC00960 expression, the nomogram prediction model was created, which showed good accuracy and precision to predict the overall survival of osteosarcoma. Single-cell and immune-related analysis showed that LINC00960 is mainly highly expressed in the tumor-exhausted CD8 T cells in osteosarcoma. In osteosarcoma, the expression of LIC00960 was favorably connected with immune checkpoint-related genes and negatively correlated with immune infiltration. TIDE analysis indicated that low LINC00960 expression patients might have a better response to immunotherapy. Drug sensitivity analysis showed that high LINC00960 expression patients might have better responses to Bleomycin and Doxorubicin.

Conclusion: LINC00960 has the potential to be a novel biomarker for predicting overall survival in osteosarcoma patients and to guide more individualized treatment and clinical decision-making.

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https://doi.org/10.1016/j.heliyon.2024.e24990
Received 15 February 2023; Received in revised form 29 November 2023; Accepted 18 January 2024
Available online 2 February 2024

1. Introduction

The most prevalent primary malignant bone tumor, osteosarcoma (OS), typically affects children and adolescents and has an annual incidence rate of 3–4 occurrences per million people worldwide [1]. Osteosarcoma typically develops from the long bones' metaphyseal area, which is close to active growth plates, especially the proximal tibia and distal femur, and originates from mesenchymal tissues [2–4]. High rates of relapse and metastasis are its main characteristics and therefore are the major causes of poor prognosis [5]. At present, surgery and combinational chemotherapy are mainly treatment methods for osteosarcoma, which can cure 70 % of osteosarcoma [6]. With the development of medical technology, increasingly novel technologies are applied to the therapy of osteosarcoma, such as preoperative chemotherapy, immunotherapy, and so on [7]. However, recent research shows that in the last 20 years, the overall survival of osteosarcoma patients has seen little to no further gains [8,9]. Patients with metastatic or relapsed osteosarcoma had a 5-year survival rate of around 20 % [6,10]. Hence, finding novel biomarkers and therapeutic targets is urgently needed to guide the individualized treatment of osteosarcoma.

Long non-coding RNAs (lncRNAs) are non-protein coding RNAs with transcript lengths longer than 200 nt [11]. Several researchers have observed that lncRNAs exhibited the characteristics of mRNAs and influenced the occurrence of tumors via various mechanisms [12,13]. With the recent developments in genomics technology, large-scale genomics and transcriptomic research of cancer have further confirmed this [14]. Long non-coding RNA 00960 (LINC00960), a novel found lncRNA, has been proven to play an important role in bladder cancer, pancreatic cancer, and lung adenocarcinoma [15–17]. Moreover, Shi et al. reported that high LINC00960 expression can adjust SALL4 by sponging miR-107, thereby promoting the proliferation of osteosarcoma [18]. Although LINC00960 has been researched in several cancers including osteosarcoma, the specific function, therapy, and prognostic values of LINC00960 in osteosarcoma are still unclear. To find it, the more efficient way, bioinformatics analysis, utilizing public information databases should be conducted. Thus, we performed the analysis on the transcriptome information and the corresponding clinical data of 88 osteosarcoma patients from the Therapeutically Applicable Research to Generate Effective Treatments (TARGET) database. Finally, we observed that LINC00960 expression, which could be a potential new therapeutic targeting, was closely connected with the immune microenvironment and overall survival of osteosarcoma.

2. Materials and methods

2.1. Data collection

The RNA sequencing (RNA-seq) and clinical data of 88 osteosarcomas were extracted from the TARGET database (https://ocg.cancer.gov/programs/target) and normalized by log2 (TPM value + 1). All available normal musculoskeletal RNA expression data in the GTEx database was included as a control group and normalized by log2 (TPM value + 1) (https://gtexportal.org/). Due to missing survival data, 3 osteosarcoma patients were removed, and, finally, 85 patients were enrolled in this study. The osteosarcoma and normal tissue datasets were merged into one dataset, and the batch effects between the two datasets were removed with the "ComBat" algorithm of the "sva" R package with default parameters [19]. The data distribution of the two datasets before and after removing the batch effect was shown in Fig. S1. Furthermore, RNA sequencing data of 33 cancers and the adjacent normal tissues were extracted from The Cancer Genome Atlas (TCGA) database (https://portal.gdc.cancer.gov/).

2.2. Survival and prognostic analysis

The X-tile software was utilized to determine the most efficient cutoff point of LINC00960 expression status based on overall survival in this study which aimed to classify patients accurately [20]. With the optimal cutoff point of '2.511' calculated by X-tile software, 85 patients were divided into low- and high-LINC00960 expression groups. The univariate and multivariate Cox regression analyses and Kaplan-Meier curves were utilized for survival and prognostic analysis. With IBM SPSS Statistics 26 software, we performed univariate and multivariate Cox regression analyses and drew Kaplan-Meier curves with "pROC" (v1.17.0.1) and "timeROC" (v0.4) R packages. In order to assess LINC00960's predictive value, we conducted a time-dependent receiver operating characteristic (ROC) curve using the survival (v3.4-0) and survivalminer (v0.4.9) R packages. We visualized all results using the "ggplot2" (v3.3.6) R package.

2.3. Developing and validating a prognostic model

LINC00960 expression and other clinical characteristics were used to establish a nomogram for predicting overall survival over one, three, and five years. The C-index and calibration curve were calculated using the R packages survival (v3.4-0) and rms (v.0.4.96.3-0) to evaluate the nomogram's performance.

2.4. Differentially expressed gene screen

Differentially expressed genes (DEGs) were screened using the R package limma (version 3.40.6) between groups of high LINC00960 expression and low LINC00960 expression [21]. An absolute value of log-fold changes greater than 1.5 and a p-value of 0.05 was the threshold for DEGs. The "ggplot2" (v3.3.6) R package was used to visualize the volcano plot of DEGs.

2.5. Functional enrichment analyses and Gene Set Variation Analysis

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis were conducted using the R package ClusterProfifiler (v3.14.3). The results were visualized by the "ggplot2" (v3.3.6) R package and the web-based tool Metascape (https://metascape.org/). Gene set enrichment analysis (GSEA) was conducted with the annotated genesets (c5.go.bp.v7.4.symbols.gmt) from the Molecular Signatures Database (http://www.gsea-msigdb.org/gsea/downloads.jsp) [22]. $|NES| > 1$ and a P value of 0.05 were used as thresholds. Meanwhile, Gene set variation analysis (GSVA) was performed to further explore the pathway alteration of patients using the "GSVA" R package (v1.44.5), and the result of GSVA was visualized with the "pheatmap" R package (v1.0.12).

2.6. Immune microenvironment and immune-related functions analysis

We measured the immune score, stromal score, ESTIMATE score, and tumor purity to compare the immune microenvironment between the two groups, using the "estimate" R package (v1.0.13) [23]. To examine the differences between the two groups in immune-related functions, the "GSVA" R package (v1.44.5)was used to perform the single-sample GSEA (ssGSEA) analysis. The association between LINC00960 expression and immune microenvironment score and immune-related functions was conducted by Pearson correlation analysis.

2.7. Infiltration of immune cells and immune checkpoints

Infiltration levels of various immune cells were evaluated and compared using the microenvironment cell populations-counter (MCP-counter) and immune cell abundance identifier (ImmuCellAI) algorithms [24,25]. Besides, with the Pearson correlation analysis, we assessed the relationship between LINC00960 expression and the abundance of immune cells and the association between LINC00960 expression and the immune checkpoint-related genes (ICGs) expression.

2.8. Single-cell analysis

Utilizing the Tumor Immunology Single Cell Hub 2 (http://tisch.comp-genomics.org/) database, we performed the single-cell analysis of 6 osteosarcoma patients in GSE162454, which was obtained from the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/), to analyze the expression pattern and immunological role of LINC00960.

2.9. Predict immunotherapy response and chemotherapy drugs sensitivity

The potential immunotherapy response of the two groups was investigated using the Tumor Immune Dysfunction and Exclusion (TIDE) online algorithm (http://tide.dfci.harvard.edu/). TIDE validation dataset GSE221173 extracted from GEO database. Additionally, to predict the chemotherapy drugs sensitivity of osteosarcoma patients with high or low LINC00960 expression, we evaluated the 50 % inhibiting concentration (IC50) values of several common chemotherapeutic drugs, with the help of the "pRRophetic" R package (v0.5).

2.10. Statistical analysis

Statistical analyses were performed applying the R software (v4.1.3) and the IBM SPSS statistics 26 software. With the Pearson

Table 1
Clinical characteristics of osteosarcoma patients with high and low LINC00960 expression.

Characteristic	Low-LINC00960 expression (n = 54)	High-LINC00960 expression (n = 31)	*p*-value
	n (%)	n (%)	
Age			0.61
< 16 years	34 (40.00)	17 (20.00)	
≥16 years	20 (23.53)	14 (16.47)	
SEX			0.36
Male	28 (32.94)	20 (23.53)	
Female	26 (30.59)	11 (12.94)	
Survival status			0.02
Dead	13 (15.29)	16 (18.82)	
Alive	41 (48.24)	15 (17.65)	
Metastasis			0.04
Non-metastasis	45 (52.94)	19 (22.35)	
Metastasis	9 (10.59)	12 (14.12)	
Tumor location			0.32
Tibia	16 (18.82)	5 (5.88)	
Femur	22 (25.88)	17 (20.00)	
Others	16 (18.82)	9 (10.59)	

correlation analysis, the correlation analysis was conducted. The *t*-test was utilized for comparison between the two groups. Especially, Kaplan-Meier curves were evaluated using the log-rank test. Statistical significance was determined by P values < 0.05, and the significance levels were set at * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

3. Result

3.1. LINC00960 expression is elevated in 17 cancers and osteosarcoma

The various expression status of LINC00960 of 85 osteosarcoma patients in the TARGET database showed different clinical characteristics and overall survival rates (Table 1). As LINC00960 increases, tumor site, metastasis status, living status, age, gender, and overall survival revealed unsymmetrical distributions in the TARGET datasets (Fig. 2a). Using the LINC00960 expression matrix of 33 cancers from the TCGA database, 85 osteosarcoma patients from the TARGET database, and normal musculoskeletal tissues from the GTEx database (Fig. 1), we discovered the expression of LINC00960 was enriched in 17 cancers compared with their corresponding adjacent normal tissues (Fig. 2b), and the LINC00960 expression was also elevated in osteosarcoma patients compared with normal musculoskeletal tissues (Fig. 2c). Besides, we found that in osteosarcoma patients with metastases, LINC00960 expression was considerably higher than in those without metastases ($p < 0.05$, Fig. 2d).

3.2. LINC00960 overexpression is related to poor prognosis of osteosarcoma

The 85 osteosarcoma patients were divided into high and low LINC00960 expression groups based on the optimal cut-off value of LINC00960. Then, K-M curves were used to calculate survival rates to investigate the effect of LINC00960 overexpression on osteosarcoma prognosis, and the results, in different clinical subgroups, suggested that high LINC00960 expression group patients showed a poor clinical prognosis (Fig. 3a–i). Meanwhile, the univariate logistic regression results revealed that status (odds ratio [OR] = 3.364, 95 % CI = 1.327–8.807, p = 0.016) and metastasis (odds ratio [OR] = 0.137, 95 % CI = 0.111–0.867, p = 0.027) do vary a lot between the two groups (Table 2). All above results demonstrated that overexpression of LINC00960 predicted poor prognosis in osteosarcoma.

3.3. High LINC00960 expression is an independent risk factor for osteosarcoma

High LINC00960 expression was evaluated by univariate and multivariate Cox regression analyses for osteosarcoma risk (Table 3). The results showed that metastasis status ($p < 0.001$, HR:4.740, 95 % CI: 2.271–9.895) and high LINC00960 expression ($p = 0.042$,

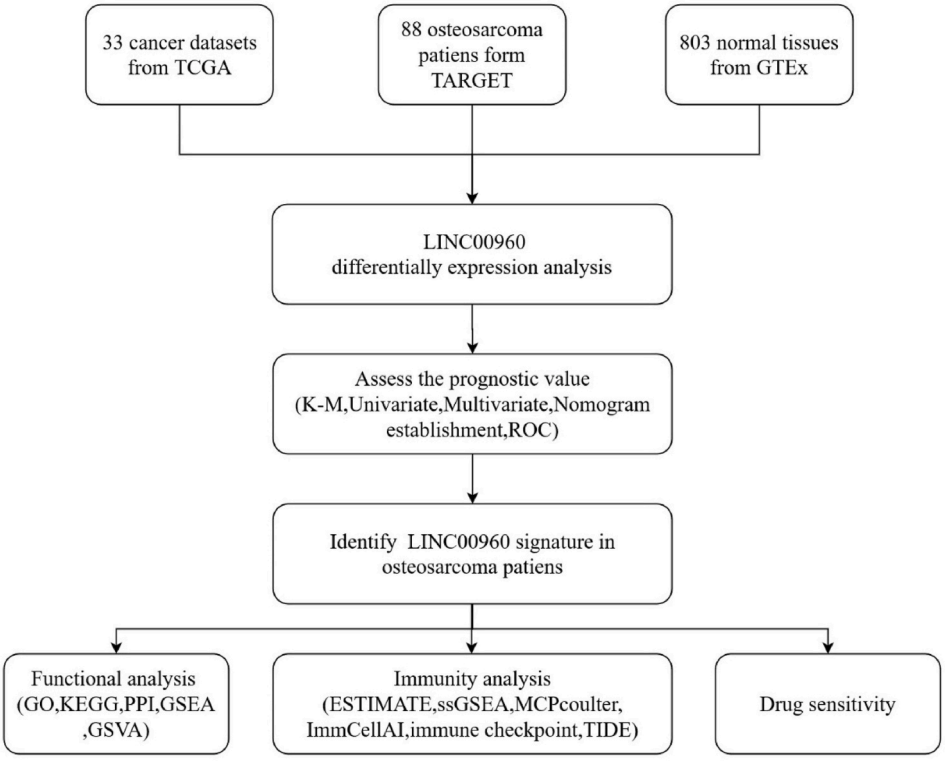

Fig. 1. Workflow chart.

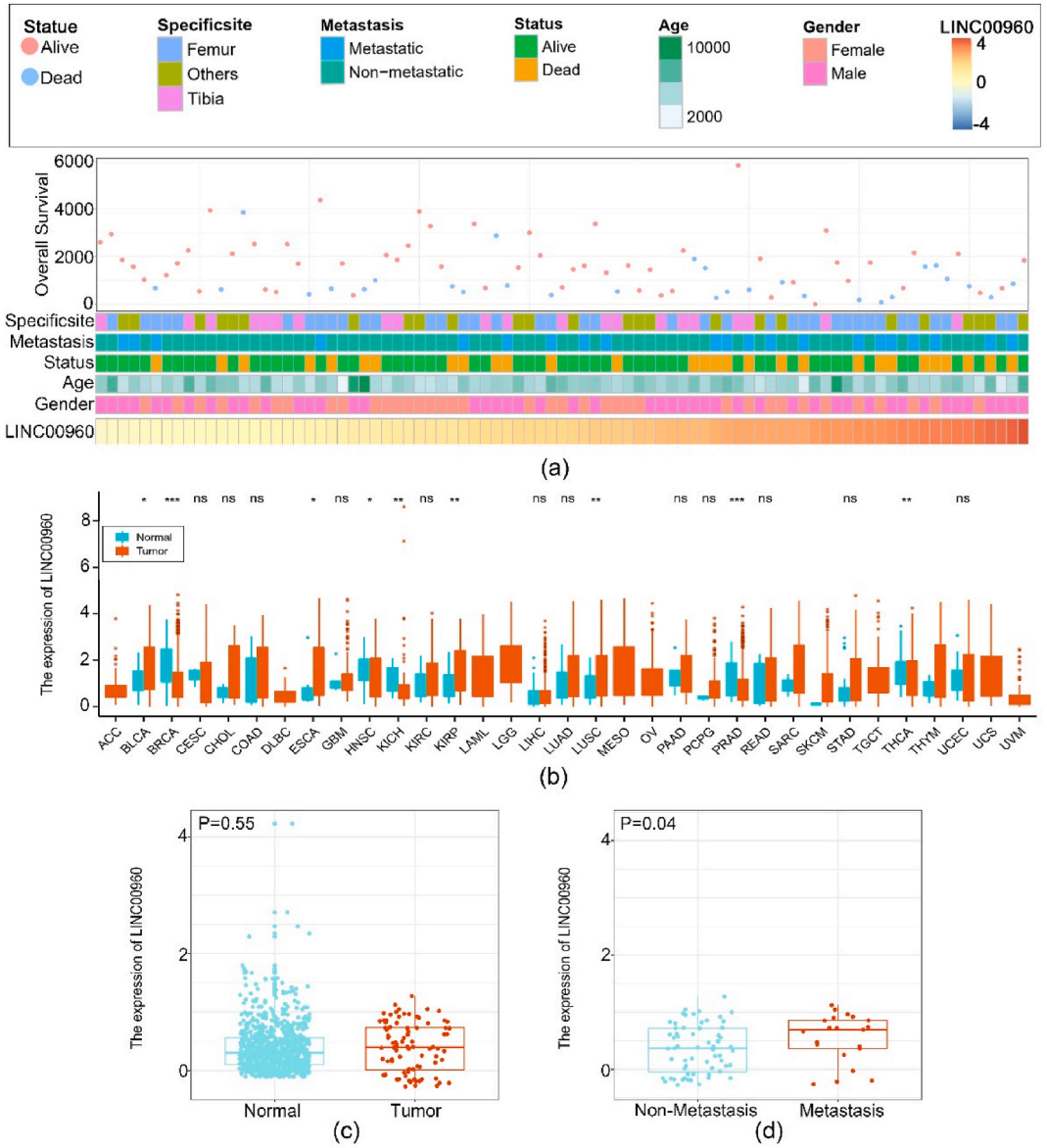

Fig. 2. LINC00960 expression in various cancers and osteosarcoma. (a) The overview of the association between LINC00960 expression and clinical features of osteosarcoma patients. (b) The expression status of LINC00960 in 33 types of tumors and their corresponding adjacent normal tissues. (c) LINC00960 expression status in normal tissues and osteosarcoma. (d) The expression of LINC00960 in osteosarcoma patients with metastasis was remarkably increased. T-tests were used to determine statistical significance. ns, $p \geq 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

HR:1.372, 95 % CI: 1.012–1.860) might be independent risk factors for osteosarcoma (Fig. 4a and b). In addition, to predict patients' survival over the next one, three, and five years more precisely, the nomogram incorporating LINC00960 expression and other clinical characteristics was created (Fig. 4c). The calibration plot was utilized to predict the effectiveness of the forecast model and the C-index of 0.731 was found in the nomogram. (Fig. 4d). Additionally, the AUC values based on the expression status of LINC00960 for predicting the patients' survival over the next one, three, and five years were 0.84, 0.64, and 0.70, as shown in the time-dependent ROC curve, which suggested the acceptable accuracy (Fig. 4e). These findings demonstrated that LINC00960 expression levels might aid clinicians in predicting the survival rates of osteosarcoma.

3.4. Functional analysis of LINC00960-related DEGs in osteosarcoma

As indicated in the volcano plot (Fig. 5a), 218 genes were upregulated and 274 genes were downregulated out of the 492 differentially expressed genes (DEGs) between the two groups that were evaluated. Gene Ontology (GO) analysis revealed that LINC00960 was most related to immune functional clusters, such as innate immune response, inflammatory response, and so on

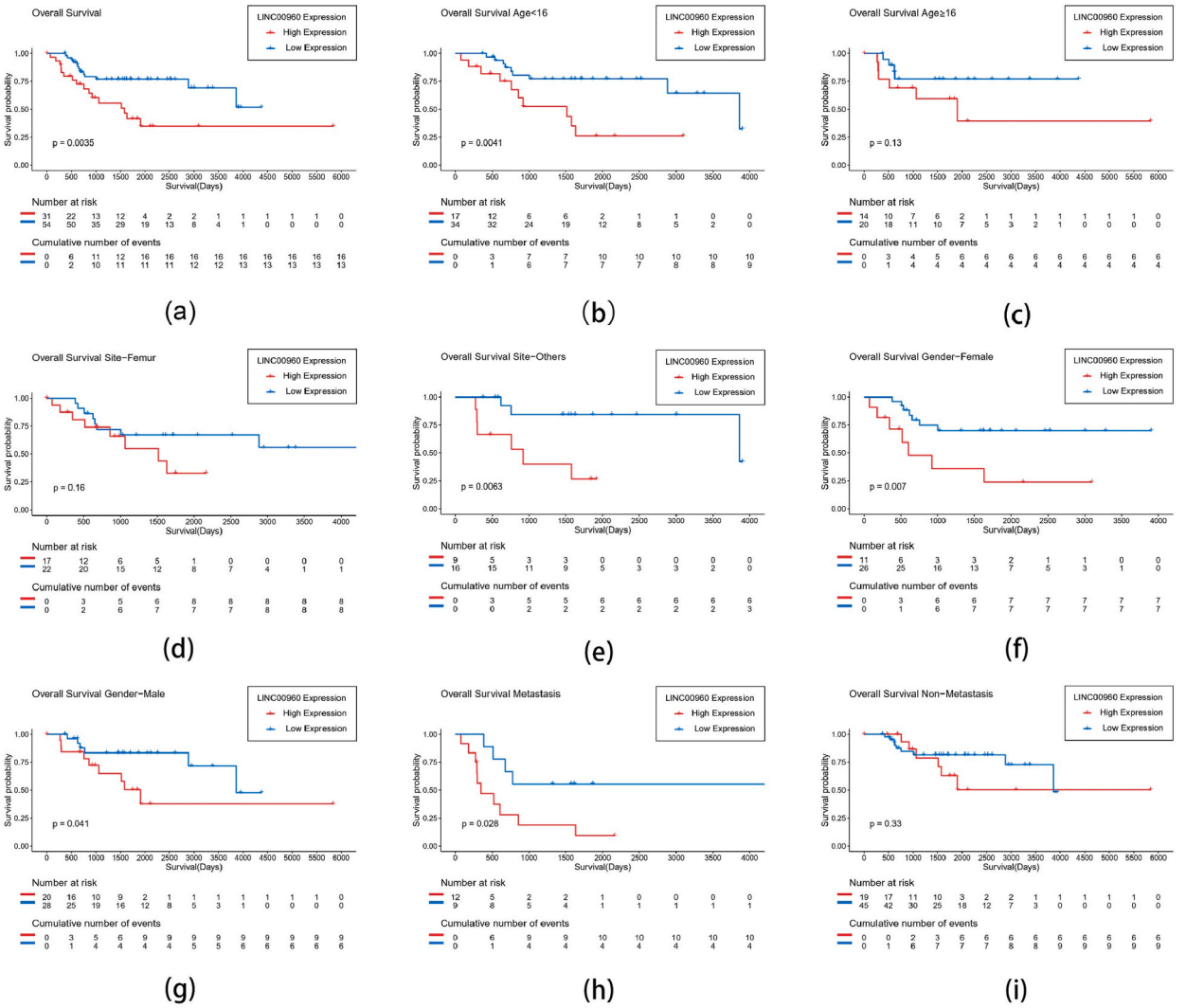

Fig. 3. Kaplan-Meier analysis indicated the relationship between LINC00960 expression and the prognosis in different clinical subgroups. Association between the low- and high-LINC00960 expression and OS of (a) all patients. (b) age <16 years subgroup. (c) age≥16 years subgroup. (d) the osteosarcoma of the Femur subgroup. (e) the osteosarcoma in other sites subgroup. (f) the female subgroup. (g) the male subgroup. (h) the metastatic subgroup. (i) the non-metastatic subgroup.

Table 2
Associations of LINC00960 expression with clinical characteristics (logistic regression).

Characteristics	Total (N)	LINC00960 expression		
		OR	95 %CI	*p*-value
Age (≥16 vs. < 16)	85	1.400	0.568–3.450	0.462
Gender (Male vs. Female)	85	1.688	0.688–4.286	0.259
Status (Alive vs. Dead)	85	3.364	1.327–8.807	0.016
Metastasis (Metastasis vs. Non- Metastasis)	85	0.317	0.111–0.867	0.027

(Fig. 5b–e). In Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway, LINC00960 was enriched in Natural killer cell mediated cytotoxicity, Osteoclast differentiation, and so on (Fig. 5f). Similarly, Protein-protein interaction (PPI) analysis showed LINC00960 was strongly linked with immune function (Fig. S2). To counteract bias caused by the functional analysis's exclusion of some linked DEGs' biological characteristics and differential exclusion filters, we also conducted GSEA and GSVA analyses. As shown in the results of GSEA, immune function and skeletal system were significantly enriched, which confirmed the conclusion of GO and KEGG analysis. (Fig. 6a and b). Consistently, GSVA analysis results revealed that LINC00960 expression was negatively correlated with the

Table 3
Univariate and multivariate Cox regression analyses of the association between clinical traits, including expression levels of LINC00960, and overall survival.

Characteristics	Univariate analysis		Multivariate analysis	
	Hazard ratio (95 % CI)	*p*-value	Hazard ratio (95 % CI)	*p*-value
Age (≥16 vs.<16)	1.000 (1.000–1.000)	0.813		
Gender (Male vs. Female)	0.687 (0.330–1.429)	0.315		
Primary site (Leg vs. Other)	0.489 (0.168–1.419)	0.188		
Specific site (Leg vs. Other)	0.569 (0.215–1.504)	0.256		
Metastasis (Metastasis vs. Non- Metastasis)	4.740 (2.271–9.895)	**< 0.001**	4.471 (2.131–9.379)	**< 0.001**
LINC00960 (High vs. Low)	1.444 (1.058–1.970)	**0.021**	1.372 (1.012–1.860)	**0.042**

inflammatory response (Fig. 6c). Collectively, these findings indicated LINC00960 may correlate with the immune functions and skeletal system in osteosarcoma.

3.5. LINC00960 correlated with the immune microenvironment and immune functions

According to the gene functional analysis results, we following explored the tumor immune microenvironment (TIME) difference between the low and high LINC00960 expression groups. Firstly, the ESTIMATE algorithm demonstrated the high-LINC00960 expression group showed a considerably lower ESTIMATE score ($p = 0.0072$), immune score ($p < 0.001$), and higher tumor purity ($p = 0.0034$) than the low-LINC00960 expression group, but the stromal score between the two groups was not statistically significant ($p = 0.31$; Fig. 7 a-d). Additionally, the Pearson correlation analysis results revealed that LINC00960 expression was positively correlated with tumor purity ($p = 0.037$, R = 0.23), but negatively correlated with ESTIMATE score ($p = 0.036$, R = −0.23), immune score ($p = 0.0013$, R = −0.34) and stromal score ($p = 0.85$, R = −0.021; Fig. 7 e-h). Furthermore, we carried out the ssGSEA analyses of the relation between LINC00960 expression and immune-related functions, and we discovered a statistically significant difference between the two groups in T_cell_co-inhibition ($p < 0.001$), Check-point ($p < 0.005$) and so on (Fig. 7i). As shown in the correlation matrix, almost all the immune-related functions were negatively correlated with LlINC00960 expression (Fig. 7j). These results indicated that LINC00960 overexpression might lead to the co-inhibition of T cells in the immune microenvironment and the over-expression of immune checkpoints.

3.6. Single-cell analysis of LINC00960

To further investigate the expression pattern of LINC00960 and its role in influencing the TIME. Using the TISCH2 database, we analyzed single-cell data from osteosarcoma patients in GSE162454 (Fig. 8a and b). Firstly, we found that LINC00960 was highly expressed mainly in Malignant Cells and Fibroblasts (Fig. 8c). Not only that, in further analysis, we found that high expression of LINC00960 was also present in the exhausted CD8 T cells (Fig. 8d). These results confirm our previous findings addition to increasing the malignancy of osteosarcoma, was associated with the exhausted CD8 T cells immune cells in the tumor immune microenvironment.

3.7. Immune cell infiltrating and immune checkpoint analysis

In terms of immune cell infiltration analysis, we found that the high-LINC00960 expression group exhibited a lower abundance of T_cells and monocytic_lineage, using the microenvironment cell populations-counter (MCP-counter) algorithm (Fig. 9a). The correlation analysis showed that LINC00960 expression was negatively correlated with the abundance of T_cells, B_lineage, and monocytic_lineage, which was consistent with the results of our immune function analysis (Fig. 9b). Additionally, the ImmuCellAI database analysis showed that γδT was abundant in the low-LINC00960 expression group, whereas the opposite was true for CD8_naive (Fig. 9c–e). The Pearson correlation analysis results indicated that LINC00960 expression was favorably connected with CD8_naive (p = 0.0052, R = 0.3) infiltrating level but negatively correlated with infiltrating level of γδT (p = 0.043, R = −0.22). Furthermore, we assessed the relationship between the expression of LINC00960 and immune checkpoint-related genes (ICGs) because of the results of immune-related function analysis. LINC00960 indicated a positive correlation with ICGs including CD80, CD47, B7-2, and B7–H (Fig. 10a). These data further confirm that the potential regulatory mechanism of LINC00960 in osteosarcoma is through activating ICGs and reducing immune cell infiltration levels.

3.8. Predict the immunotherapy response and the drug sensitivity

Tumor immune dysfunction and exclusion (TIDE) analysis was used to compare the clinical therapeutic efficacy of LINC00960 in two groups and predict how well patients with osteosarcoma will respond to immunotherapy. As shown in the violin diagram, the high-LINC00960 expression group patients had a higher T-cell exclusion score and TIDE score than the low-LINC00960 expression group while the T-cell dysfunction score was lower, although there was no statistical difference in TIDE score between the two groups (Fig. 10b–d). In addition, percentage bar graphs showed that the low-LINC00960 expression group responded better to immunotherapy (Fig. 10e). The results of the TIDE analysis were further confirmed in the validation dataset GSE221173 (Fig. S3). Besides, the

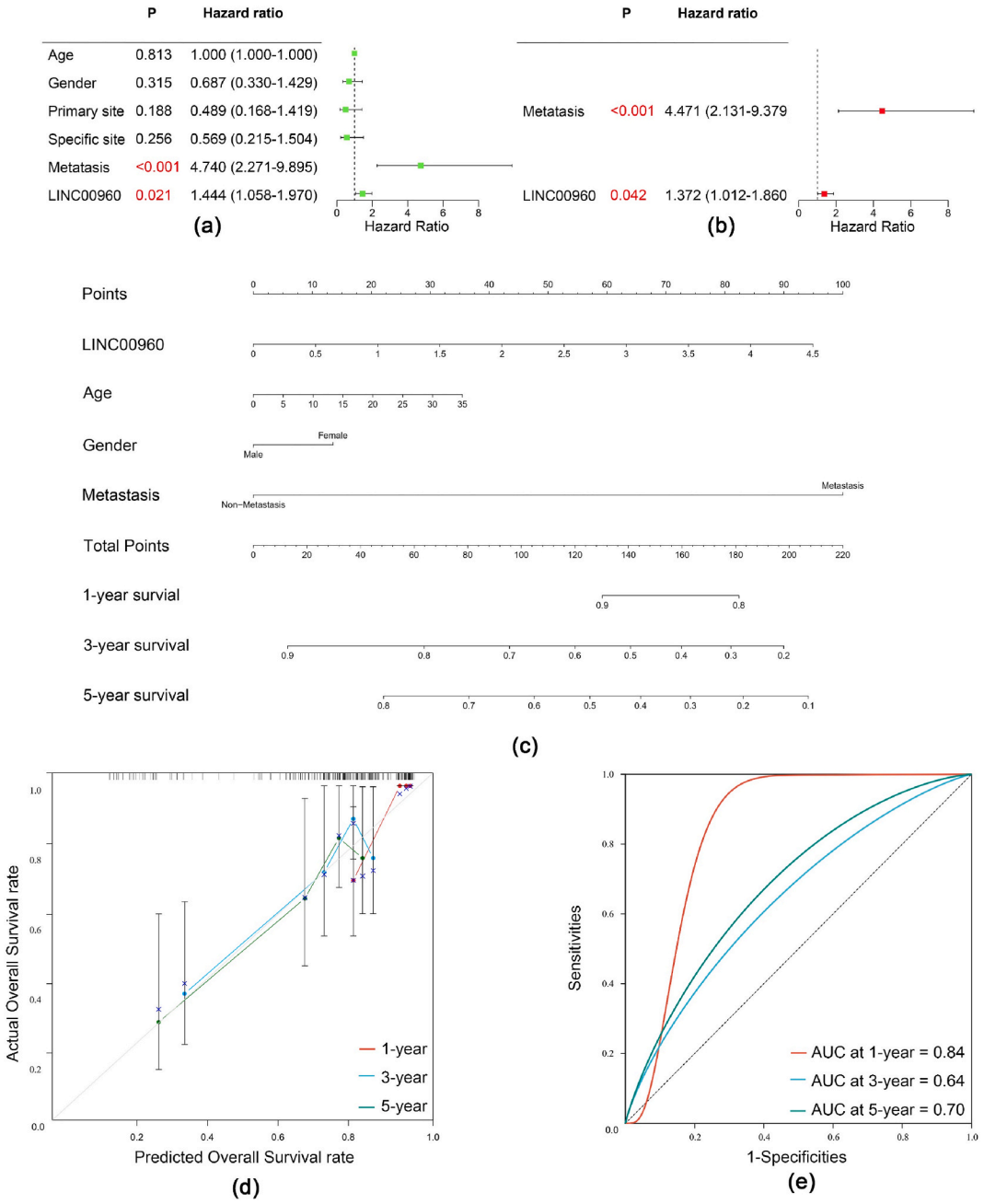

Fig. 4. High LIN00960 expression is an independent risk factor in osteosarcoma. Forest plots show the results of (a)univariate and (b) multivariate cox analysis (c) The nomogram model based on the expression level of LIN00960 and other clinical characteristics. (d) The calibration of the nomogram shows that the overall survival rate at 1, 3, and 5 years was consistent with the expected overall survival rate. (e)Time-dependent ROC curve based on the LINC00960 expression level.

results of drug sensitivity analysis showed that 8 drugs displayed high sensitivity for the low- LINC00960 expression group, including Temsirolimus, CI.1040, AZD6244, BMS.536924, JW.7.52.1, Sorafenib, PF.02341066, and PD.0325901 (Fig. 11a–h). Simultaneously, 4 drugs displayed high sensitivity for the high- LINC00960 expression group including GSK.650394, AG.014699, Bleomycin, and Doxorubicin (Fig. 11i-l). According to these results, LINC00960 may be utilized as a predictive biomarker to choose the appropriate chemotherapy drugs for osteosarcoma patients in the future.

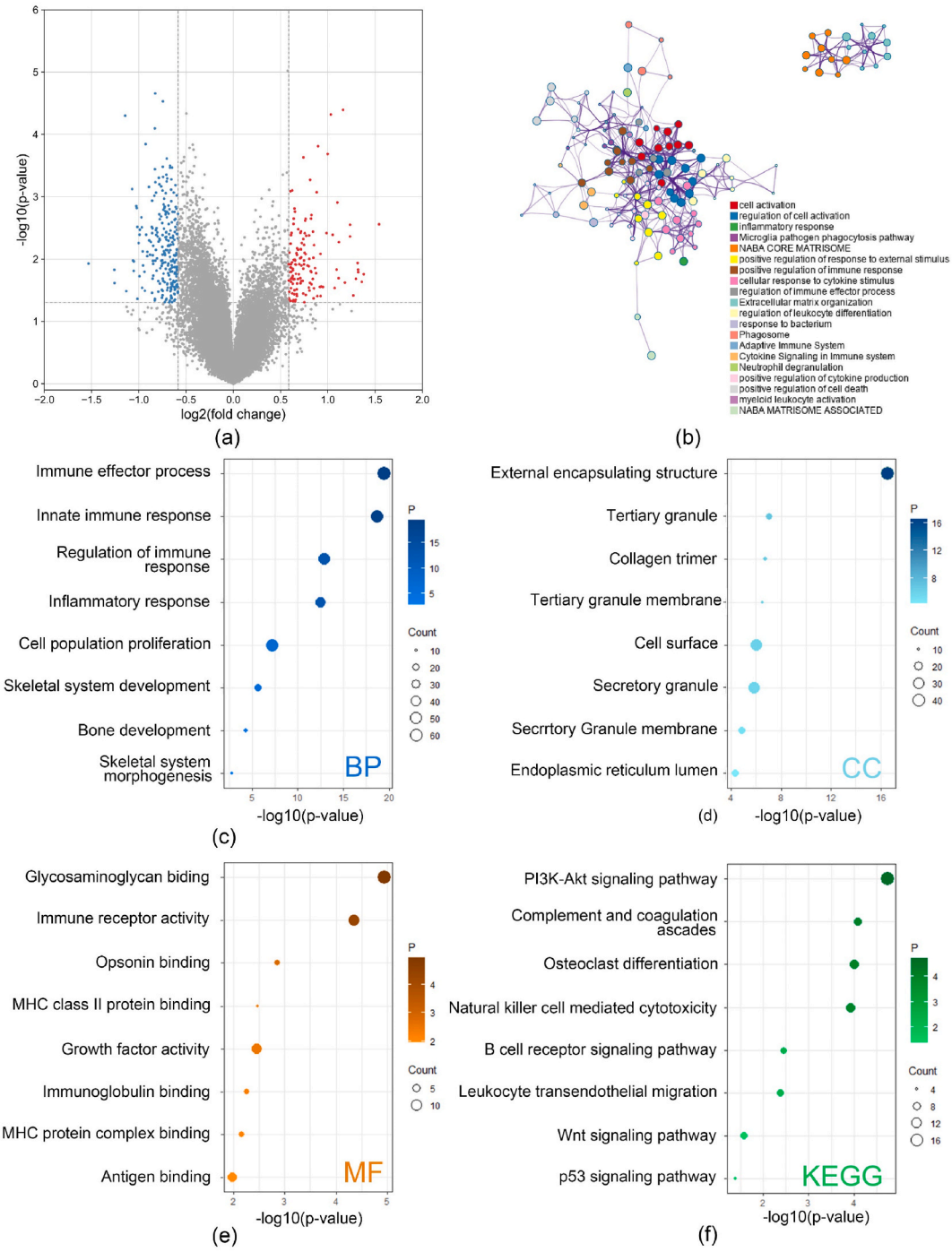

Fig. 5. GO and KEGG analysis of DEGs reveal LINC00960 is associated with immune functions and skeletal system in osteosarcoma (a) The volcano plot shows DEGs (b) GO functional clusters visualized by network picture (c) Biological processes (BP) enrichment analysis. (d) Cellular components (CC) enrichment analysis. (e) Molecular functions (MF) enrichment analysis. (f) KEGG pathway enrichment analysis. GO, Gene Ontology; DEGs, differentially expressed genes; KEGG, Kyoto Encyclopedia of Genes and Genomes.

4. Discussion

Osteosarcoma is the most common primary malignant bone tumor. Although 70 % of people with osteosarcoma can have their overall survival prospects improved by surgery and chemotherapy, recurrence and metastasis of osteosarcoma and resistance to conventional chemotherapy drugs are still the main reasons for the poor prognosis of patients. Therefore, it is urgently needed to

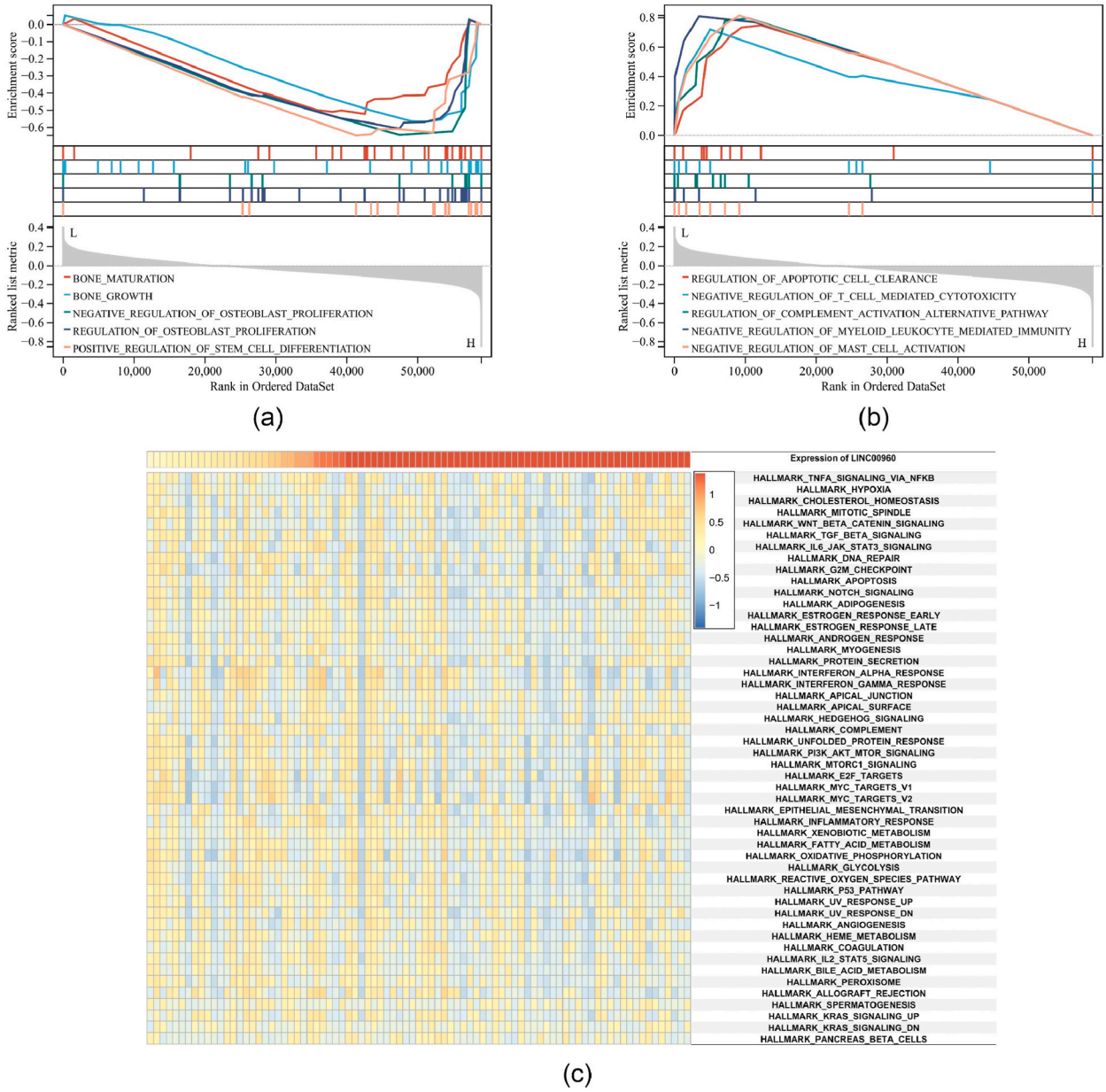

Fig. 6. GSEA and GSVA analysis of DEGs (a, b) BP enrichment analysis based on GSEA. (c) The heatmap shows the hallmark enrichment analysis based on GSVA. GSVA, Gene Set Variation Analysis; GSEA, Gene Set Enrichment Analysis.

identify new therapeutic targets which can offer more individualized and effective treatment to osteosarcoma patients [2,26,27]. We conducted this study and discovered the correlation between LINC00960 expression and osteosarcoma metastasis, prognosis, and treatment. As a result, it could be employed as a novel osteosarcoma predictive and therapeutic target.

Human cancer incidence and progression are highly correlated with lncRNAs. Several researchers have proved the effects of lncRNAs on tumor cellular activity, immune microenvironment, drug resistance, and overall survival [28–34]. LINC00960, a newly discovered long intergenic non-protein coding RNA, has been shown in several prior investigations to be significantly expressed in lung adenocarcinoma, pancreatic cancer, and bladder cancer [15–17]. Additionally, a substantial overexpression of LINC00960 in osteosarcoma was discovered. and it is linked to promoting the growth of osteosarcoma cells through the miR-107/SALL4 axis [18]. However, the specific functions of LINC00960 in osteosarcoma and the association between the expression of LINC00960 and the diagnosis, treatment, and prognosis of osteosarcoma patients have not been further investigated. Hence, in this research, we carried out the research on the effect of LINC00960 expression on the TIME of osteosarcoma using bulk RNA sequencing data and single-cell data from the TARGET database and the GEO database.

First of all, we discovered that 17 tumors had enhanced expression of LINC00960 as compared to their corresponding nearby

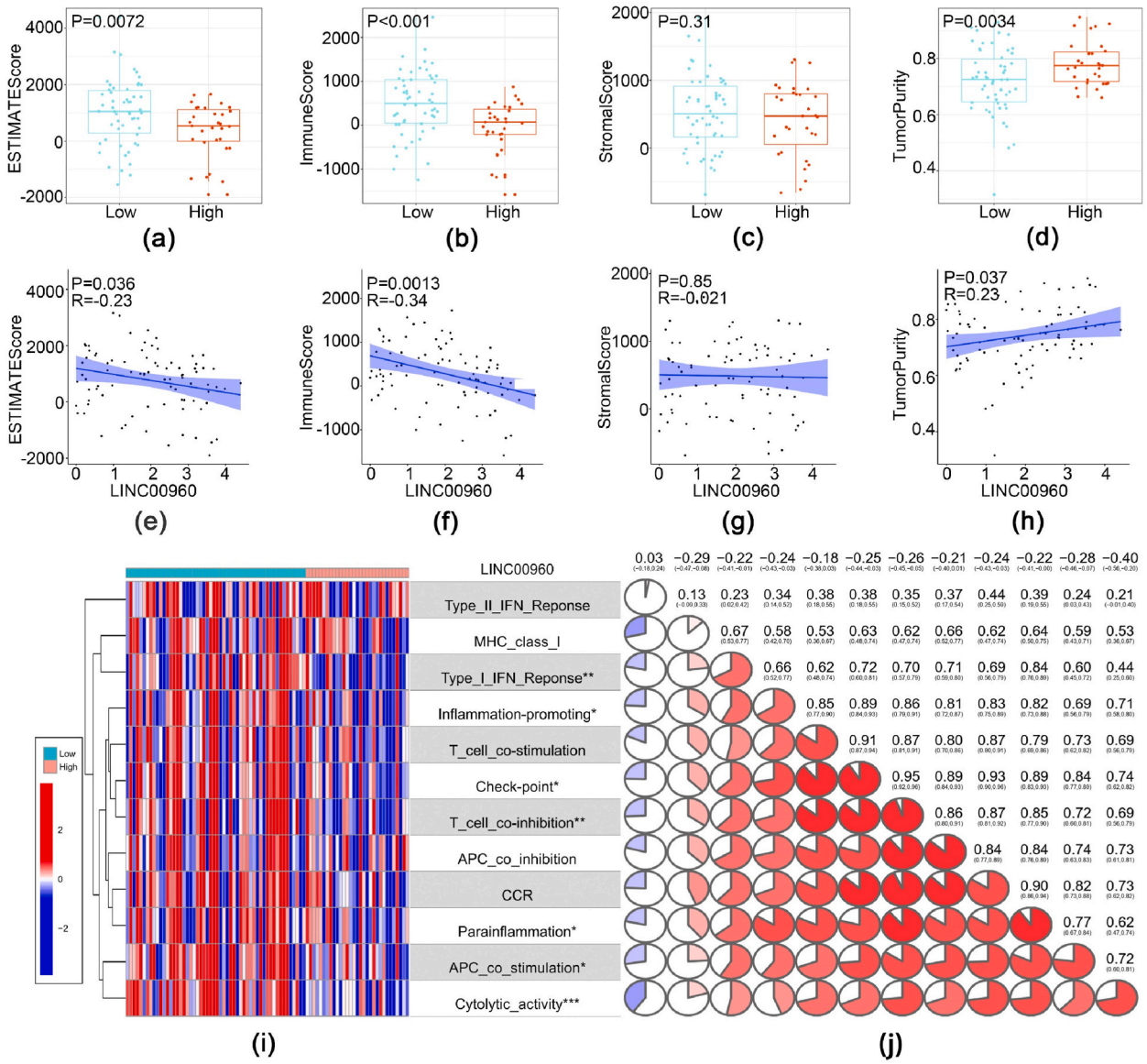

Fig. 7. The relationship between the expression of LINC00960 and tumor immune microenvironment and immune functions. ESTIMATE analysis shows the tumor immune microenvironment (TIME) difference between low- and high-LINC00960 expression groups in osteosarcoma, including (a) ESTIMATE, (b) immune, (c)stromal score, and (d) tumor purity. The relationship between LINC00960 expression and ESTIMATE analysis in osteosarcoma, including (e) ESTIMATE, (f) immune, (g)stromal score, and (h) tumor purity. (i) heatmap and (h) correlation matrix show the relationship between LINC00960 expression and immune functions. Positive and negative correlations are denoted by the colors red and blue, respectively. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

normal tissues using the RNA sequencing of 33 malignancies from the TCGA database. Then, with the data from TARGET and GTEx databases, we discovered LINC00960 was expressed more abundantly in osteosarcoma than in healthy musculoskeletal tissues, and it was in line with the results observed by Shi et al. However, the expression differences were not statistically significant in our study. The reason may be that there are more samples of healthy musculoskeletal tissues than osteosarcoma. Moreover, by comparing metastatic patients with non-metastatic patients, we discovered that patients with metastatic osteosarcoma had significantly greater levels of LINC00960 expression. This suggested high LINC00960 expression may be associated with poor prognosis in patients with osteosarcoma. To verify that high expression of LINC00960 leads to poor prognosis in patients with osteosarcoma, we divided the patients into two groups (high- and low-LINC00960 expression) and conducted a Kaplan-Meier analysis. The findings demonstrated that as compared to the low LINC00960 expression group, the high LINC00960 expression group had worse overall survival. Additionally, the results of Cox regression revealed that high expression of LINC00960 was an independent risk factor for the prognosis of osteosarcoma.

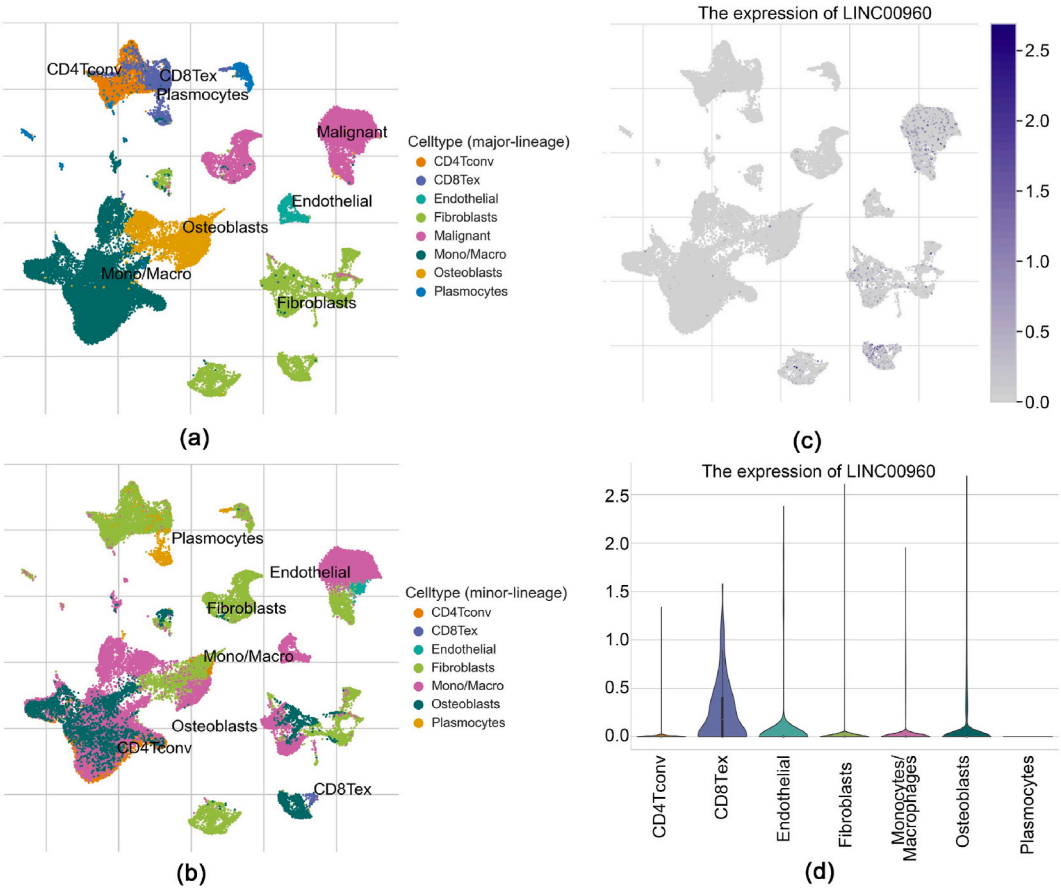

Fig. 8. Single-cell analysis. (a, b) Single-cell UMAP. (c, d) The expression pattern of LINC00960. CD4Tconv, Conventional CD4 T Cells; CD8Tex, Exhausted CD8 T Cells; Mono/Macro, Monocytes or Macrophages.

All above results demonstrated that the poor prognosis of osteosarcoma patients was associated with increased expression of LINC00960. Osteosarcoma is characterized by high malignancy and rapid progression, so clinically predicting a patient's total survival is challenging [35]. Therefore, we used the nomogram combining LINC00960 expression and other clinical features to build prediction models with good accuracy and precision to help clinicians predict the overall survival of patients with osteosarcoma.

Considering that the specific mechanisms by which high expression of LINC00960 leads to a poor prognosis of osteosarcoma remain unclear, we further conducted the GO, KEGG, GSEA, and GSVA functional analysis. The results indicated that the DEGs were significantly enriched in immune-linked biological processes, cell proliferation, and skeletal system, such as immune effector process, immune receptor activity, cell population proliferation, growth factor activity, skeletal system development, osteoclast differentiation, PI3K-Akt signaling pathway, Wnt signaling pathway, and so on. These results showed that LINC00960, in addition to increasing the malignancy of osteosarcoma, was associated with immune responses.

Studies have reported that the deregulation of the immune system, such as immune cells and immune microenvironment, is closely related to tumor progression, prognosis, and oncotherapy [36–38]. Therefore, with the ESTIMATE algorithm, we first performed the immune microenvironment analysis and the results uncovered that the high-LINC00960 expression group showed considerably lower ESTIMATE score, immune score, and higher tumor purity than the low-LINC00960 expression group. In terms of immune-related functions, almost all the immune-related functions were negatively correlated with LIINC00960 expression. Especially, significant variations existed in T_cell_co-inhibition and checkpoint between the two groups. To investigate the expression pattern of LINC00960 in osteosarcoma patients at the single-cell level, we performed single-cell analysis and found that LINC00960 was predominantly expressed in malignant cells and the exhausted CD8 T cells. In addition, analyzing the bulk mRNA sequencing data, we observed that the LINC00960 high expression group showed the lower infiltration of T cells, especially γδT, and monocyte lines. Not alone, in the correlation analysis of inhibitory immune checkpoints, we found that the expression of LINC00960 was significantly positively correlated with CD28, B7-2, and B7–H3. Interestingly, it was shown that the exhausted CD8 T cells inhibit tumor immune response through upregulation of PD-L1 to limit immune-mediated pathological damage leading to continued tumor progression and deterioration, and was a major target for tumor immune checkpoint suppression therapy [39]. However, PD-L1 suppresses T cells mainly through co-stimulation of the CD28/B7 pathway [40,41]. In contrast, γδT has an extremely strong ability to kill cancer cells [42–44]. We, therefore, speculate that high LINC00960 expression affects the osteosarcoma progression, prognosis, and therapy by reducing T

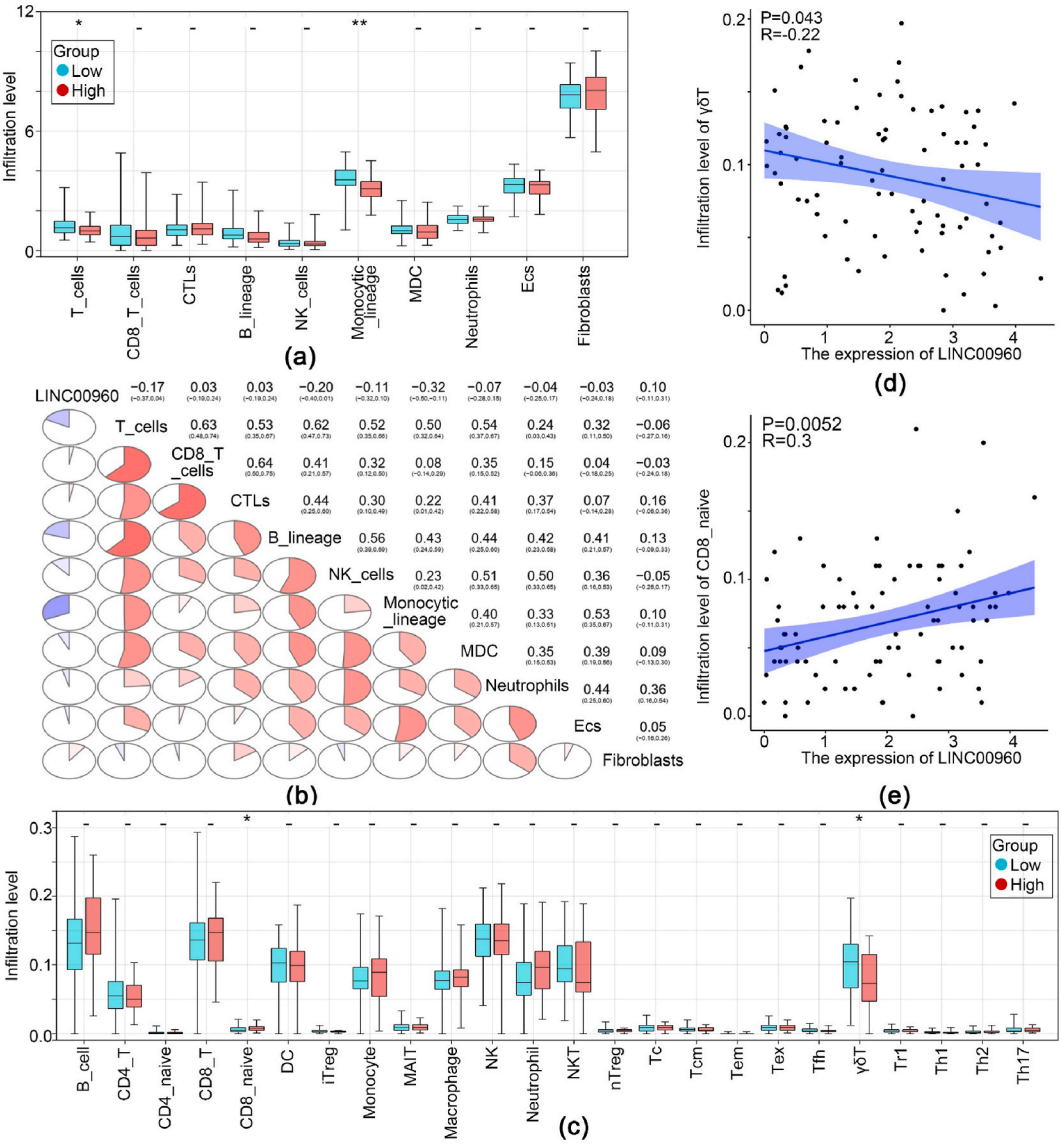

Fig. 9. Immune infiltration analysis (a) The infiltration levels of 10 immune-related cells between low- and high-expression groups based on the MCPcoulter algorithm (b) Correlation matrix of LINC00960 expression and 10 immune-related cells enriching degrees. (c) 24 immune cells' abundance between the two groups using the ImmCellAI algorithm. Pearson's association analysis between the expression of LINC00960 and the abundance of (d) γδT and (e) CD8_naive. Positive and negative correlations are denoted by the colors red and blue, respectively, in the correlation matrix -, $p \geq 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

cell infiltration, especially γδT, and increasing the expression of B7-2 and B7–H3 [45–47]. Based on the results of the above analysis, it can be hypothesized that LINC00960 is mainly highly expressed in the exhausted CD8 T cells in osteosarcoma and suppresses T cells, especially γδT, through co-stimulation of the CD28/B7 pathway, resulting in high malignancy and low prognosis of osteosarcoma.

In recent years, immunotherapy has become a novel form of treatment for osteosarcoma patients with poor prognosis [48–51]. Therefore, TIDE analysis was done to compare the two groups and anticipate how osteosarcoma patients will react to immunotherapy. The results showed that osteosarcoma patients in the low-LINC00960 expression group had a better response to immunotherapy [52]. Importantly, based on our previous findings, high LINC00960 expression leads to overexpression of the immunosuppressive checkpoints CD28 and B7, and thus CD28 and B7 could be used as immunotherapeutic targets for high LINC00960 expression osteosarcoma patients. Interestingly, Zhang et al. have proved that the B7–H3 targeted therapy, which showed a potential therapeutic effect for osteosarcoma patients [53].

At present, the main chemotherapy drugs for osteosarcoma are cisplatin, methotrexate, and doxorubicin, but patients with metastasis and recurrence showed high resistance to these drugs. Thus, it is necessary to find new chemotherapeutic drugs for

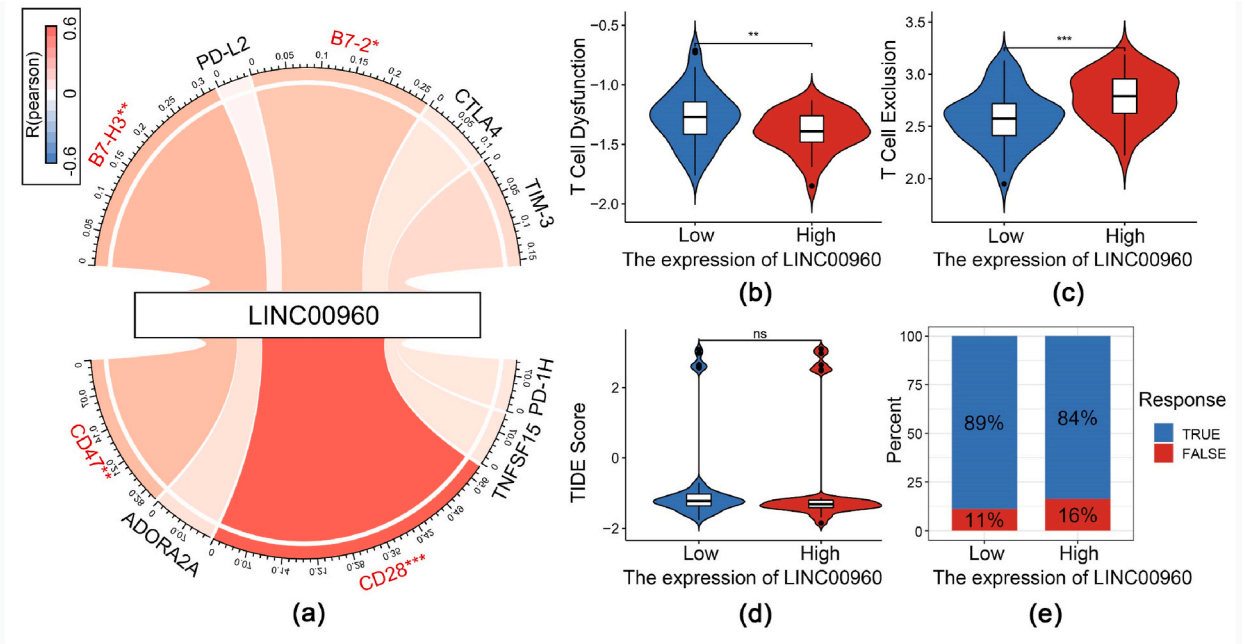

Fig. 10. Immune checkpoints association and immunotherapy response analysis. (a) Association analysis between inhibitory immune checkpoints related genes expression and LINC00960 expression. (b) Tumor immune dysfunction, (c) tumor immune exclusion, and (d) TIDE scores of high- and low-expression groups were investigated by the TIDE algorithm. ns, $p \geq 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

chemotherapy-resistant osteosarcoma [54,55]. Considering this, we performed the drug sensitivity analysis and obtained 8 drugs that displayed high sensitivity for the low- LINC00960 expression group including Temsirolimus, CI.1040, AZD6244, BMS.536924, JW.7.52.1, Sorafenib, PF.02341066, and PD.0325901, and 4 drugs that displayed high sensitivity for the high- LINC00960 expression group including GSK.650394, AG.014699, Bleomycin, and Doxorubicin. However, these drugs have only been screened by computational methods, and their application to osteosarcoma therapy should be validated by experimental studies or clinical trials to confirm their effectiveness and guide therapeutic decisions.

This study researches the connection between LINC00960 expression and osteosarcoma, but several limitations remain. Firstly, as a result of the study's limited sample size and potential for selection bias, the findings may have been impacted. We need to address this by expanding the sample data. Secondly, because the patient's clinical information was obtained from the TARGET databases, some clinical information was missing, such as the tumor stage. Thirdly, cell and animal experiments are needed to validate the results and conclusions of our study, and we plan to conduct this in future experiments. Finally, given the complexity of therapeutic decision-making and the multifactorial nature of drug response, the utility of LINC00960 as a novel biomarker for prognosis and treatment of osteosarcoma should be evaluated in the context of other clinical factors and validated in a large-scale clinical cohort before being implemented for clinical decision-making.

5. Conclusions

In summary, we revealed that LINC00960 is mainly highly expressed in the exhausted CD8 T cells in osteosarcoma and suppresses T cells, especially γδT, through co-stimulation of the CD28/B7 pathway, resulting in high malignancy and low prognosis of osteosarcoma. Therefore, it could be used as a new therapeutic target to guide clinical decision-making and more individualized treatment.

Data availability statement

Online public databases contain the datasets that were used for the investigation. The websites can be found in the article.

Funding

Project supported by: National Natural Science Foundation of China (Grant No. 81874125 and 82072966).

CRediT authorship contribution statement

Yiwei Zhang: Conceptualization. **Guanghua Lu:** Writing – review & editing. **Yonghao Guan:** Formal analysis. **Tianyang Xu:** Data

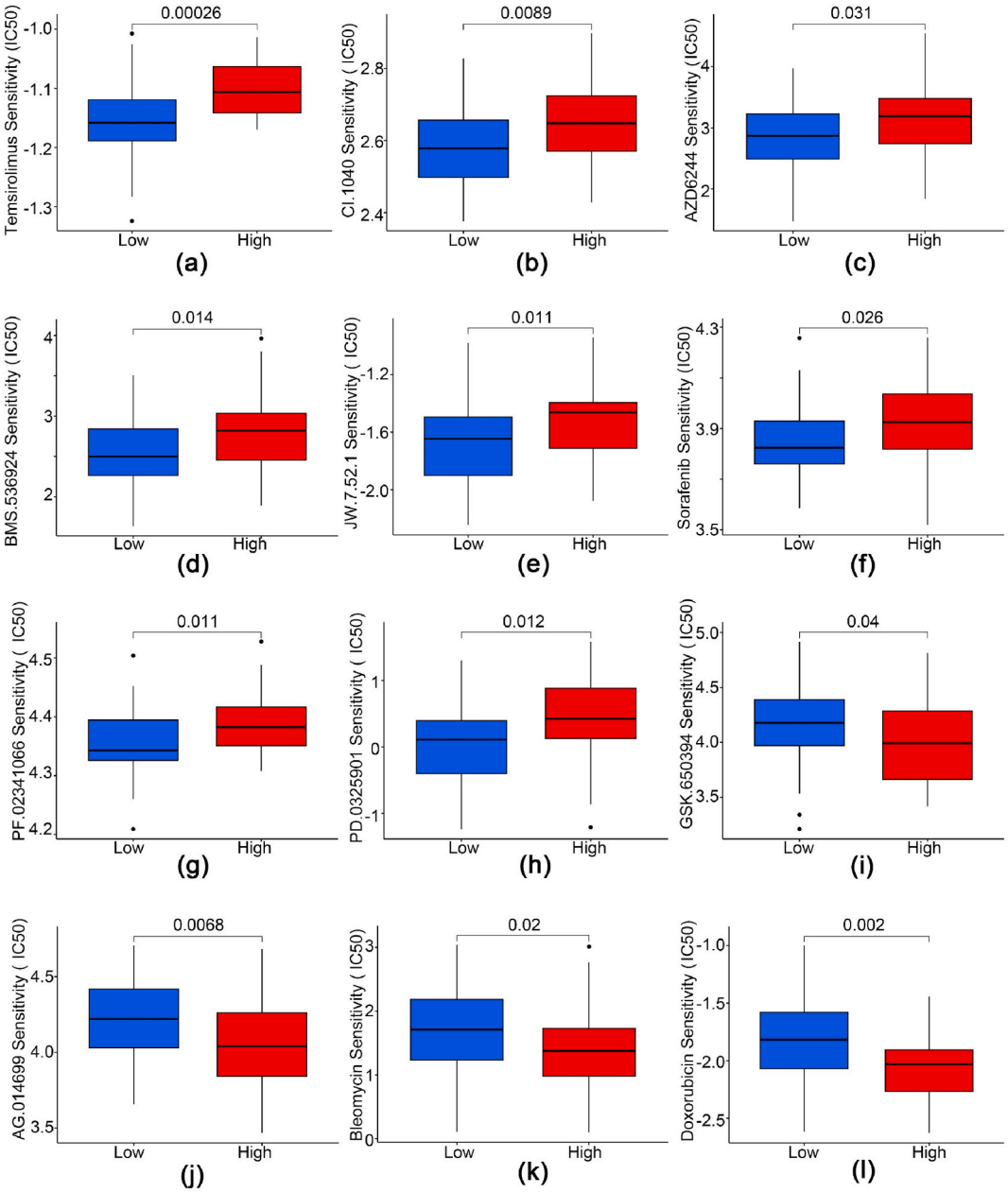

Fig. 11. Prediction of drug sensitivity. The IC50 of candidate anticancer drugs with remarkable treatment outcomes for the (a–h) low and (i–l) high LICN00960 expression osteosarcoma patients.

curation. **Zhengwei Duan:** Writing – original draft. **Guodong Li:** Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e24990.

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