Contents lists available at ScienceDirect

Journal of Bone Oncology

CD44 expression is correlated with osteosarcoma cell progression and immune infiltration and affects the Wnt/β-catenin signaling pathway

Hairu Ji [a],[1], Lingwei Kong [b],[1], Yu Wang [b],[*], Zhiping Hou [a], Wei Kong [a], Jiemin Qi [a], Yu Jin [b]

[a] Department of Pathology, Chengde Medical University, Chengde 067000, China
[b] Department of Traumatology and Orthopaedics, Affiliated Hospital of Chengde Medical University, Chengde 067000, China

HIGHLIGHTS

- CD44 promotes the proliferation, migration, and invasion of Osteosarcoma in vivo and in vitro.
- CD44 plays a role in promoting the malignant progression of osteosarcoma through the Wnt/β-catenin signaling pathway.
- CD44 is associated with immune infiltration in osteosarcoma and can be used as a candidate biomarker for immune infiltration-related prognosis.

ARTICLE INFO

ABSTRACT

CD44 is associated with a variety of human diseases and plays a potential role in tumorigenesis, however, the mechanism of its role in osteosarcoma remains unclear. We analyzed the expression of CD44 in the Cancer Genome Atlas (TCGA) and genotype-tissue expression pan-cancer data and found that it was highly expressed in most tumors, including sarcoma. The expression of CD44 in osteosarcoma cell lines was higher than that in human osteoblast cell line in the results of the Western blot and Immunohistochemical staining assay. The results of colony formation assay and CCK 8 showed that CD44 improved the proliferation capacity of osteosarcoma cells, transwell assay and wound healing assay showed that CD44 improved the migration capacity of osteosarcoma cells. Further studies revealed that CD44 exerts its influence on the biological behavior of osteosarcoma cells through the Wnt/β-catenin signaling pathway. Since CD44 may be involved in the immune response, we analyzed the correlation between CD44 expression and immune cell infiltration in TCGA database using the previous cluster analyzer R software package, TIMER2.0 database and, GEPIA2 database, and found its involvement in the immune infiltration of osteosarcoma. Therefore, we believe that CD44 could be a potential target for the treatment of osteosarcoma patients and may be a candidate biomarker for immune infiltration-related prognosis.

1. Introduction

Osteosarcoma is the most common malignant bone tumor in children and adolescents with an annual incidence of about 3.4 per million worldwide [1], Osteosarcoma cells often arise from the primitive mesenchymal-derived bone-forming cells, and it usually occurred in the bone that is growing quickly such as the bones near the ends of the leg or arm, it is highly malignant, prone to early metastasis, and the prognosis of patients is extremely poor [2–4]. Although the treatment modality of neoadjuvant chemotherapy surgical resection-chemotherapy has been adopted, however, because of drugs resistance, metastasis, or recurrence, the survival rate for osteosarcoma patients is still not very high [5,6], the second chief cause of cancer-related deaths in children and adolescents, accounting about 5% of childhood cancers [7]. Thus, identifying prognostic biomarkers that associate with the biological heterogeneity of osteosarcoma is urgently needed to improve prognosis.

About 10–20% of patients with osteosarcoma have measurable metastasis before diagnosis [8]. Proliferation is the basis for the existence and invasion of tumor cells [9], which is currently a hot topic in oncology research, therefore, it is crucial to search for markers related to the proliferation and migration of osteosarcoma [10]. Tumor microenvironment (TME) refers to the occurrence, growth, and metastasis of

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tumors that are closely related to the internal and external environment of tumor cells [11], it not only includes the structure, function, and metabolism of the tissue where the tumor is located but also is related to the internal environment of tumor cells themselves (nuclear and cytoplasm) [12]. The tumor microenvironment (TIME) contains a large number of immune cells, which have diverse and complex functions and hijack key physiological pathways to promote the survival and proliferation of tumor cells [13]. In the tumor microenvironment, tumor-infiltrating immune cells account for the primary non-tumor constituents, which have been demonstrated to play an important role in the prognostic prediction of osteosarcoma patients[14]. Multiple cytokines and growth factors were released from these cells, and immune-related genes and immune cell infiltration act as indispensable roles in tumor development and progression [15].

CD44 is a cell surface receptor for hyaluronate and regulates the interaction of cells with substrates and involve in lymphocyte homing, cell adhesion and aggregation, cell migration, leukocyte activation, lymphopoiesis, and myelopoiesis, angiogenesis and cytokine release [16]. Numerous studies have shown that CD44 is highly expressed in gastric cancer [17], breast cancer [18], colorectal cancer [19], lymphoma [20], and osteosarcoma [21], and correlates with aggressive biological behavior and a poor prognosis. The role of CD44 in tumors is not well defined, the physiological functions of CD44 indicate that it is involved in the metastasis of tumors, a large number of studies have shown that CD44 can promote the proliferation of breast cancer, non-small cell lung cancer, and gastric cancer, and can be used as an immune checkpoint for gastric cancer [22], lung cancer, and breast cancer [23], and is related to the prognosis of cancer.

Wnt signaling is involved in numerous fundamental processes essential for embryonic development and normal adult homeostasis [24]. This signaling pathway has already been shown to be involved in many cellular functions essential for normal organ development including cell proliferation, survival, self-renewal/differentiation, etc. multiple dysfunctions and mutation of these pathways were shown to be related to several diseases, especially in tumors [25], it is involved in the proliferation, migration and drug resistance of liver cancer [26], rectal cancer [27], and osteosarcoma [28]. Recent findings regarding the Inhibition of the Wnt/β-catenin signaling pathway promote the immune response in cancer, and this signaling pathway regulates the inflammatory effects of T cells in the tumor microenvironment [29].

In the present study, we comprehensively analyzed CD44 to investigate its effects on the proliferation, migration, and immune infiltration of osteosarcoma, Moreover, we also explored the regulatory role of CD44 along with the Wnt/ β-catenin signaling pathway in osteosarcoma. Our work may provide a new clue for exploring the underlying molecular mechanisms of osteosarcoma, shed a novel light on the targeting therapy strategy of osteosarcoma, and promote the individual-based treatment of osteosarcoma patients.

2. Materials and methods

2.1. Data collection

Expression of mRNA and related clinical data were retrieved from TCGA and Genotype-Tissue Expression (GTEx) clinical pan-cancer data were downloaded from the University of California, Santa Cruz (UCSC) Xena database (https://xenabrowser.net/datapages/). Meanwhile, CD44 expression profiles between sarcoma and normal tissues were analyzed on the GEPIA2 (Gene Expression Profiling Interactive Analysis) (https://gepia2.cancer-pku.cn/#index). The cell line mRNA expression matrix of tumors was obtained from the Broad Institute Cancer Cell Line Encyclopedia (CCLE) dataset (https://portals.broadinstitute.org/ccle).

2.2. Cell lines and cell culture

Human OS cells MG-63, U-2 OS, Saos-2 and the human osteoblast cell line hFOB 1.19 were obtained from the Cell Bank of Shanghai Institute of Cell Biology (Shanghai, China). MG63 was cultured in MEM medium supplemented with 10% fetal bovine serum, U-2 OS and Saos-2 were cultured in McCoy's 5A medium supplemented with 10% and 15% fetal bovine serum respectively, Osteoblastic hFOB 1.19 was cultured in D-MEM/F-12 supplemented with 10% fetal bovine serum at 37C in a humidified atmosphere with 5% carbon dioxide.

2.3. Plasmids and si-RNA

pCMV-CD44(human)-3 × Myc-Neo and Vector plasmid was acquired from MiaoLingBio, China (P40982). The small interfering RNA of CD44 was purchased from RiboBio, China, the si-CD44 sequences used in this paper is GCAGTCAACAGTCGAAGAA.

2.4. RNA extraction and quantitative real-time PCR

Total RNA was extracted using TRIzol from MG-63, U-2 OS, Saos-2, and osteoblastic hFOB 1.19 cells after CD44-interfering, reverse transcription was performed using the ABScript II RT Master Mix for qPCR according to the manufacturer's instructions. Real-time RT-PCR analysis was performed as in manual 2 × Universal SYBR Green Fast qPCR Mix (ABclonal, China). The PCR was performed under the following conditions: 95 °C for 3 min, 42 cycles of 95 °C for 5 s, and 60 °C for 32 s. The relative mRNA expression levels were calculated by using the $2 - \Delta\Delta Ct$ method. The forward (F) and reverse (R) primers used for the indicated genes are as follows: CD44(forward, 5′-ACCGACAGCACAGACAGAATC-3′, reverse, 5′-GTTTGCTCCACCTTCTTGACTC-3′) and GAPDH(forward, 5′-GTCTCCTCTGACTTCAACAGCG-3′, reverse, 5′-ACCACCCTGTTGCT GTAGCCAA-3′).

2.5. Western blot

The proteins were extracted from the cells using RIPA lysis buffer (Ruipate, Hebei, China). BCA Protein Assay Kit (MultiSciences, Jiangsu, China) was utilized to measure the protein concentrations. Proteins were separated by 10% SDS-PAGE gels and transferred onto a polyvinylidene difluoride (PVDF) of 0.45 μm membranes. The membrane was blocked in 5% skim milk for 1.5 h and then incubated with CD44 antibody (1:1000, ABclonal, China) at 4 °C overnight. After 3 times washing with Tris-buffered saline with Tween 20 (TBST), the membrane was incubated at room temperature for 1 h with an anti-rabbit secondary antibody (1:5000, Sera care). The β-actin was used as endogenous control. The intensity of each band was measured with Image J, and each experiment was repeated 3 times.

2.6. Immunohistochemical staining (IHC)

Osteosarcoma tissue microarrays adopted in this study were purchased from Bioaitech (L714901, Xi'an, China), included 70 osteosarcoma and 1 normal tissue. The tissue microarrays were rehydrated and incubated with primary anti-CD44(Proteintech, USA 1:800) antibody, then treated with secondary antibody. Finally, the slides were colored with the DAB Kit (ZSGB-BIO, China) and photographed using the microscope.

2.7. Colony formation assay

A colony formation assay was performed to detect cell proliferation. After the cells were transfected siRNA or plasmid for 24 h, 400–600 cells were incubated in 6-well plates for 1–2 weeks. 6-well plates were washed 3 times with PBS after the medium was removed, fixed in prechilled methanol solution for 20 min, then stained with crystal violet solution for 15 min and analyzed by Image J statistical photography, and each experiment was repeated 3 times.
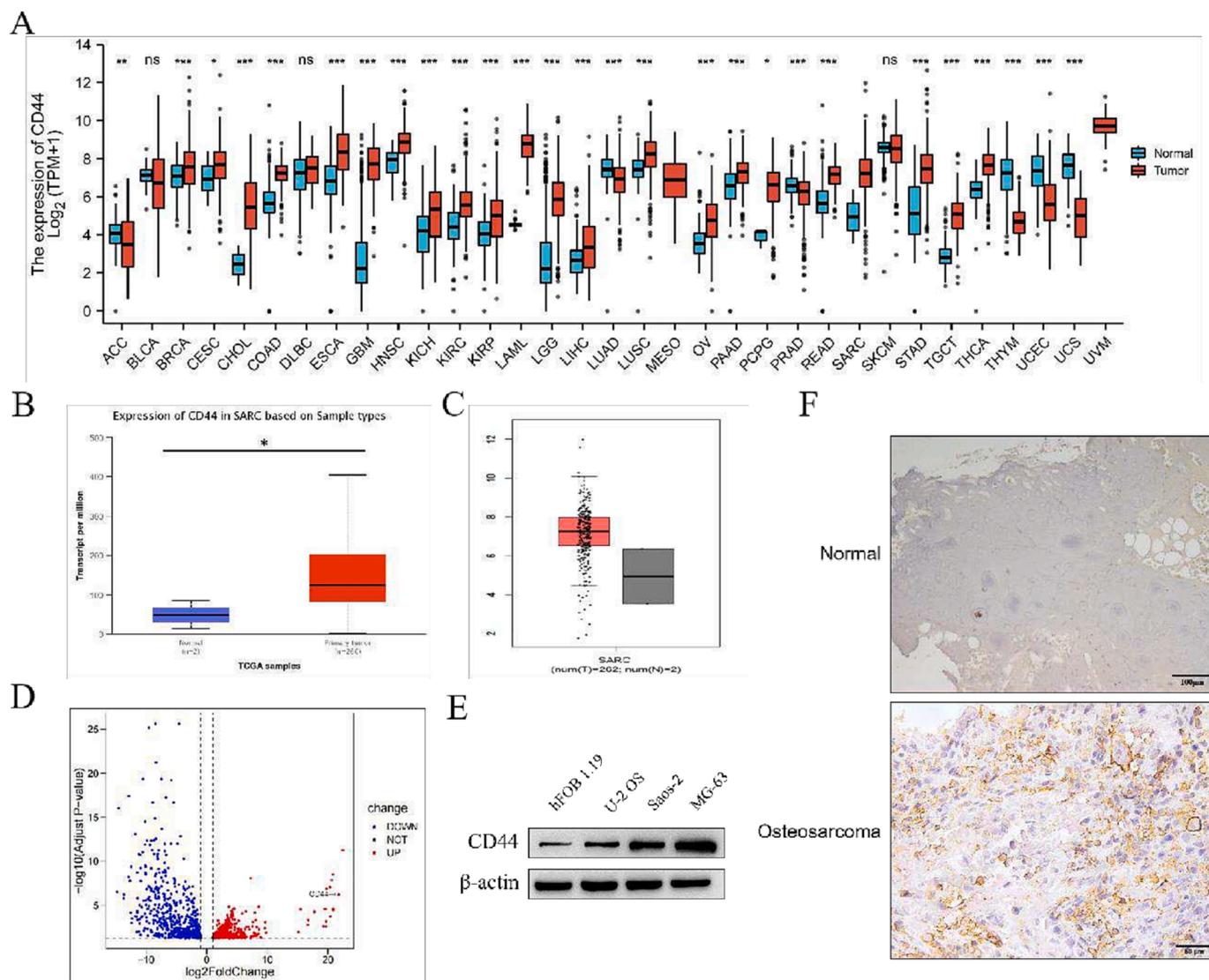
Fig. 1. Expression of CD44 in sarcoma and osteosarcoma. (A) CD44 expression in different types of cancer was investigated with the XENA-TCGA_GTEx RNAseq database. (B) Increased or decreased expression of CD44 in sarcoma compared to normal tissues in the GEPIA database. (C) CD44 expression in sarcoma was examined by using the UALCAN database. (D) Differential gene analysis of sarcoma in TCGA showed that CD44 appeared as a highly expressed red dot in the sarcoma differential gene. (E) Expression of CD44 protein in osteosarcoma cells and normal human osteoblasts. (F) IHC demonstrated that CD44 was more highly expressed in osteosarcoma tissues than innormal tissues ×200. *p < 0.05, **p < 0.01, ***p < 0.001. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.8. CCK8 assay

CCK8 assay was performed to detect cell proliferation. Osteosarcoma cells were seeded into 96-well plates at a density of 2000 cells/well. At 0,24,48, and 72 h, 10 µl CCK8 of the solution was added to each well and culture plates continued at 37℃ for 1.5 h to detect the spectral absorbance values at 450 nm, and each experiment was repeated 3 times.

2.9. Nude mouse xenograft assay

The use of all animals in this study was approved by the Institutional Animal Care and Use Committee of China Medical University. Log-stage tumor cells (5×10^6) were then subcutaneously injected into the right posterior back of 6-week-old female nude mice (Beijing Victoria Lihua Experimental Animal Co., Ltd., Beijing, China). Tumor length (a) and width (b) were monitored with calipers every 3 days after injection. The tumor volume (V) was calculated as follows: $V = ab^2 / 2$. At the end of the experiment, mice were sacrificed and xenograft weights were measured.

2.10. Wound healing assay

A wound-healing assay was performed to detect cell migration. MG-63 and U-2 OS cells (0.3×106) were seeded into 6 well plates. Transfection was done with CD44 siRNA or plasmid along their respective controls. Cells were allowed to grow till 100% confluency. A Scratch was made in the plate using a P200 pipette tip. Images were collected at 0 h and 24 h, under an inverted microscope (Olympus, Japan). Cell migration was analyzed using Image J statistical photography, and each experiment was repeated 3 times.

2.11. Transwell assay

A Transwell assay was performed to detect cell migration. A 200ul cell suspension containing 40,000 cells was added dropwise to the upper chamber of the chamber and 600ul of medium containing 20% fetal bovine serum was in the lower chamber. After 20 h hours, the medium in the small chamber was discarded, washed with PBS, fixed with pre-chilled methanol for 15 min, stained with crystal violet solution for
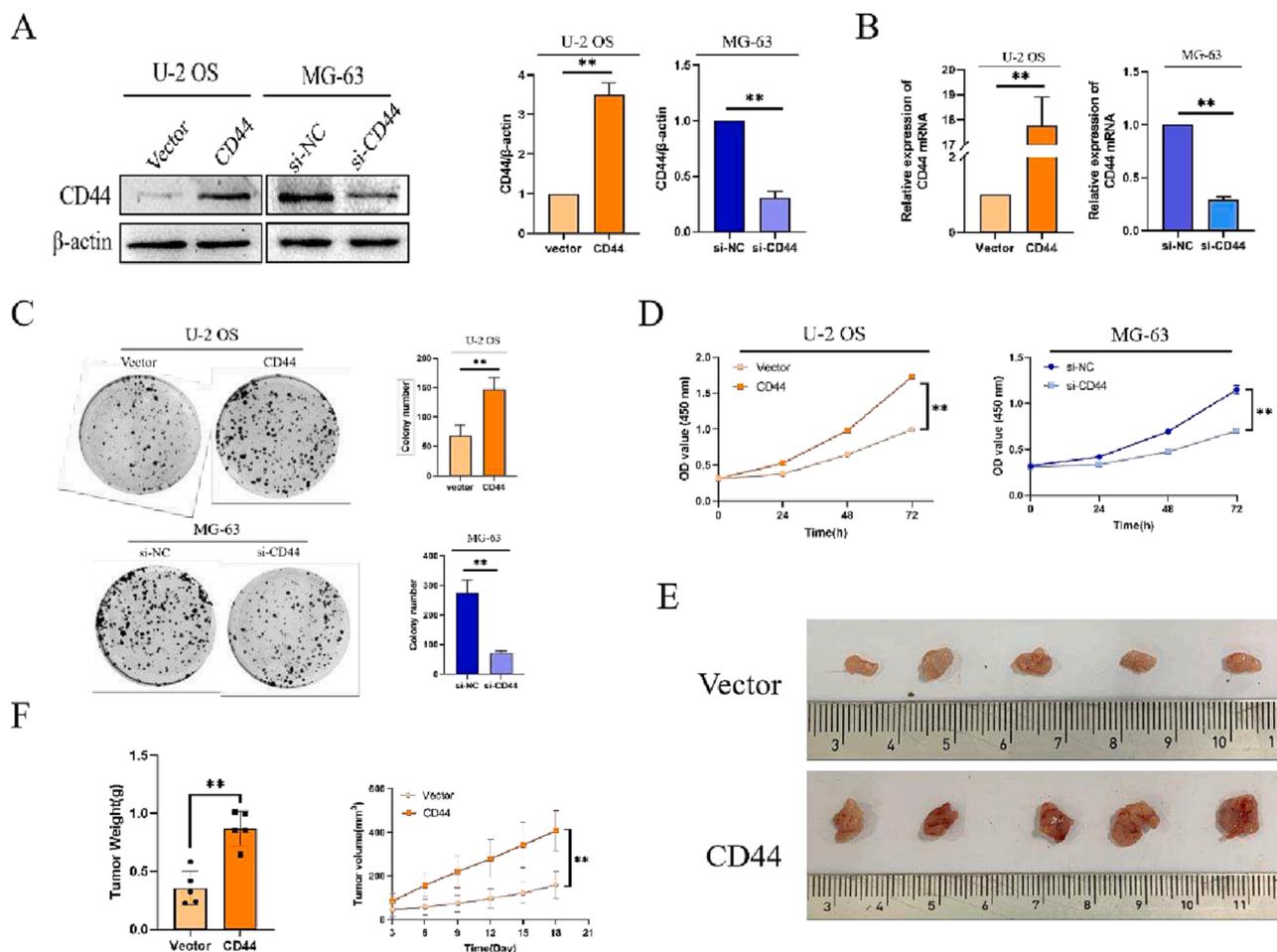
Fig. 2. CD44 promotes the proliferation of osteosarcoma in vitro and in vivo. (A) Protein expression levels after CD44 overexpression and knockdown in U-2 OS and MG-63 cells respectively. (B) The mRNA expression levels after CD44 overexpression and knockdown in U-2 OS and MG-63 cells respectively. (C) CD44 affects cell clone formation ability in U-2 OS and MG-63 cells. (D) The effect of CD44 on the growth of U-2 OS and MG-63 cells was examined by CCK 8 at 0 h, 24 h, 48 h, and 72 h. € Subcutaneous tumor formation after CD44-overexpression and Vector. (E) Xenograft tumors in nude mice. (F) Tumor growth curves and tumor weights were shown for CD44-overexpression and Vector groups. The CCK-8 indicates Cell Counting Kit-8. *p < 0.05, **p < 0.01.

15 min, rinsed with distilled water, and counted under the microscope, and each experiment was repeated 3 times.

2.12. Gene-related networks and enrichment analyses

The STRING database (https://string-db.org/) is a database that analyses protein interactions. The protein–protein interaction (PPI) network of CD44 was constructed by using this database.

The Linked Omics database (https://www.linkedomics.org) is a database of multiple omics and clinical data for 32 cancer types. Data types include miRNA, mRNA data, methylation data, mutation sites, etc. We used this database to obtain heat maps of genes positively and negatively correlated with CD44 in sarcoma.

Gene ontology (GO) analysis was performed using the EnrichGO function in the cluster profile R software package R with the following parameters: ont = all, pvalue-Cutoff = 0.05, and qvalue-Cutoff = 0.05. Gene set enrichment analysis was performed using the gseKEGG and gsePathway functions in cluster profile with the following parameters: nPerm = 1,000, minGSSize = 10, maxGSSize = 1,000, and pvalue-Cutoff = 0.05.

2.13. Tumor immune correlation analysis

The TIMER2.0 database (https://cistrome.shinyapps.io/timer) is a database related to tumor immunity. It includes immunity, exploration, and assessment. We first performed a comprehensive assessment of CD44 expression in multiple types of cancer. In addition, we investigated the correlation between CD44 and immune cell inflammation. the correlation between CD44 and immune cell inflammation, such as B cells, CD8 + T cells, CD4 + T cells, neutrophils, macrophages, and dendritic cells, was analyzed by using the "Genes" module. In addition, the correlation module was used to analyze the relationship between CD44 and immune cell markers used to analyze.

2.14. Statistical analysis

Statistical analyses were carried out using GraphPad Prism (Version 9.0). R language was used for the different analyses based on the sample data obtained from the TCGA and GEO databases. The volcano map of genes in TCGA sarcoma cancer samples was drawn by using the R software package ggscatter, and |log2Fold Change|>1 was defined as the differentially upregulated and downregulated genes. Spearman correlation analysis of GEPIA genes was performed. Dates in the figures
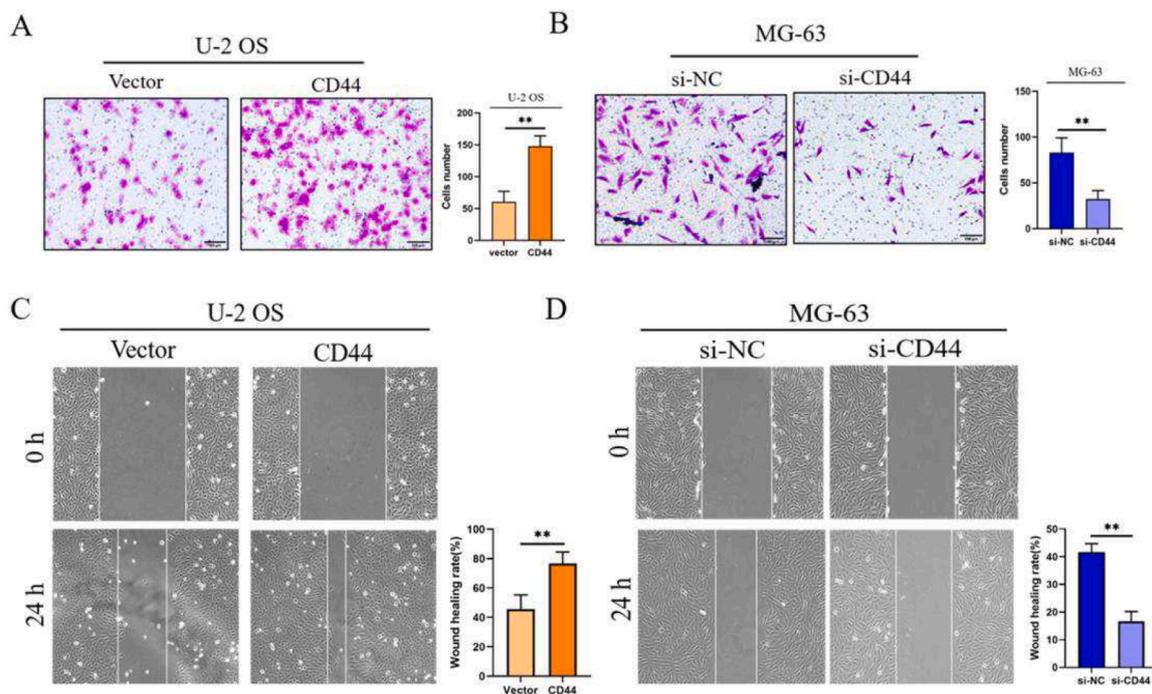
Fig. 3. CD44 promotes the migration of osteosarcoma cells. (A) Cell migration in CD44 overexpressing in U-2 OS cells was determined by a transwell migration assay. (B) Cell migration in CD44 knockdown in MG-63 cells was determined by transwell migration assay. (C) Cell migration in CD44 overexpressing in U-2 OS cells was also determined by wound healing assay. (D) Cell migration in CD44 knockdown in MG-63 cells was determined by wound healing assay. *p < 0.05, **p < 0.01.

are the means ± SD. For all tests in this study, a P value of <0.05 was considered statistically significant.

3. Results

3.1. CD44 is upregulated in sarcoma and osteosarcoma

We first evaluated CD44 expression in TCGA and Genotype-Tissue Expression (GTEx) clinical pan-cancer database and found higher CD44 expression in 27 tumors compared with the corresponding normal tissues, including ACC, BRCA, CESC, CHOL, COAD, ESCA, GBM, HNSC, KICH, KIRC, KIRP, LAML, LGG, LIHC, LUAD, LUSC, OV, PAAD, PCPG, PRAD, READ, STAD, TGCT, THCA, THYM and UCS (Fig. 1A). Consistently, we also found that higher mRNA of CD44 was expressed in sarcoma tissues than in normal tissues in the gene expression profiling interactive analysis (GEPIA) and UALCAN databases (Fig. 1B, C).

Next, we analyzed the TCGA data set of sarcomas to explore the up-/down-regulated genes, which might be therapeutic targets for sarcoma patients, Interestingly, we observed CD44 among the overexpressed genes (Fig. 1D). Since sarcomas still contain a variety of tumors, how does CD44 behave in osteosarcoma? We examined the expression of CD44 in human osteoblasts hFOB 1.19 and osteosarcoma cells MG-63, U-2 OS, and Saos-2, and found that the protein expression of CD44 protein in osteosarcoma cells was higher, with a statistically significant difference (Fig. 1E).

IHC demonstrated that CD44 was more highly expressed in osteosarcoma tissues than innormal tissues(Fig. 1F).We searched the mRNA expression of CD44 in sarcoma cells through the Cancer Cell Line Encyclopedia database and found that CD44 mRNA expression in osteosarcoma cells was generally high (Supplementary Fig. 1A), which is also consistent with the results of our test.

3.2. CD44 promotes the proliferation of osteosarcoma in vitro and in vivo

To determine the biological function of CD44 in osteosarcoma cells, we transfected MG-63 and U-2 OS cells with the siRNA and over-expression plasmid for CD44, respectively, followed by qRT-PCR and Western blot assays. As shown in Fig. 2A-B, all P < 0.01, CD44 levels in MG-63 cells transfected with si-CD44 were significantly lower than in si-NC and control cells, with significantly high CD44 expression levels in U-2OS cells transfected with CD44 overexpression plasmid Vector group, confirming the successful transfection. Then, the effect of CD44 on cell proliferation was detected by Colony formation assay and CCK8 assay, which showed that upregulation of CD44 significantly promoted the proliferation of U-2 OS cells, and downregulation of CD44 significantly inhibited the proliferation capacity of MG-63 cells (Fig. 2C-D, all P < 0.05). To evaluate the role of CD44 in the tumor formation ability in vivo, the CD44-modified osteosarcoma cells and their control cells were injected subcutaneously into nude mice. The xenograft tumors in the CD44-overexpression group were larger and heavier than those in the control group (Fig. 2E, F, all P < 0.05).

3.3. CD44 promotes the migration of osteosarcoma cells

To explore whether CD44 affects the motility of osteosarcoma cells, we applied transwell and wound healing assays after transfection and si-RNA with the CD44 in U-2 OS and MG-63 cells. Transwell assay results showed that overexpression of CD44 significantly promoted the migration capacity of U-2 OS cells (Fig. 3A), while knockdown of CD44 significantly inhibited the migration capacity of MG-63 cells (Fig. 3B). In addition, we also assessed the migration of osteosarcoma in a wound-healing assay. We found that overexpression of CD44 promoted the migratory capacity of U-2 OS cells (Fig. 3C), while knockdown of CD44 dramatically suppressed MG-63 cell migratory abilities (Fig. 3D). Taken together, our results suggest that CD44 plays an important role in promoting osteosarcoma cell migration.
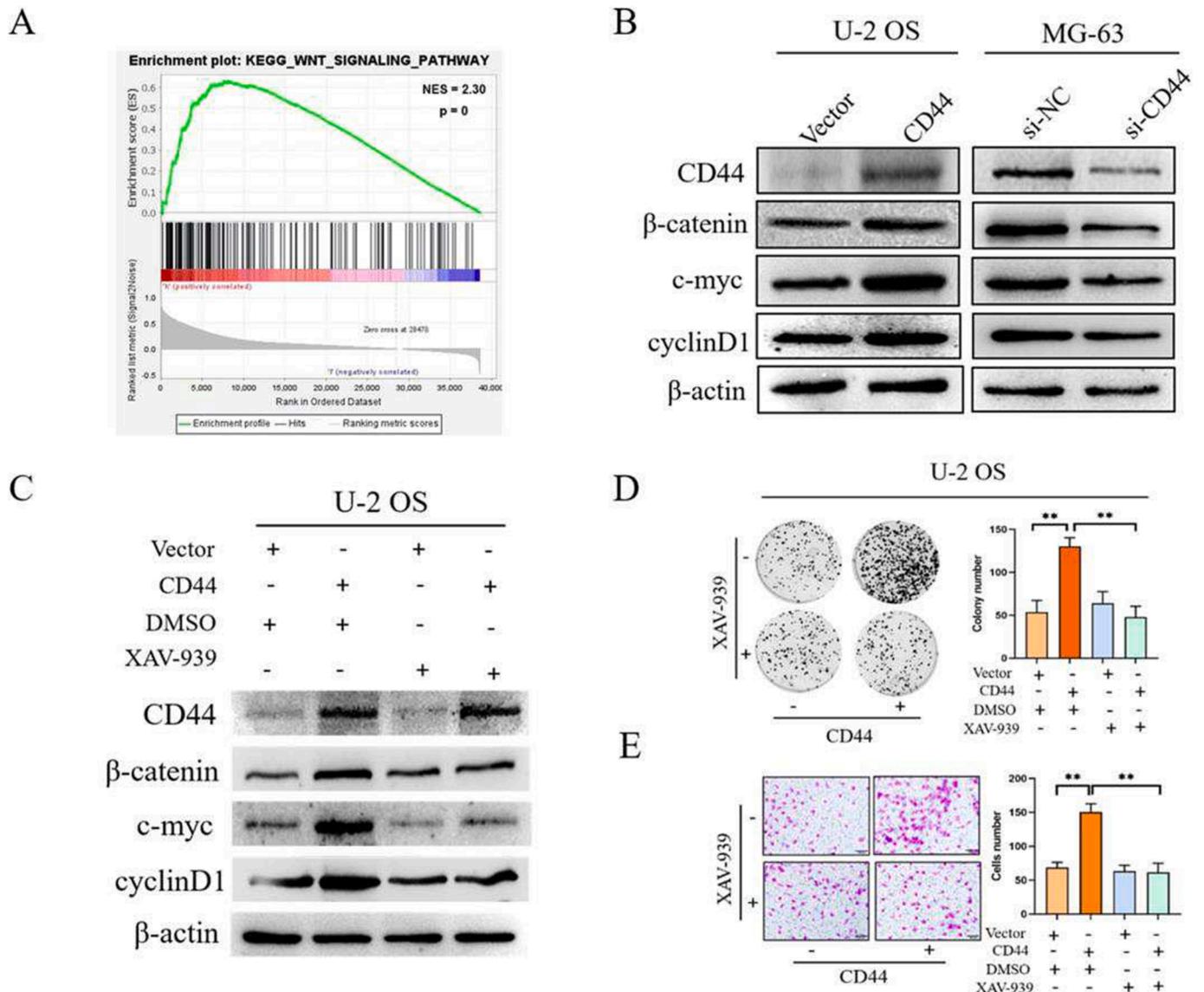
Fig. 4. CD44 plays a role in promoting the proliferation and invasion of osteosarcoma cells through the Wnt signaling pathway. (A) The Wnt signaling pathway was enriched. (B) CD44 regulated the expression of Wnt signaling pathway-related proteins. (C) XAV-939 reversed the activation of the Wnt signaling pathway by CD44. (D) XAV-939 reversed the proliferative effect of CD44 on osteosarcoma cells. (E) XAV-939 reversed the invasive effect of CD44 on osteosarcoma cells. *p < 0.05, *p < 0.05, **p < 0.01.

3.4. CD44 promotes osteosarcoma progression via Wnt signaling pathway activation

To investigate how CD44 regulates osteosarcoma cell proliferation and invasion, enrichment of KEGG pathway enrichment analysis was performed in sarcomas, and the Wnt/β-catenin signaling pathway was enriched(4A), to verify whether CD44 functions through this pathway, after our knockdown of CD44, it is found that β-catenin, a key factor in the Wnt signaling pathway, and its downstream target gene cyclinD1, c-myc and Axin2 were downregulated. Opposite results were obtained after overexpression of CD44 (4B). To further verify that CD44 does act through regulation of the Wnt pathway, we applied the Wnt pathway inhibitor XAV-939 concomitantly after CD44 overexpression, which reversed the activation of Wnt signaling by CD44 (4C) and promoted osteosarcoma proliferation (Fig. 4D) and invasion (4E).

3.5. Network-interacting protein of CD44 and GSEA, KEGG enrichment analysis for CD44

A protein–protein interaction (PPI) network for CD44 was generated using the string database, including 103 edges and 21 nodes (Fig. 5A). The HiSeq RNA database in the TCGA secondary database Linked Omics was used to analyze and predict the correlation between the target gene CD44. The "Pearson correlation test" was selected as the statistical method. The two heatmaps generated indicated genes positively correlated with CD44, including PLAU, VEGFC, and CDCP1 (Fig. 5B), and genes negatively correlated with CD44, including LPHN1, ZNF682, and SEMA6A (Supplementary Fig. 5B).

To extract RNAseq data in level 3 HTSeq-Counts format from the SARS RC (Sarcoma) project within TCGA, a List of differential analysis with CD44 as a single gene, GSEA analysis of the list containing 589 genes yielded the mountain map, REACTOME KERATINIZATION, REACTOME FORMATION OF THE CORNIFED ENVELOP, REACTOMA HEMOSTASIS Statistical significance, KEGG NEUROACTIVE LIGAND RECEPTOR INTERACTION, REACTOME G ALPHA I SIGNALLING
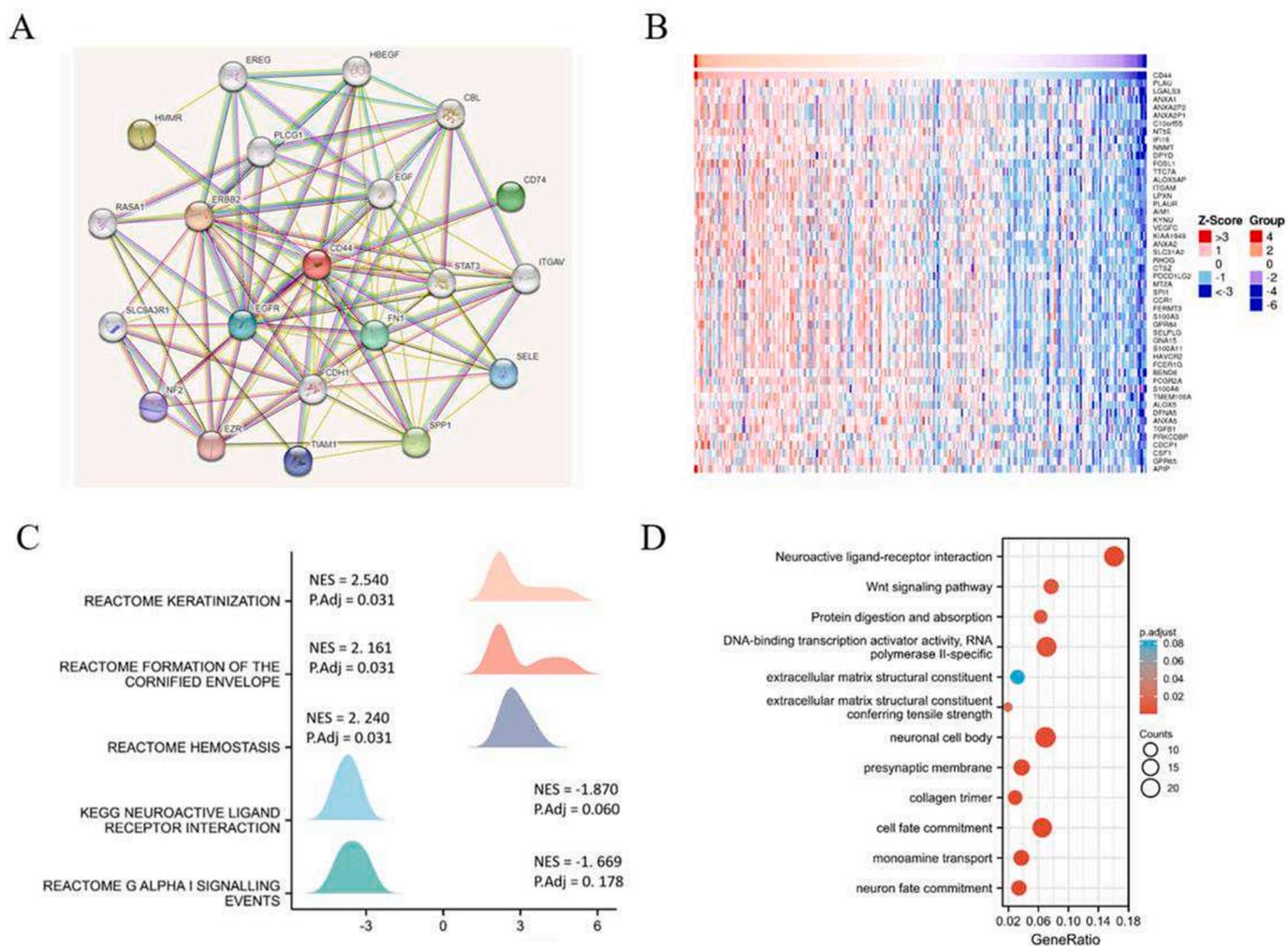
Fig. 5. Network interaction proteins of CD44 and GSEA, KEGG enrichment analysis for CD44. (A) The PPI network of CD44 was generated using STRING. (B) Heat maps showing the expression-pattern-of-in-sarcoma Genes positively correlated with CD44 in SARC. (C) GSEA enrichment analysis for CD44. (D) GO and KEGG enrichment analysis for CD44.

EVENTS Not statistically significant (Fig. 5C). According to GO/KEGG enrichment analysis, CD44 was enriched to the Neuroactive ligand-receptor interaction and Wnt signaling pathway and Protein digestion and absorption after differential expression (Fig. 5D).

3.6. Correlation analysis between CD44 expression and infiltrating immune cells

The correlation between CD44 expression and six types of infiltrating immune cells was used in the TIMER database, including B cells, CD8 + T cells, CD4 + T cells, macrophages, neutrophils, and dendritic cells. The results showed that CD44 expression levels had a significant positive correlation with the infiltration of B cells, CD8 + T cells, macrophages, neutrophils, and dendritic cells and no significant correlations with CD4 + T cells in Sarcoma (Fig. 6A). To further assess the effect of CD44 on the tumor microenvironment (TME), We applied the GSVA R package to analyze the RNAseq data in level 3 HTSeq-FPKM format in the TCGA SARC project and analyze the correlation between CD44 and immune infiltration. Notably, CD44 was positively correlated with the infiltration levels of macrophages, Neutrophils, Th2 cells, Th1 cells, T cells, DC, aDC, iDC, TReg, NK CD56dim cells, B cells, T helper cells, Cytotoxic cells, Th17 cells but negatively correlated with the infiltration levels of NK cells, pDC and Tgd in Sarcoma (Fig. 6B). The literature and the presence of multiple landmark immune checkpoints in osteosarcoma are

instructive for evaluating the immune microenvironment of osteosarcoma. We applied the GEPIA2.0 database to analyze the association of CD44 with osteosarcoma immune checkpoint-related proteins CD274, CD47, CTLA 4, and CD276 and found that CD44 has a positive relationship with them (Fig. 6C). These findings further support that CD44 expression is significantly related to immune infiltration and suggest that CD44 plays an important role in immune escape in the osteosarcoma microenvironment.

3.7. Correlation between CD44 expression and various immune markers

To gain a deeper understanding of the relationship between CD44 and immune responses, we verified the correlation between CD44 expression and different immune features in sarcomas. We obtained Table 1 through the TIMER database, where the listed genes were used to characterize immune cells, including B cells, T cells (general), CD8 + T cells, Monocyte, tumor-associated macrophages (TAMs), M1, M2, Neutrophils, Natural killer cell, and Dendritic cell. Tumor purity is an important aspect affecting the dissection of immune infiltration in clinical cancer biopsies. After adjusting for tumor purity, CD44 expression was significantly associated with most immune markers in divergent types of immune cells in Sarcoma (Table 1).

The correlation between CD44 expression and various functional T cells was also examined, including Th1, Th1-like, Th2, Treg, Resting
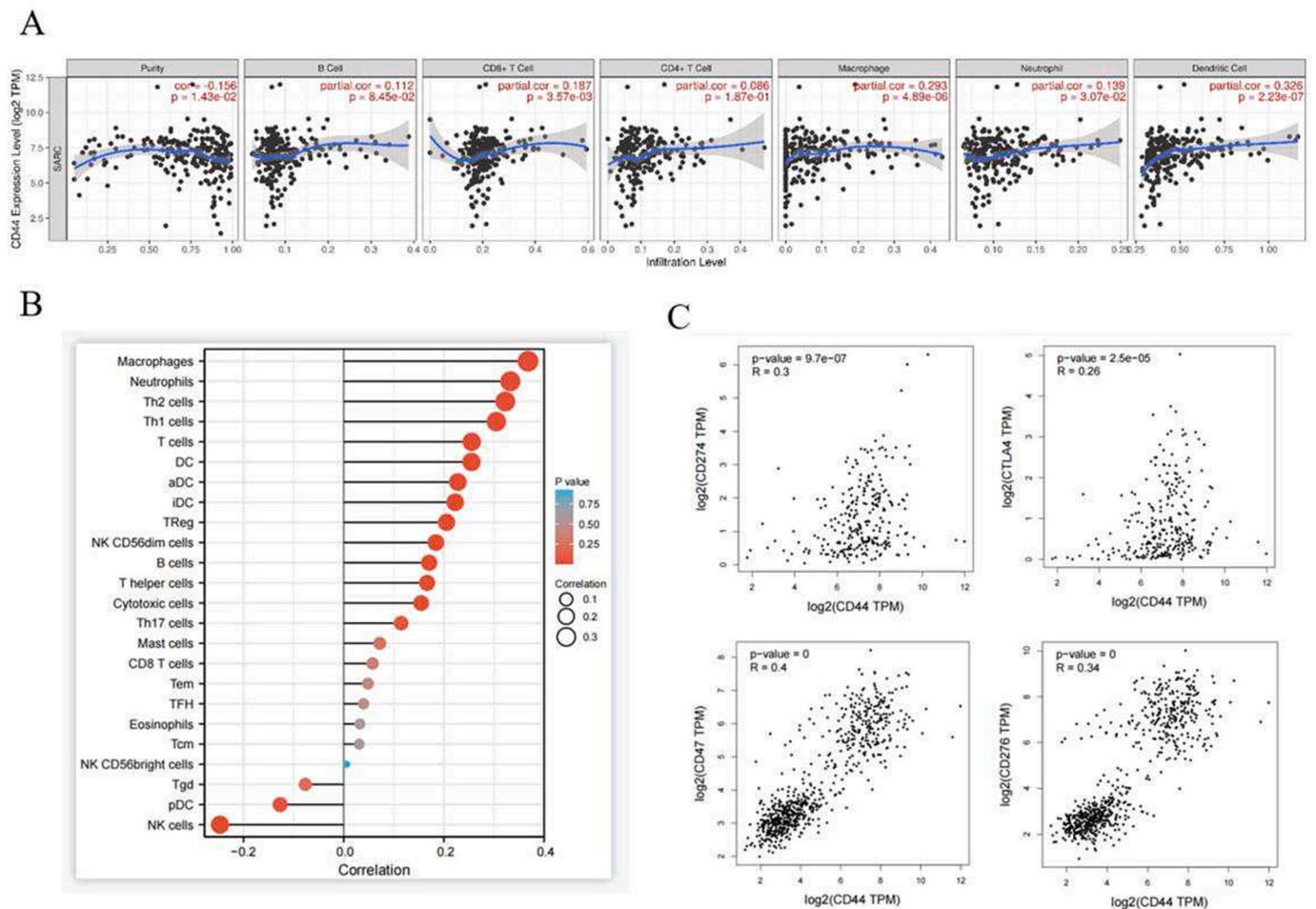
Fig. 6. Correlation of CD44 expression with immune infiltration level. (A) CD44 is significantly associated with tumor purity and is positively correlated with the infiltration of different immune cells using the TIMER database. (B) CD44 expression has a significant correlation with the infiltration of immune cells in. (C) Scatterplots of the correlations between CD44 expression and CD274, CTLA-4, CD47, and CD276 in Sarcoma using the GEPIA database.

Tregs, Effector Treg T-cell, Effector T-cells, Native T-cell, Effector memory T-cell, Resistant memory T-cell, General, Memory T-cell, and Exhausted T-cell. Moreover, the CD44 expression level was significantly correlated with 32 of 38 T cell markers in Sarcoma after adjusting for tumor purity in the TIMER database (Table 2).

4. Discussion

Osteosarcoma is characterized by high aggressiveness, early metastasis, and easy recurrence, which leads to a low survival rate and poor prognosis of patients [30]. Although the treatment of neoadjuvant chemotherapy has improved the survival rate, the overall treatment effect and prognosis of patients are not optimistic, which seriously harms the health of adolescents [5]. It is urgent to find the mechanism for the development of osteosarcoma and try to improve patient survival rate and improve prognosis through targeted molecular therapy. In the present study, we identified CD44 is highly expressed in osteosarcoma cell lines, suggesting a possible pro-cancer role in osteosarcoma, consistent with predictions from the TCGA and GTEx databases, we analyzed CD44 expression in sarcoma cell lines by CCLE database and found that CD44 was generally highly expressed in osteosarcoma cell lines, and previous studies found that CD44 was up-regulated in tumors including osteosarcoma [21], such as lung cancer [31], head and neck squamous carcinoma [32], and ovarian cancer [33], which is consistent with our validation results in osteosarcoma cell lines and patient tissue specimens (Fig. 1). CD44 is one of the key cancer stem-like cell (CSC)

markers and may have a potential role in tumorigenesis, previous studies have found that both, downregulation of CD44 inhibited the proliferation of glioblastoma [34], silencing of CD44 decreased the proliferation, migration, and invasion of breast cancer cells [35], while the knockdown of CD44 promotes proliferation and migration in Claudin-Low MDA-MB-231 and Hs 578 T Breast Cancer Cell Lines [36], we previously found that after disrupting CD44 expression in osteosarcoma using siRNA, proliferation, the migration and invasion capacity of OS cells has decreased [21]. In this study, we bidirectional regulated CD44, and again verified that CD44 promoted the malignant progression in osteosarcoma in vivo and in vitro (Figs. 2 and 3).

How does CD44 play a role in tumors? Mechanistically, CD44 promoted lung cancer cell proliferation by activating Kras-mediated signaling through the mitogen-activated protein kinase (MAPK) pathway [37]. In A549 and HepG2 cells, downregulation of CD44 significantly promoted cell apoptosis and inhibited cell proliferation, cell cycle progression, and migration, in which CD44 could act through RhoA signaling to regulate YAP expression was consistent with the effects of RNAi-mediated YAP [38]. CD44 activation-induced expression of EGFR and activation of phosphatidylinositol 3′kinase(PI3K)/Akt and expression of glycogen synthase kinase-3 β(GSK-3β) promote colon cancer cell invasion and migration [39]. Previous studies also showed that the inhibition of CD44 sensitizes cisplatin resistance and affects Wnt/β-catenin signaling in head and neck squamous cell carcinoma cells [40]. In our study, it is the first time found that CD44 can promote the proliferation and migration of osteosarcoma cells through the regulation

Table 1
Correlation analysis between CD44 and gene markers of immune cells in TIMER.

Description Gene markers SARC		None		Purity	
		Cor	P	Cor	P
B cell	CD19	0.157	*	0.131	*
	CD79A	0.095	0.125	0.051	0.426
T cell (general)	CD3D	0.259	***	0.221	***
	CD3E	0.264	***	0.233	***
	CD2	0.266	***	0.23	***
CD8+ T cell	CD8A	0.235	***	0.203	**
	CD8B	0.221	***	0.186	**
Monocyte	CD86	0.373	***	0.332	***
	CSF1R	0.318	***	0.265	***
TAM	CCL2	0.253	***	0.205	**
	CD68	0.414	***	0.396	***
	IL10	0.358	***	0.319	***
M1	IRF5	0.278	***	0.272	***
	PTGS2	−0.128	*	−0.109	0.088
	NOS2	−0.073	0.243	−0.116	0.07
M2	CD163	0.373	***	0.338	***
	VSIG4	0.362	***	0.317	***
	MS4A4A	0.318	***	0.267	***
Neutrophils	CEACAM8	−0.034	0.586	−0.034	0.597
	ITGAM	0.425	***	0.397	***
	CCR7	0.16	**	0.125	0.052
Natural killer cell	KIR2DL1	0.18	**	0.136	*
	KIR2DL3	0.116	0.062	0.081	0.207
	KIR2DL4	0.239	***	0.218	***
	KIR3DL1	0.116	0.063	0.066	0.306
	KIR3DL2	0.16	**	0.124	0.053
	KIR3DL3	0.123	*	0.096	0.135
	KIR2DS4	0.118	0.058	0.089	0.165
Dendritic cell	HLA-DPB1	0.318	***	0.271	***
	HLA-DRA	0.366	***	0.325	***
	HLA-DPA1	0.319	***	0.276	***
	CD1C	0.168	**	0.139	*
	NRP1	0.107	0.084	0.037	0.57
	ITGAX	0.325	***	0.287	***

*p < 0.05, **p < 0.01, ***p < 0.001.

Table 2
Correlation analysis between CD44 and gene markers of different types of T cells in TIMER.

Description	Gene markers	SARC			
		None		Purity	
		Cor	P	Cor	P
Th1	TBX21	0.192	**	0.147	*
	STAT4	0.203	**	0.138	*
	STAT1	0.266	***	0.246	***
	TNF	0.298	***	0.258	***
	IFNG	0.188	**	0.143	*
Th1-like	HAVCR2	0.406	***	0.383	***
	IFNG	0.188	**	0.143	*
	CXCR3	0.283	***	0.251	***
	BHLHE40	0.282	***	0.27	***
	CD4	0.367	***	0.331	***
Th2	STAT6	−0.038	0.537	−0.014	0.832
	STAT5A	0.147	*	0.122	0.056
Treg	FOXP3	0.2	***	0.154	***
	CCR8	0.309	***	0.277	***
	TGFB1	0.384	***	0.34	***
Resting Treg	FOXP3	0.2	***	0.154	***
	IL2RA	0.373	***	0.338	***
Effector Treg T-cell	FOXP3	0.2	***	0.154	***
	CCR8	0.309	***	0.277	***
	TNFRSF9	0.352	***	0.308	***
Effector T-cell	CX3CR1	0.056	0.365	0.024	0.707
	FGFBP2	−0.027	0.668	−0.081	0.207
	FCGR3A	0.35	***	0.298	***
Native T-cell	CCR7	0.16	***	0.125	**
	SELL	0.179	**	0.123	0.055
Effector memory T-cell	DUSP4	0.108	0.082	0.08	0.216
	GZMK	0.237	***	0.198	**
	GZMA	0.239	***	0.196	**
Resident memory T-cell	CD69	0.201	**	0.178	**
	CXCR6	0.256	***	0.226	***
	MYADM	−0.03	0.629	0.012	0.857
General	CCR7	0.16	***	0.125	**
Memory T-cell	SELL	0.179	**	0.123	0.055
	IL7R	0.242	***	0.201	**
Exhausted T-cell	HAVCR2	0.406	***	0.383	***
	LAG3	0.077	0.219	0.041	0.527
	CXCL13	0.267	***	0.234	***
	LAYN	0.151	*	0.103	0.108

*p < 0.05, **p < 0.01, ***p < 0.001.

of the Wnt/β-catenin signaling pathway (Fig. 4). The PPI network of CD44 was generated using STRING, including EGFR, CD74, EGF, etc., and the expression-pattern-of-in-sarcoma Genes positively correlated with CD44 including PLAU, LGALS3, ANXA1etc. (Fig. 5), and negatively correlated with CD44 include LPHN1, EMILIN3 and NTN3 etc. (Supplementary Fig. 5b).

The tumor microenvironment (TME) is an integral part of cancer, and the discovery of new targets within TME could help guide and improve the role of various cancer therapies, particularly immunotherapies that work by enhancing the host's anti-tumor immune response [11]. Previous studies have shown that GDF-15 might facilitate ovarian cancer immune escape by interacting with CD44 in DCs to inhibit their function [41]. Data from Klement et al. indicated that myeloid and tumor cell-expressed OPN/CD44 acted as an immune checkpoint to suppress T cell activation and conferred host tumor immune tolerance [42]. A strong association between tumor-infiltrating CD4+ cells and the presence of CD44 expression in osteosarcoma samples was observed [43]. We found a positive correlation of CD44 with B cells, CD8+ T cells, CD4+ T cells, Macrophage, Neutrophils, and Dendritic Cell by database analysis, with the common immune checkpoint CD274, CTLA 4, CD47, and CD276, so we speculated that osteosarcoma patients with higher CD44 expression may be associated with strong immune cells, undeniably, some relationship between CD44 and osteosarcoma immune infiltration was added in our study. Accordingly, CD44 can be used as an immune checkpoint or immunotherapy target for osteosarcoma, which provides ideas for the immunotherapy of osteosarcoma.

5. Conclusion

In summary, we have demonstrated that CD44 Promotes the proliferation and invasion of osteosarcoma acts through the Wnt/β-catenin signaling pathway, and CD44 is associated with the immune infiltration of osteosarcoma, which will provide new ideas for the treatment of osteosarcoma. What is insufficient is that our experiment on CD44 affecting the immune infiltration of osteosarcoma is still ongoing, and no richer conclusion is being reached, which will be the focus of our future work, and strive to further solve the answer to this puzzle.

Author contributions

HJ and LK designed the experiments, YW and ZH performed in vitro studies, WK performed nude mouse xenograft assay, JQ and YJ performed statistical analysis of the relevant information on the database. HJ wrote the manuscript, LK reviewed and corrected the manuscript, HJ and LK arranged the funds. All authors approved the final version of the manuscript.

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.jbo.2023.100487.

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