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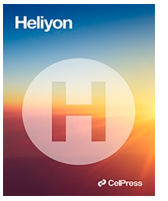

Research article

Prognostic signature and immunotherapeutic relevance of Focal adhesion signaling pathway-related genes in osteosarcoma

Zhiqiang Wu [a,b,1], Zhiqing Wang [c,1], Zhanqiang Hua [d,1], Yingzheng Ji [e], Qingrong Ye [a,b,**], Hao Zhang [e,***], Wangjun Yan [a,b,*]

[a] Department of Musculoskeletal Oncology, Fudan University Shanghai Cancer Center, Shanghai, 200032, China
[b] Department of Oncology, Shanghai Medical College, Fudan University, Shanghai, 200032, China
[c] Zhabei Central Hospital, No. 619, Zhonghuaxin Road, Jing'an District, Shanghai, 200070, China
[d] Department of Orthopedics, Shanghai Electric Power Hospital, Shanghai, 200050, China
[e] Department of Orthopedics, Naval Medical Center of PLA, Second Military Medical University, China

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ABSTRACT

Background: As the most common primary malignant bone tumor in children and adolescents, osteosarcoma currently lacks an effective clinical cure. Focal adhesion plays a crucial role in tumor invasion, migration, and drug resistance by mediating communication between the extracellular matrix and tumor cells. This study investigated the prognostic features and immunotherapeutic relevance of focal adhesion pathway-related genes in osteosarcoma to aid in the development of new therapeutic options.

Methods: We obtained mutational, transcriptomic, gene expression, and clinical data of osteosarcoma patients from the Gene Expression Omnibus (GEO) and Therapeutically Applicable Research to Generate Effective (TARGET) databases. Differentially expressed genes were screened, followed by the Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) analyses. Kaplan-Meier survival analysis was performed for genes related to the focal adhesion pathway, and multivariate Cox regression analysis was employed to construct a prognostic signature model. Genes such as SIGLEC15, TIGIT, CD274, HAVCR2, PDCD1, CTLA4, and LAG3 were extracted from the TARGET and CCLE databases for osteosarcoma patients and osteosarcoma cell lines, respectively , to observe the expression of immune checkpoint-related genes. Finally, qRT-PCR was used to verify the expression of these immune checkpoint-related genes in osteosarcoma cell lines.

Results: In our study, 376 samples were analyzed, including 369 osteosarcoma samples and 7 normal tissue samples. We identified 50 up-regulated and 28 down-regulated differentially expressed genes. Among these, 10 Candidate genes relative to focal Adhesion were selected , and CAV1, ZYX, and ITGA5 were found to have a significant prognostic role based on survival analysis of osteosarcoma samples from the TARGET database. A predictive signature model related to the focal adhesion signaling pathway was constructed using these genes, and the AUCs of the 1-year, 3-year, and 5-year ROC curves were 0. 647, 0. 712, and 0. 717, respectively. The overall survival

* Corresponding author. Department of Musculoskeletal Oncology, Fudan University Shanghai Cancer Center, Shanghai, 200032, China.
** Corresponding author.
*** Corresponding author.
E-mail addresses: wangzhiqing1203@163.com (Z. Wang), 16301050171@fudan.edu.cn (Q. Ye), doctor_zhanghao@163.com (H. Zhang), yanwj@fudan.edu.cn (W. Yan).
[1] These authors contributed equally to this work.

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(OS) rate of osteosarcoma patients with high-risk scores was poorer than those with low-risk scores. Then, samples were divided into two subgroups based on the expression of the three genes, revealing significant differences in the expression of certain immune checkpoint-related genes between the subgroups. Additionally, above three genes and immune checkpoint-related genes in osteosarcoma cell lines were extracted from the CCLE database, showing high expression levels in eight osteosarcoma cell lines. We observed that CD274 and PDCD1LG2 were highly expressed in some osteosarcoma cell lines. Finally, the expression of CAV1, ZYX, ITGA5, CD80, CD274, and PDCD1LG2 in osteosarcoma cell lines was verified by qRT-PCR.

Conclusions: Our study validated the prognostic role of three focal adhesion pathway-related genes (ZYX, CAV1, and ITGA5) in patients with osteosarcoma and constructed a prognostic signature model associated with the focal adhesion signaling pathway. We identified significant differences in the expression of multiple immune checkpoint-related genes among subgroups defined by the three genes. Additionally, CD274 and PDCD1LG2 showed higher expression in osteosarcoma cell lines characterized by these genes. These findings may aid in the selection of effective immunotherapy for specific osteosarcoma patients.

1. Introduction

Osteosarcoma is a malignant bone tumor primarily affecting children and adolescents and is a leading cause of amputation and tumor-related death in this age group [1,2]. Osteosarcoma is highly malignant, aggressive, and prone to early lung metastasis [3]. Recent updates and optimizations in clinical treatment protocols have increased the OS rate of patients with non-metastatic osteosarcoma from less than 20 % to approximately 70 %. However, the 5-year survival rate for patients with recurrent or metastatic osteosarcoma remains below 30 % [4,5]. Current research emphasizes early diagnosis and treatment [6]. Reliable predictors are crucial for monitoring disease progression, guiding treatment decision, and assessing prognosis.

The tumor microenvironment (TME) comprises non-immune cells, immune cells, and extracellular components. Non-immune cells primarily consist of cancer-associated fibroblasts (CAFs), while immune cells include T regulatory (Treg) cells, myeloid-derived suppressor cells (MDSCs), natural killer (NK) cells, tumor-associated macrophages (TAMs), and dendritic cells (DCs). The extracellular components consist of cytokines, growth factors, and the extracellular matrix (ECM) [7,8]. Previous studies on osteosarcoma primarily focused on the molecular biological changes of tumor cells, often overlooking the role of the microenvironment, which consists of many non-tumor cells in tumorigenesis. Various studies have demonstrated that the TME plays a crucial role in tumor occurrence, progression, and metastasis. As a component of the TME, the ECM is rich in biochemical molecules, including fibrin, glycoproteins, proteoglycans, and polysaccharides [9]. Focal adhesion regulates cell signaling including cell differentiation, migration, growth, and proliferation by interacting with cell cytoskeleton and ECM [10,11]. It has also been implicated in tumor development, migration, invasion, and drug resistance [12]. Researchers have found that the focal adhesion signaling pathway significantly impacts other components of the TME [13]. Additionally, some studies have shown that tumor response to treatment is closely related to the activity of focal adhesion signaling [14,15]. For example, focal adhesion kinase (FAK) is highly expressed in various malignancies such as ovarian, lung, melanoma, and breast cancers [16], and can influence tumor therapy by promoting cell adhesion and metastasis [17]. Studies have indicated that using FAK inhibitors can reduce the resistance of melanoma cells to targeted drugs [18]. However, the relation between focal adhesion signaling pathway and osteosarcoma has been less studied.

Treatment options for osteosarcoma remain limited, particularly for recurrent and metastatic cases where surgical interventions and conventional chemotherapy often yield unsatisfactory results. In recent years, tumor immunotherapy has garnered significant attention from researchers. Under pathological conditions, cancer cells can evade immune detection, a phenomenon known as immune escape. Tumor immunotherapy aims to enhance the body's anti-tumor immune function to inhibit and destroy tumor cells [19]. The cellular and molecular components of the TME can influence the effectiveness of immunotherapy, making it crucial to study the TME in the context of immunotherapy. The lymphocyte-mediated immune response requires recognition by the T-cell receptor (TCR) and stimulation by various cell surface molecules. While costimulatory proteins enhance the antigenic response of lymphocytes, a series of inhibitory cell surface proteins within the TME act to suppress this response. Tumor antigens can inhibit T cell activity by expressing inhibitory ligands and receptors, thereby avoiding recognition by immune cells. Consequently, blocking or suppressing immune checkpoints can enhance the anti-tumor immune response, providing a promising strategy for treating osteosarcoma patients. In this study, we investigated the prognostic significance and immunotherapeutic relevance of genes related to the focal adhesion signaling pathway in osteosarcoma, aiming to uncover potential mechanisms underlying osteosarcoma development and progression. Our findings also offer insights into selecting effective immunotherapy options for specific osteosarcoma patients.

2. Materials and methods

2.1. Data acquisition and screening for differential genes

All microarray data were downloaded from the GEO database (http://www. ncbi. nih. gov/geo,GSE11127,GSE14359,GSE16091, GSE19276,GSE21257,GSE30699,GSE32981,GSE33382,GSE36001,GSE37552,GSE39055 and GSE39057). The raw data were obtained as MINiML files. Our study included 376 samples, consisting of 369 osteosarcoma samples and 7 normal tissue samples. The limma

package in R software was used to analyze differentially expressed mRNA. The adjusted P-value was calculated to correct for false positive results in the GEO datasets. Differential expression of mRNAs was defined using the thresholds of adjusted $P < 0.05$ and Log 2 (Fold Change) > 1 or Log 2 (Fold Change) < -1. ".

2.2. Functional enrichment analysis

Gene Ontology (GO) is a widely-used tool for annotating genes with functions, including molecular function (MF), biological processes (BP), and cellular components (CC). The Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis is a valuable resource for studying gene functions and associated high-level genome functional information. To gain a better understanding of the carcinogenesis of mRNA, the "clusterProfiler" R package (version 3.18.0) was used to analyze the GO functions of potential targets and to enrich KEGG pathways. The "pheatmap" package in R was utilized to create heatmaps [20].

2.3. Pathway-associated gene selection and correlation analysis

We used KEGG Mapper (https://www. kegg. jp/kegg/mapper/search. html) to select genes associated with the focal adhesion

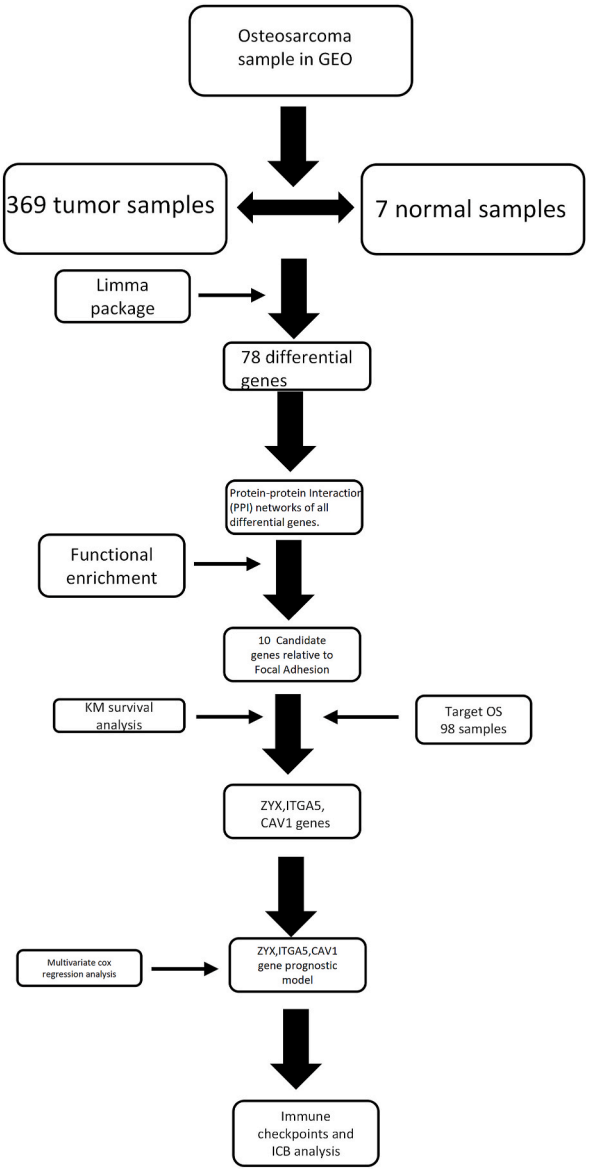

Fig. 1. Flow chart of the research study. **GEO**, Gene Expression Omnibus; **KM**, Kaplan-Meier; **TARGET**, Therapeutically Applicable Research to Generate Effective Treatments; **OS**, overall survival; **ICB**, immune checkpoint blockade.

signaling pathway from the differentially expressed genes. Spearman's correlation analysis was conducted to describe the correlation between quantitative variables without a normal distribution. P values less than 0. 05 were considered statistically significant (*P < 0. 05).

2.4. Survival analysis and construction of prognostic signature model

The Therapeutically Applicable Research to Generate Effective Treatments (TARGET) database is a public resource for pediatric cancer research. Gene expression and clinical data for osteosarcoma patients were obtained from the TARGET database. The log-rank test was used to assess survival differences between groups. The R package "timeROC" (version 0.4) analysis was employed to compare the predictive accuracy of each gene. For Kaplan-Meier curves, log-rank test and univariate Cox proportional hazards regression were utilized to generate p-values and hazard ratios (HR) with 95 percent confidence intervals (CI). Multivariate Cox regression analysis was performed to construct a prognostic model, and the "survival" package in R was used for the analysis. All analyses and R packages were implemented using R (Foundation for Statistical Computing, 2020) version 4. 0. 3. A p-value of <0. 05 was considered statistically significant.

2.5. Analysis of the expression of immune checkpoint-related genes

SIGLEC15, TIGIT, CD274, HAVCR2, PDCD1, CTLA4, LAG3, and PDCD1LG2 are genes associated with immune checkpoints. The expression values of these eight genes were extracted from osteosarcoma data in the TARGET database to assess the expression of immune checkpoint-related genes. All the aforementioned analysis methods and R packages were implemented using R (Foundation for Statistical Computing, 2020) version 4. 0. 3, including the ggplot2 and pheatmap packages.

The CCLE dataset (https://portals. broadinstitute. org/ccle) was utilized to obtain the mRNA expression matrix in tumor cell lines. The analysis was conducted using the ggplot2 package (v3. 3. 3) in R software version 4. 0. 3.

2.6. RNA extraction and qRT-PCR assay

According to the protocol, total RNA was extracted from osteosarcoma cell lines using TRIzol reagent. The isolated RNA was then

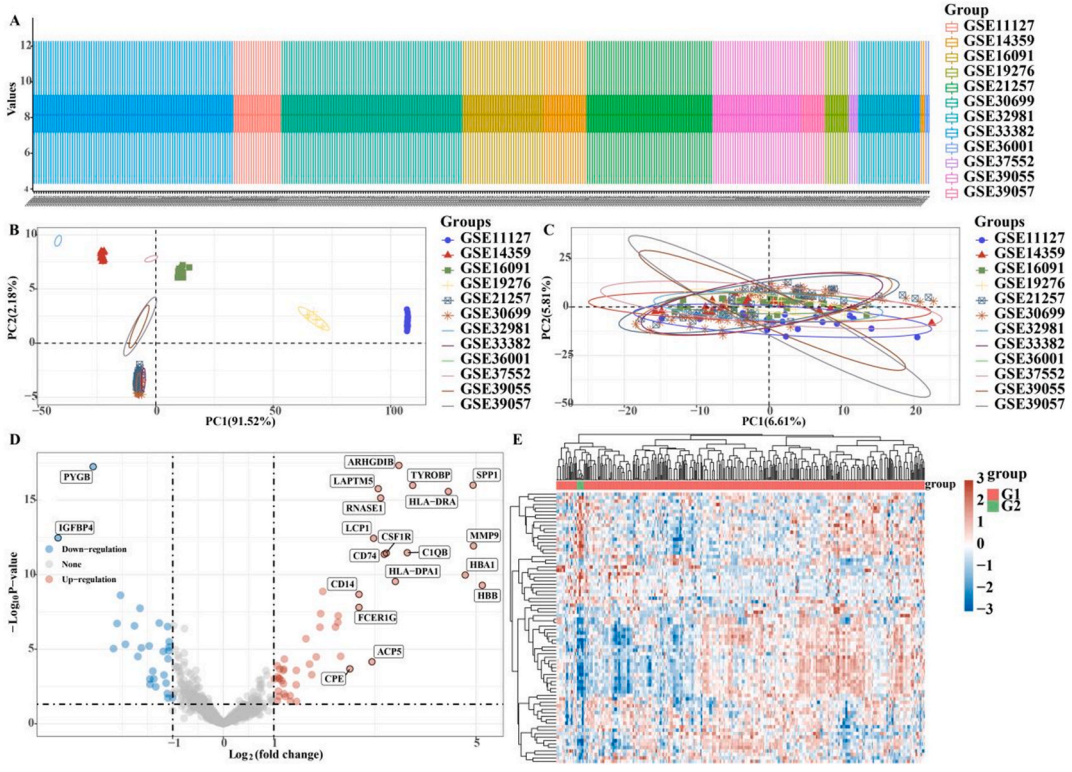

Fig. 2. Differentially expressed genes (DEGs) in patients with osteosarcoma. **(A)** The boxplot of the normalized data. Different colors represent different osteosarcoma datasets. Rows represent samples, and columns represent the gene expression values in the samples; **(A)**PCA results before batch removal for multiple datasets; **(C)** PCA results after batch removal;**(D)** DEGs between tumor and normal tissues; **(E)** heatmap for DEGs in all the osteosarcoma samples.

reverse transcribed into complementary DNA (cDNA) using HiScript III RT SuperMix (Vazyme, Nanjing, China) and specific primers. Quantitative PCR (qPCR) was performed with the qPCR Master Mix (SYBR Green, Vazyme, Nanjing, China) on a CFX96 Real-Time PCR detection system (Bio-Rad, USA). The relative expression of genes was analyzed using the $2^{-\Delta\Delta Ct}$ method.

3. Results

3.1. Screening of differential genes in osteosarcoma

The flow chart of this study was shown in Fig. 1. Fig. 2A showed the 12 datasets used in this study analyzed. After conducting a principal component analysis (PCA) on these datasets, we observed partial overlap among them (Fig. 2B). We subsequently removed batch effects from the PCA results and proceeded with further analysis (Fig. 2C). This analysis encompassed 376 samples, including 369 osteosarcoma samples and 7 normal tissue samples. Ultimately, we identified 50 up-regulated and 28 down-regulated differentially expressed genes (Fig. 2D and E).

3.2. Construction of protein-protein interaction network

After constructing a protein-protein interaction (PPI) network of differentially expressed genes using the STRING database, we

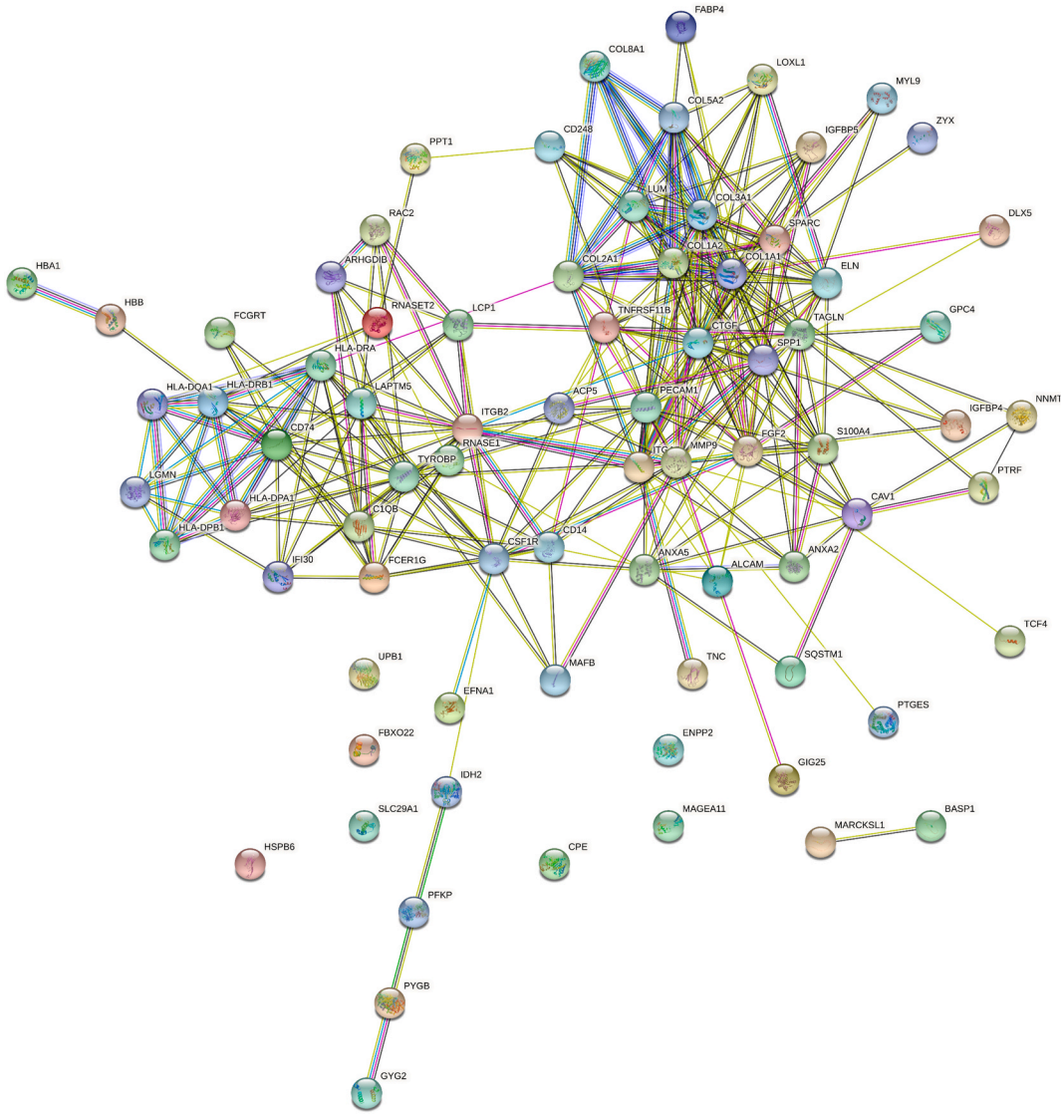

Fig. 3. Protein-protein Interaction (PPI) networks of all differential genes.

observed that most of these genes were interconnected at the protein level (Fig. 3).

3.3. Enrichment analysis of GO and KEGG pathways

We conducted GO and KEGG analyses for the up-regulated and down-regulated differentially expressed genes. For the up-regulated genes, the top two KEGG pathways were identified as Tuberculosis and Antigen processing and presentation. The top two GO pathways were extracellular structure organization and extracellular matrix organization. For the down-regulated genes, the leading KEGG pathways included Focal adhesion, Shigellosis, Salmonella infection, Regulation of actin cytoskeleton, and Proteoglycans in cancer. The top two GO pathways for these genes were extracellular structure organization and extracellular matrix organization (Fig. 4).

3.4. Correlation analysis of focal adhesion signaling pathway-related differential genes

We utilized KEGG Mapper to select 10 genes from the 78 differentially expressed genes associated with Focal adhesion. These genes are COL1A1, COL2A1, COL1A2, SPP1, MYL9, RAC2, TNC, ZYX, CAV1, and ITGA5 (Fig. 5A, Table 1). In osteosarcoma samples, most of these focal adhesion-associated genes showed positive associations, while a few exhibited negative associations (Fig. 5B).

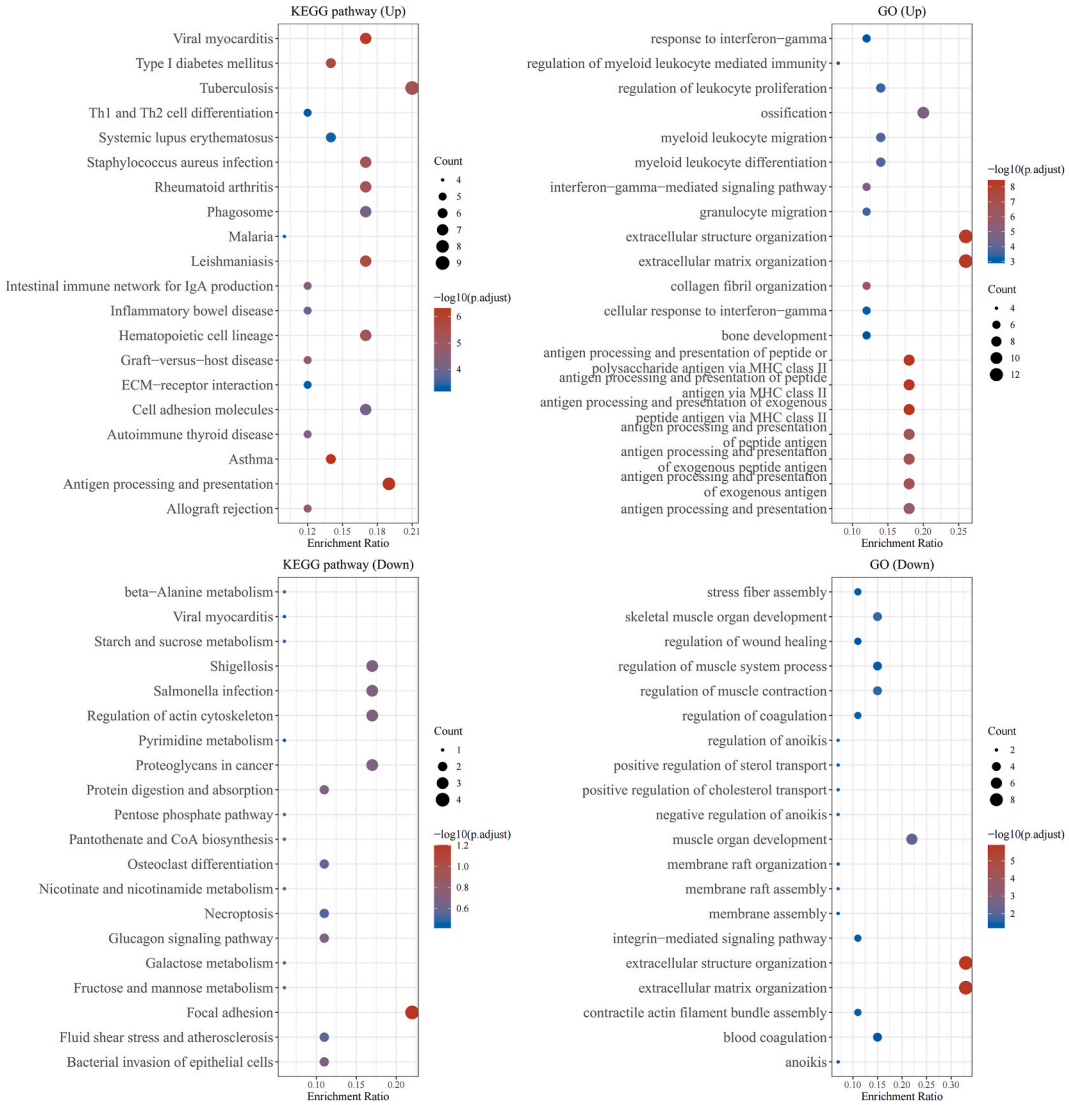

Fig. 4. The Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses for up- and down-regulated DEGs, respectively.

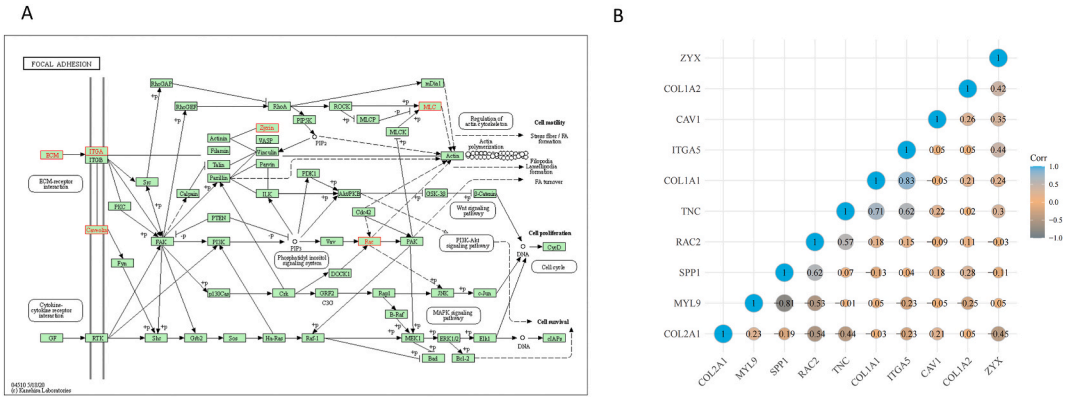

Fig. 5. **(A)** Focal adhesion pathway diagram. The selected pathway-related differential genes are highlighted in red;**(B)** Correlation analysis among pathway-related differential genes in osteosarcoma patients.

Table 1
Genes associated with Focal adhesion selected by KEGG Mapper.

Genes	logFC	P. Value	adj. P. Val
SPP1	4. 941588134	9. 98E-17	2. 50E-14
COL1A1	1. 188034704	0. 000272	0. 004065
COL1A2	1. 075302014	0. 00072	0. 009076
TNC	1. 442313964	0. 002489	0. 023925
RAC2	1. 101624135	0. 0149	0. 099095
COL2A1	1. 331894131	0. 032654	0. 157746
ZYX	−1. 072406499	7. 29E-06	0. 000191
ITGA5	−1. 647976954	1. 80E-08	8. 32E-07
CAV1	−1. 756815426	2. 73E-07	9. 46E-06
MYL9	−1. 129890155	0. 001901846	0. 020072749

3.5. Survival analysis of hub genes in focal adhesion signaling pathway-

After obtaining 98 osteosarcoma samples from the TARGET database, we conducted univariate Cox regression analysis to determine the p-value, HR, and CI for the expression and prognostic characteristics of selected genes. ZYX (log-rank p = 0. 0092, HR = 0. 4095, 95 % CI = 0. 2092–0. 8018), CAV1 (log-rank p = 0. 0313, HR = 0. 4819, 95 % CI = 0. 2479–0. 9367), and ITGA5 (log-rank p = 0. 018, HR = 0. 4501, 95 % CI = 0. 2324–0. 8719) were identified as significant independent prognostic factors for OS (Fig. 6A). Further survival analysis revealed that ZYX, CAV1, and ITGA5 play crucial prognostic roles in osteosarcoma. Kaplan-Meier survival curves demonstrated that the probability of survival was significantly higher in the high expression groups of ZYX (p = 0. 00921), CAV1 (p = 0. 0313), and ITGA5 (p = 0. 018) compared to their low expression groups (Fig. 6B–D).

3.6. Construction of a focal adhesion signaling pathway-related prognostic signature model

We then employed multivariate Cox regression analysis on the three genes—ITGA5, CAV1, and ZYX—to construct a prognostic model associated with the focal adhesion signaling pathway in osteosarcoma. The risk score for each osteosarcoma patient was calculated the following formula: risk score= (−0.1367) × (expression value of ITGA5) + (−0.0602) × (expression value of CAV1) + (−0.6113) × (expression value of ZYX). Based on the median of risk scores, we categorized all osteosarcoma samples into low- and high-risk groups. We created a figure that depicts the survival status, the distribution of risk scores, and gene expression profile of the prognostic signature (Fig. 7A). Survival analysis revealed that patients with low-risk scores had significantly higher OS rates compared to those with high-risk scores, with median survival times of 9. 3 years and 2. 9 years, respectively (Fig. 7B). The area under the curve (AUC) for the 1-year, 3-year, and 5-year ROC curves was 0. 647, 0. 712, and 0. 717, respectively (Fig. 7C), indicating that this prognostic signature performed a good ability in predicting the prognosis of osteosarcoma patients, especially in predicting the 5-year survival rate.

3.7. Validation of the expression of immune checkpoints in osteosarcoma tissues and osteosarcoma cell lines

We divided the samples into high and low expression groups based on the expression levels of the three genes (CAV1, ZYX, and ITGA5) to investigate differences in the expression levels of eight immune checkpoint-related genes between these groups. The results showed statistically significant differences in the expression of CD274 (p < 0. 05) and PDCD1LG2 (p < 0. 001) between the two CAV1 subgroups; HAVCR2 (p < 0. 001), LAG3 (p < 0. 05), and SIGLEC15 (p < 0. 05) between the two ZYX subgroups; and SIGLEC15 (p < 0.

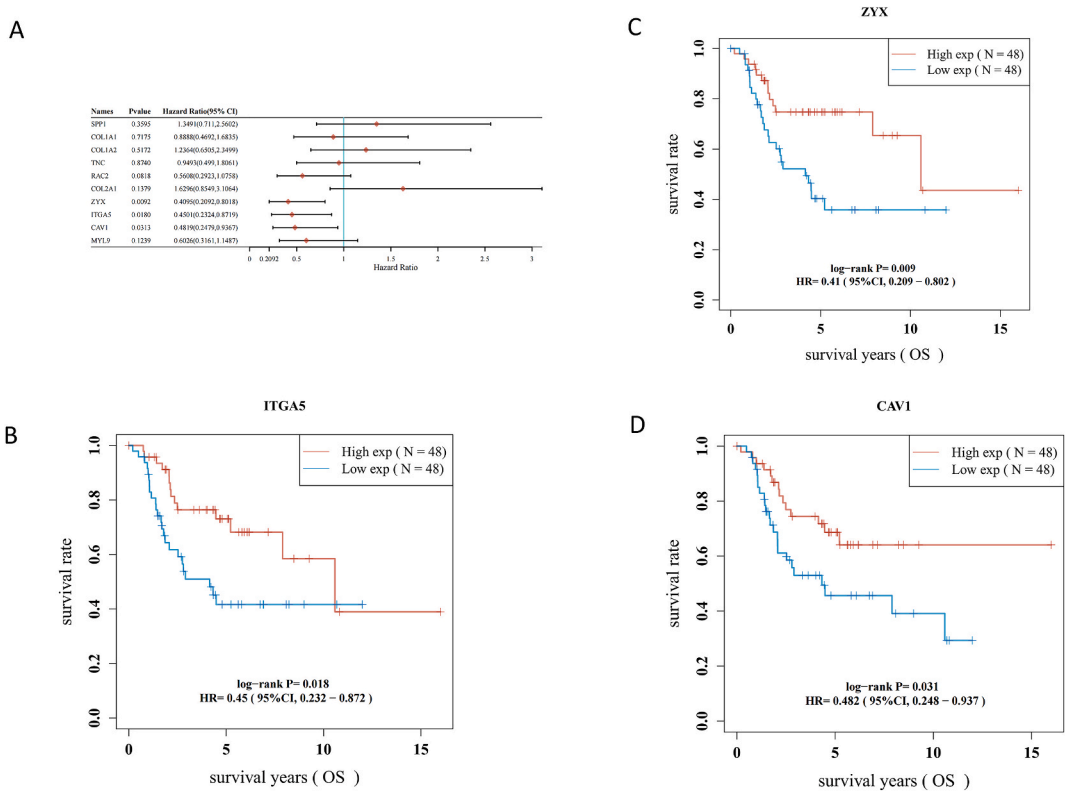

Fig. 6. The identification of prognostic factor for OS. **(A)** Univariate Cox analysis**; (B)** Survival curves according to the expression of ZYX;**(C)** Survival curves according to the expression of CAV1;**(D)** Survival curves according to the expression of ITGA5.

001), CD274 ($p < 0.001$), HAVCR2 ($p < 0.001$), PDCD1 ($p < 0.05$), LAG3 ($p < 0.01$), and PDCD1LG2 ($p < 0.001$) between the two ITGA5 subgroups. The expression levels of these checkpoint-related genes were higher in the high expression group compared to the low expression group (Fig. 8).

By analyzing the gene expression data of osteosarcoma cell lines in the CCLE database, we found that these checkpoint-related genes were also highly expressed in three osteosarcoma cell lines, especially CD274 and PDCD1LG2 (Fig. 9). We verified the expression of CAV1, ZYX, ITGA5, CD80, CD274, and PDCD1LG2 in osteosarcoma cell lines using qRT-PCR (Fig. 10).

4. Discussion

The current standard treatment strategy for patients with osteosarcoma includes neoadjuvant chemotherapy (preoperative), surgical resection (either amputation or limb-sparing surgery), and adjuvant chemotherapy (postoperative) [21]. Significant advances in the research and treatment of osteosarcoma in recent years have led to an increase in the overall 5-year survival rate for patients. However, the cure rate has not improved significantly, primarily due to local recurrence and distant metastases after surgical resection [22,23]. The advancement in osteosarcoma treatment has reached a frustrating plateau [24]. In addition, the resistance of osteosarcoma patients to chemotherapeutic drugs significantly impacts the effectiveness of treatment. Previous research on the mechanisms of drug resistance in osteosarcoma primarily focused on tumor metabolism. However, recent studies have increasingly highlighted the crucial role of the TME in contributing to drug resistance [25]. A significant presence of immune cells is observed in the TME of osteosarcoma. However, these immune cells often fail to perform their role in immune surveillance effectively. Osteosarcoma cells can evade immune recognition and attack by altering their surface antigens and modifying the surrounding TME, thereby achieving immune escape [26]. Because the molecular mechanisms of osteosarcoma remain unclear, traditional clinical markers provide very limited predictive power [27]. A study has developed a prognostic model based on methylation by performing a comprehensive analysis of DNA methylation and examining mRNA and miRNA gene expression in samples from osteosarcoma patients [28]. Therefore, it is crucial to identify new diagnostic and prognostic biomarkers to more accurately diagnose osteosarcoma and predict patient survival. Additionally, there is an urgent need to investigate the molecular mechanisms underlying the development and progression of osteosarcoma, as well as to identify new and more effective therapeutic targets. Among the many genes implicated in osteosarcoma, SPP1 (Secreted Phosphoprotein 1), also known as Osteopontin (OPN), has been extensively studied. SPP1 is a secreted glycoprotein that plays a significant role in various physiological and pathological processes, particularly in bone metabolism and tumor development. It promotes cell proliferation and survival by binding to its receptors (such as integrins and CD44) [29] and

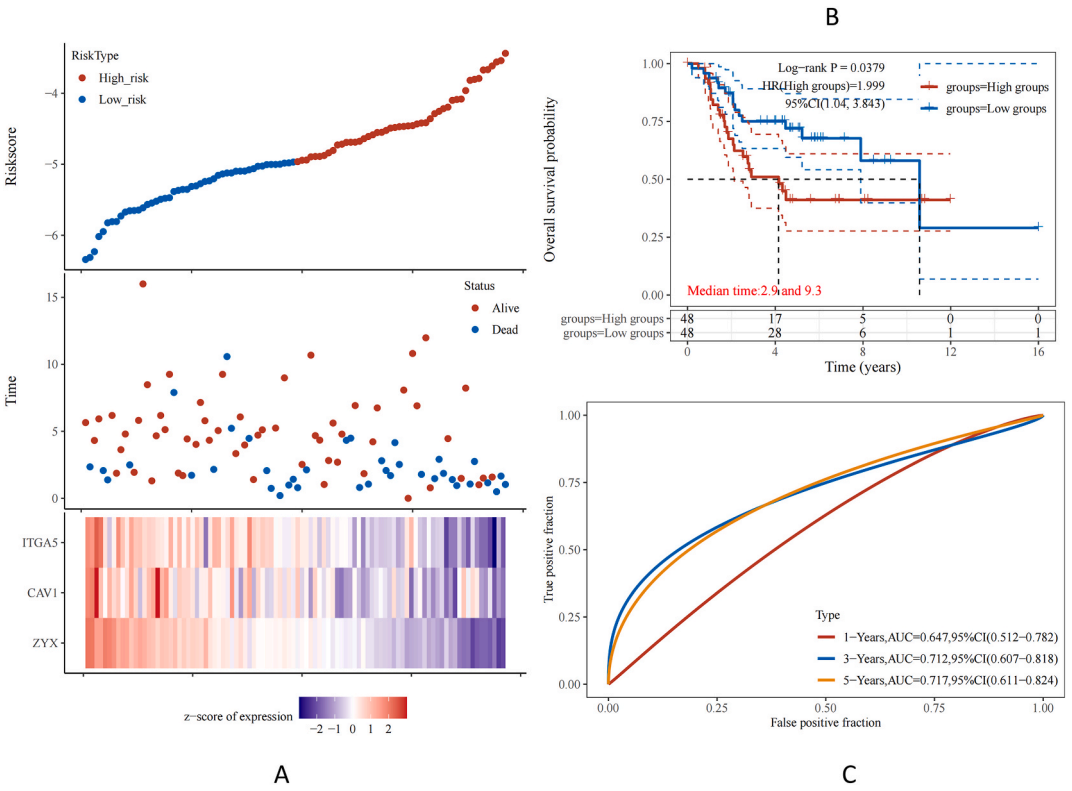

Fig. 7. Construction of a Focal adhesion signaling pathway-related prognostic signature. **(A)** The risk score distribution, survival status of osteosarcoma cases and gene expression profile of this prognostic signature;**(B)** Overall survival curve in the high-/low-risk group. **(C)** The ROC curve evaluating prognosis predicting performance of LUSC patients.

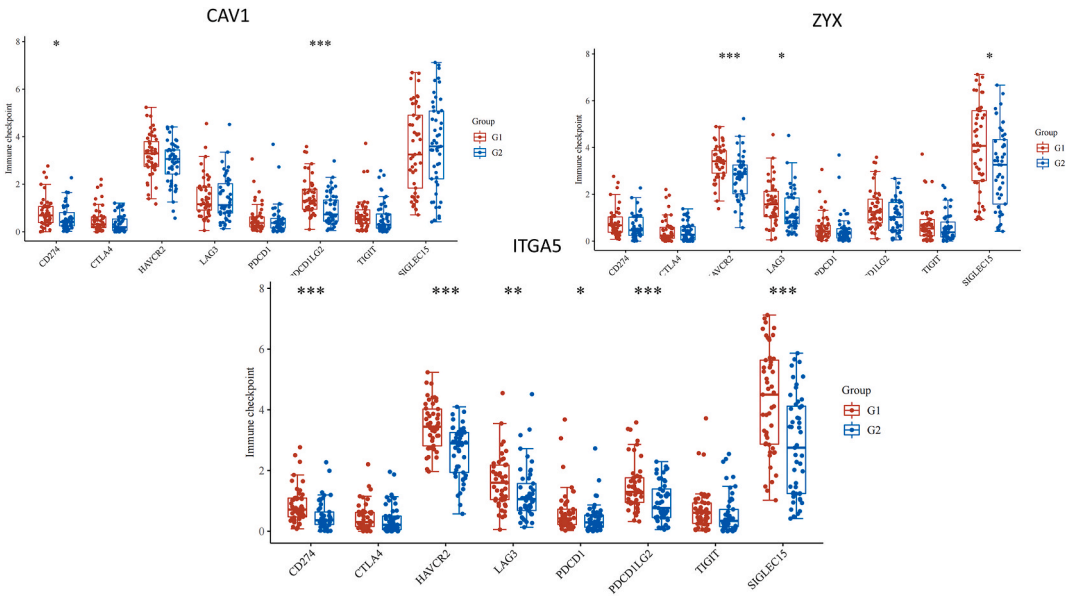

Fig. 8. The expression distributions of 8 immune checkpoints-related genes in osteosarcoma subgroups. *p < 0.05, **p < 0.01, ***p < 0.001.

activating multiple signaling pathways such as PI3K/Akt and MAPK [30]. SPP1 can activate anti-apoptotic signaling pathways and inhibit the activity of apoptosis-related proteins (such as Bax and Caspase), thereby enhancing the survival ability of tumor cells [31]. SPP1 also promotes tumor angiogenesis, providing the necessary blood supply for tumor growth. SPP1 also induces the proliferation

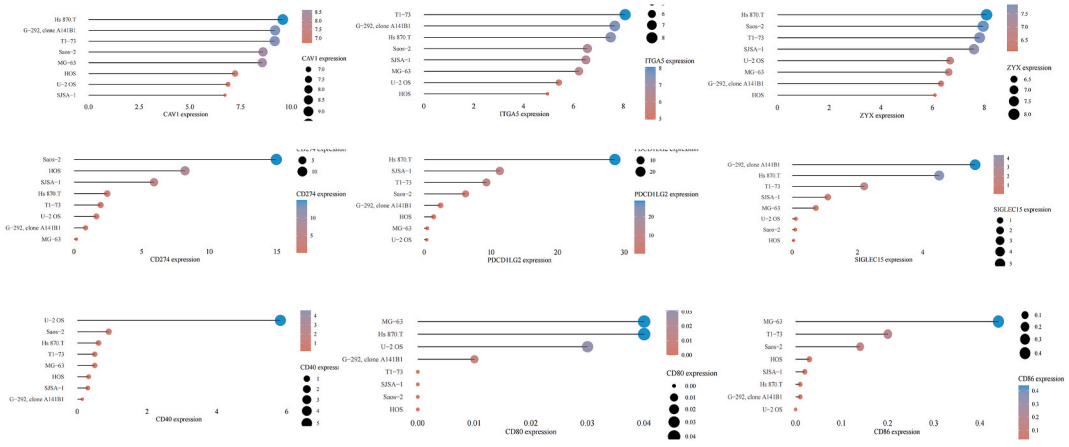

Fig. 9. Expression of ZYX, CAV1, ITGA5 and immune checkpoint-related genes in osteosarcoma cell lines.

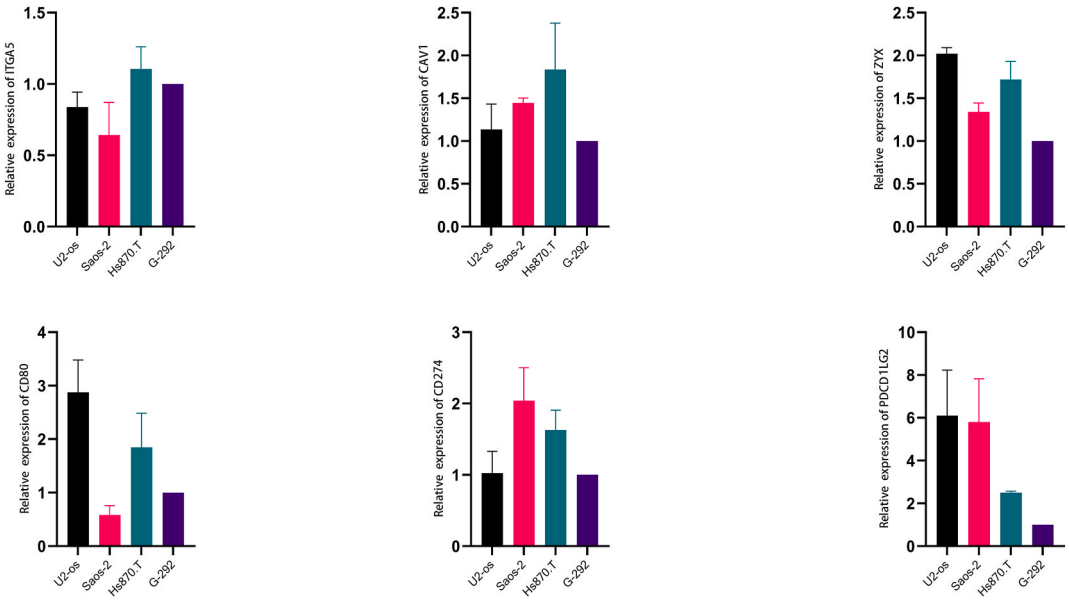

Fig. 10. Validation of gene expression in osteosarcoma cell lines using qRT-PCR.

and migration of endothelial cells, facilitating the formation of new blood vessels. Furthermore, SPP1 enhances the invasion and metastasis capabilities of osteosarcoma cells by regulating the expression of extracellular matrix (ECM) degrading enzymes [32,33]. In our study, we analyzed data from GEO and TARGET database, focusing on osteosarcoma and normal samples. We identified three key genes (ZYX, CAV1, and ITGA5) related to the focal adhesion signaling pathway through functional enrichment analysis, correlation analysis, and survival analysis of the differentially expressed genes. Based on these three genes, we performed multivariate Cox regression analysis to construct a prognostic signature associated with the focal adhesion signaling pathway, which may serve as potential immunotherapeutic targets for specific osteosarcoma patients.

In the functional enrichment analysis, we found that the most significant KEGG pathway, with the highest number of down-regulated genes, was focal adhesion. Focal adhesions are composed of integrin receptors linked to the actin cytoskeleton through focal adhesion-associated proteins, such as Crk, p130Cas, paxillin, Src, and FAK. These proteins work together to regulate ECM interactions [34,35]. Malignant tumor cells possess the ability to invade surrounding tissues and spread to other parts of the body. These invasive characteristics are closely associated with the ECM in the TME. The ECM plays a crucial role in regulating the proliferation, localization, and function of myeloid cells within the TME, contributing to tumor-related immunosuppression [36]. Moreover, researchers have found that the excessive deposition of collagen fibers in the ECM within the TME can act as a barrier that hinders the spread of tumor cells. However, this barrier effect also obstructs the penetration of chemotherapeutic drugs into tumor cells. Therefore, to improve drug delivery, normal ECM must be considered during chemotherapy [37]. Adhesion to ECM has been found to significantly contribute to tumor cell resistance to chemotherapy, radiation [38,39] and targeted therapies [40].

Focal adhesion signaling hubs consist of a variety of survival signaling molecules, including growth factor receptors, intracellular molecules, and integrins. These components influence cell activity, impact tumor cell survival, and can serve as targets for cancer therapy. Focal adhesion signaling plays a crucial role in osteosarcoma. It involves a multi-protein complex formed by the interaction of cells with the ECM through integrins, fulfilling dual functions of mechanical connection and signal transduction. These signals are essential for cell adhesion, migration, proliferation, and survival. In osteosarcoma, focal adhesion signals enhance the migration and invasion capabilities of cells by regulating cytoskeleton reorganization and adhesion dynamics. They also promote cell proliferation and resistance to apoptosis through the activation of various downstream signaling pathways. Additionally, focal adhesion signals modify the TME by affecting the interactions between tumor cells and the surrounding stroma and cells, thereby facilitating tumor progression. The mechanisms of focal adhesion in osteosarcoma include the regulation of these processes [41,42]. This also provides us with a direction for research.

In survival analyses, ZYX (log-rank p = 0. 0092, HR = 0. 4095, 95 % CI = 0. 2092–0. 8018), CAV1 (log-rank p = 0. 0313, HR = 0. 4819, 95 % CI = 0. 2479–0. 9367), and ITGA5 (log-rank p = 0. 018, HR = 0. 4501, 95 % CI = 0. 2324–0. 8719) were found to have significant prognostic roles. ZYX is a key factor in focal adhesions, capable of shuttling between the nucleus and cytoplasm. It can interact with various transcription factors to regulate the synthesis of other proteins [43,44]. ZYX has been shown to play an important role in the progression of many tumors. Wagner [45] et al. discovered that ZYX binds to Wilms Tumor Protein (WT1), a melanoma proliferation-associated protein, thus promoting melanoma growth. Ma [46] et al. found that ZYX was able to respond to transforming factor-β and hypoxia by promoting Lats2 degradation and ubiquitination. This resulted in the dephosphorylation of YAP, which subsequently inhibited the Hippo pathway, thereby enhancing the tumorigenic, proliferative, and migratory abilities of breast cancer cells. Additionally, researchers have identified a potential role for ZYX as a tumor suppressor in prostate, bladder, Ewing sarcoma, and lung cancers [47–50]. However, there are limited studies on the involvement of ZYX in osteosarcoma progression. Our study found that patients with high ZYX expression had a better prognosis compared to those with low ZYX expression. The protein Caveolin-1, encoded by the CAV1 gene, is a major component of caveolae on cell membranes. Caveolin-1 plays various crucial roles in cellular activities, including material transport, signaling, and is involved in processes such as cell proliferation, differentiation, and apoptosis [51–53]. One study found that the expression of CAV1 significantly increased during the budding stage of tumors, suggesting a close association with tumor metastasis and prognosis [54,55]. Wang [56]et al. found that down-regulation of CAV1 expression can lead to over-activation of the phosphatidylinositol 3-kinase (PI3K) signaling pathway, promoting cell survival, and the mitogen-activated protein kinase (MAPK) signaling pathway, which promotes cell proliferation and is involved in early tumor transformation and development [57]. Researchers found that high expression of CAV1 was detected in 5-fluorouracil-resistant colorectal cancer cell lines, cisplatin-resistant ovarian cancer cell lines, and multidrug-resistant lung adenocarcinoma cell lines, suggesting that CAV1 may be closely associated with increased tumor drug resistance [58–60]. However, Chatterjee [61] et al. found that high CAV1 expression facilitated the entry of albumin-bound paclitaxel into H23 lung cancer cells, thereby promoting apoptosis. The mechanism why CAV1 influences tumor chemoresistance is not fully understood, but the aforementioned studies collectively suggest that CAV1 expression is associated with tumor chemoresistance. Ren [62] et al. found that low expression of CAV1 makes breast cancer more aggressive, suggesting that CAV1 acts as a tumor suppressor gene in breast cancer. In the present study, we found that high CAV1 expression was a protective factor for OS in osteosarcoma patients. ITGA5, a member of the integrin protein family, is considered to be one of the most abundant proteins in the ECM [63]. ITGA5 plays a role in various tumors by promoting cell adhesion and migration through the activation of FAKs after binding to integrin β1 [64,65]. For example, Zhang [66] et alfound found that miR-31 could synergize with ITGA5 to influence the invasive metastasis of gastric cancer cells. However, the role of ITGA5 varies across different tumors, and its function in osteosarcoma remains unclear. It has been demonstrated that ITGA5 is highly expressed in ovarian, breast, and gastric cancers, where it can serve as a prognostic marker closely associated with poor patient prognosis [67–69]. However, the expression of ITGA5 in osteosarcoma patients and its prognostic impact have not been well established. In our study, we found that ITGA5 expression was downregulated in osteosarcoma patients, and those with low ITGA5 expression had a better prognosis compared to those with high ITGA5 expression. We constructed and validated a risk model associated with the Focal adhesion signaling pathway, analyzing the expression of ITGA5, CAV1, and ZYX to predict OS in osteosarcoma patients. According to Kaplan-Meier survival analysis, patients in the low-risk group had significantly better outcomes than those in the high-risk group. This prognostic model demonstrated strong predictive performance, with a 5-year ROC curve showing a high AUC value of 0. 717. These findings highlight the model's reliability and applicability in predicting the survival of osteosarcoma patients.

In recent years, immunotherapy has emerged as a new strategy in oncology treatment, with its remarkable efficacy demonstrated in various malignancies [70,71]. This also offers a new approach for the treatment of osteosarcoma. In the TME, immune checkpoints play an inhibitory role in anti-tumor immunity. One key mechanism by which tumors inhibit the host's anti-tumor immune response involves up-regulated immune checkpoints in the TME binding to receptors on lymphocytes. This interaction promotes tumor growth, proliferation, and invasion [72]. Tumor cells utilize immune checkpoints expressed on their surface to evade host immune surveillance, leading to immune escape. Regulatory T cells (Tregs), a subgroup of $CD4^+$ T cells with potent immunosuppressive properties, contribute to the formation of a highly immunosuppressive TME through the expression of immune checkpoints on their surface [73]. Furthermore, researchers found that immune checkpoints play roles in promoting the formation of tumor-initiating cells, the EMT, the maintenance of tumor stem cells, and the metastasis of tumor cells within the TME. Additionally, immune checkpoints are associated with clinical characteristics such as chemotherapy resistance and poor prognosis [74]. We analyzed osteosarcoma data from the TARGET database to determine the expression values of eight immune checkpoint-associated genes. The results showed elevated expression levels of several immune checkpoint-related genes in the high expression groups of ZYX, CAV1, and ITGA5. In the CCLE database, we examined the expression levels of these genes in eight osteosarcoma cell lines with high expression of all three genes. It was found that CD274 (programmed death-ligand 1, PD-L1) and PDCD1LG2 (programmed death-ligand 2, PD-L2) were notably

expressed. Finally, qRT-PCR was used to validate these findings. PD-L1 and PD-L2 are ligands for PD-1, and their binding inhibits the activation and proliferation of T lymphocytes, enabling tumor cells to establish immune tolerance and evade detection by T cells, thereby weakening the anti-tumor immune response [75]. Blocking the PD-1/PD-L1 pathway has been shown to improve the prognosis of patients with non-small cell carcinoma, melanoma, kidney cancer, and other malignancies [76–78]. Some studies found that PD-1/PD-L1 pathway inhibitor therapy was not effective and did not completely inhibit the progression of osteosarcoma. However, this may be related to the high heterogeneity of osteosarcoma [79]. According to our results, anti-PD-1 therapy may be a promising treatment option for osteosarcoma patients with high expression of ZYX, CAV1, and ITGA5.

Ethical approval

Review and/or approval by an ethics committee was not needed for this study because this research utilized data from online database and classic osteosarcoma cell lines, and did not involve human or animal organs, tissues, or primary cells in the experiments.

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Consent

Not available.

Data availability statement

The datasets used in this investigation are available in internet repositories. This article contains the names of the repository/repositories as well as the accession numbers(see Fig. 2 or Fig. 3).More raw data and code can be obtained by contacting the authors via email.

CRediT authorship contribution statement

Zhiqiang Wu: Resources, Project administration, Funding acquisition, Data curation. **Zhiqing Wang:** Methodology. **Zhanqiang Hua:** Investigation. **Yingzheng Ji:** Formal analysis. **Qingrong Ye:** Supervision, Software. **Hao Zhang:** Writing – review & editing, Writing – original draft. **Wangjun Yan:** Writing – original draft, Conceptualization.

Declaration of competing interest

The authors declare that they have no competing interests.

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