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## Research Article

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# **Identification of Driver Genes and Key Pathways of Osteosarcoma Shows K-7174 as a Novel Anti-osteosarcoma Drug by inhibiting VCAM1**

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## **Abstract**

### **Background**

Osteosarcoma is one of the leading causes in cancer-related death of children and adolescents. However current standard therapeutic strategy, surgery combined with neoadjuvant chemotherapy is very limited in effects. As big data mining and analysis using bioinformatics method has been applied in the diagnosis and treatment of many cancers, we want to use bioinformatic combined with experimental assays to found new molecular targets and test new drug for osteosarcoma.

### **Methods**

The gene chip of osteosarcoma samples constructed by Richter GH et al were downloaded from the Gene Expression Omnibus (GEO) database, Gene Ontology analysis (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis were performed on differential expression genes which screened by bioinformatics methods.

Protein-protein interaction network was constructed by using STRING database to find hub gene. Combined with pertinent literature, genes of interest and corresponding drug was selected. Series of experiments were performed on the osteosarcoma cell lines in vitro, involved cell viability test, colony formation assay, migratory and invasive tests, western blot as well.

## **Results**

A total of 1069 DEGs were obtained from data, including 375 up-regulated genes and 694 down-regulated genes. Differentially expressed genes mainly involve biological processes such as cellular immune function, such as interferon-gamma-mediated signaling pathway and antigen processing and presentation of peptide. Among top 20-ranked degree hub gene evaluated by PPI network, vascular cell adhesion molecule-1 (VCAM1) was picked out. An VCAM1 inhibitor K-7174 was treated in U2OS and MG63 cell lines. In vitro experiments have shown that K-7174 can inhibit the proliferation, migration and invasion of osteosarcoma, the protein expression of VCAM1 was also decreased by K-7174.

## **Conclusions**

VCAM1 could be a potential target for osteosarcoma and K-7174 promises to be a therapeutic drug after more nuanced evaluation in animal and clinical trials.

## **Keywords**

Osteosarcoma, Bioinformatics, VCAM1, K-7174, migration

## **Introduction**

Osteosarcoma which originated from primitive mesenchymal cells is a kind of primary malignant tumor. Most patients diagnosed with osteosarcoma are children and adolescents under 20 years old. Despite its low morbidity, the mortality rate and incidence of metastasis of osteosarcoma are extremely high [1]. In the past few decades, surgeries were the main methods for the treatment of osteosarcoma, however the overall outcome is poor with high disability rate. With new progress in medical technology, surgery combined with neoadjuvant chemotherapy has become the standard treatment

[2]. Chemotherapy drugs mainly include adriamycin, methotrexate, ifosfamide and cisplatin. Since the combination of surgery and neoadjuvant chemotherapy, the 5-year survival rate for OS patients has been improved [3]. However, osteosarcoma prognosis is still suboptimal, distant metastasis especially lung metastasis is common in early stage and patients are prone to relapse after treatment [4]. The long-term use of chemotherapy drugs is often accompanied by drug toxicity also cause adverse effects on multiple organs in patients. Therefore, it is very important to identify new treatment methods of osteosarcoma.

In recent years, with the development and application of bioinformatics and microarray technology, Bioinformatics data-mining of gene has been applied in the diagnosis and treatment of many diseases [5]. And it is possible for researchers to better understand the mechanism of osteosarcoma at molecular level. Many bioinformatics analysis techniques based on genomics, transcriptomics and proteomics have revealed the high genetic heterogeneity of osteosarcoma [6]. Many genes, such as EGR1, CXCL10 and MYC have been found to be associated to the progression and metastasis of osteosarcoma [7]. Since database updated and new data uploaded, there has been a continual increase in the number of new targets gene available for osteosarcoma treatment, which suggests that additional genetic factors remain to be found.

In this study, bioinformatics method was used to further investigate OS microarray data. A total of 1069 differential expression genes (DEGs) between metastatic human osteosarcoma tissue and non- metastatic human osteosarcoma tissue were selected by screening. Gene Ontology (GO) analysis and pathway enrichment were performed for functional and pathway analysis of DEGs, and protein-protein interaction (PPI) network was also constructed for DEGs. Finally, hub genes with top 20 ranked degree in the PPI network were picked out. Among top 20-ranked hub genes, there were various gene that had been known to development, progression and relapse of osteosarcoma, such as TP53 and MYC. We notice vascular cell adhesion molecule-1 (VCAM1) which has been reported closely associated with distant metastasis and poor

prognosis of patients with breast and colorectal cancer. However, VCAM1 was not fully understood in tumorigenesis and metastasis of osteosarcoma. And it was still unknown if VCAM1 could be a potential therapeutic target of osteosarcoma. So, aiming at VCMA1 we used K-7174 as potential drug in subsequent in-vitro experiments. In osteosarcoma cell lines U2OS and MG63, we found that k-7174 could inhibit the proliferation, migration and invasion of osteosarcoma cells by CCK-8, colony formation, scratch and Transwell assay. Further western blot confirmed that VCAM1 could indeed inhibit the expression of VCAM1 in osteosarcoma cell. Our results showed that k-7174 could effectively inhibit the progression and metastasis of osteosarcoma in vitro, and VCAM1 could become a potential treatment target for osteosarcoma.

## **Methods**

### Microarray Data

The gene expression profiles of osteosarcoma named GSE73166 were downloaded from Gene Expression Omnibus database (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE73166>). Microarray Data contain samples from metastatic and non-metastatic osteosarcoma patients.

### Identification of DEGs

GeneSpring software (version 11.5) was used to analyze raw data from microarray and identify DEGs. hierarchical clustering analysis was used to classify and identify the group metastatic and non-metastatic osteosarcoma samples. The quality control of probe in GeneSpring was performed using virtue of principal component analysis. Probes with intensity values smaller than 20th were discarded by choosing “filter probe sets by expression” option. T test with a P value  $<0.05$  and a change  $>2$ -fold as a statistically significance was used to identify DEGs.

### GO and Pathway Enrichment Analysis of DEGs

The main goal of KEGG (Kyoto Encyclopedia of Genes and Genomes) is to establish

links from genomic information to the functions of cells and organisms. While the GO is mainly used to integrate genes with their products from different species, analyze relevant biological process and molecular function as well. In our study, the function of DEGs were analyzed using DAVID database which considered to be a comprehensive set of functional annotation tool of various gene. GSEA (Gene Set Enrichment Analysis) was used to determine statistical significance and conduct GO and KEGG pathway enrichment.

#### PPI Network Construction and Modules Selection

STRING (Search Tool for Retrieval of Interacting Genes) was used to provided PPI analysis. Then, hub genes and modules were identified by Cytoscape software with MCODE (Molecular Complex Detection). Function and pathway enrichment analysis of DEGs in modules was conducted to identify the hub genes and their degrees subsequently.

#### Cell culture, agents and antibodies

U2OS and MG63 was purchased from Shanghai Zhong Qiao Xin Zhou Biotechnology Co., Ltd. Cells were cultured with Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) at 37 °C in a 5% CO<sub>2</sub> atmosphere. K-7174 was purchased from MedChemExpress LLC, and drugs are in the form of solutions in water reserved in -20°C. The antibodies of VCAM1 and GAPDH were purchased from Abcam Plc.

#### Cell viability assay

Osteosarcoma cells with a density of 1000 cells/well were grown in 96-well plates, and K-7174 with designed concentration was treated on cells for 24h or 48h. Then CCK-8 was added to each well, incubated at 37 °C for 2 h were following. Next, the optical density (OD) values of samples were measured by using an EnSpire plate reader (PerkinElmer) when light absorbance was fixed at 450 nm. The cells viability was calculated by the following equation: viability = (the OD values of treatment groups/the

OD values of control group)  $\times$  100%.

#### Colony formation assay

Osteosarcoma cells were plated at a density of  $1 \times 10^3$  cells/well in 6-well plates and treated with 20 $\mu$ M K-7174 for 24h. Then, previous medium was replaced by fresh culture medium and cells were incubated for another 7 days. When colonies were visible, they were washed with PBS and stained with 1% crystal violet for 15 min. Then photographing and counting of colonies were performed.

#### Scratch Assay

Osteosarcoma cells were cultured in 6-well plates. When 80–90% of the plate bottom was covered by the cells, a 1000 $\mu$ L pipette tip was used to make an artificial wound on bottom of plate. The detached cells were washed with PBS, and 24h was waiting for scratch healing under 20 $\mu$ M K-7174 or not. The results of cells migration were observed and the wound was photographed, and the area of wound and repaired region were measured with image J software.

#### Transwell assay

The invasion of osteosarcoma cells was performed using Transwell chambers of which upper surface coated with Matrigel (2.5 mg/L). After osteosarcoma cells incubated with 20 $\mu$ M K-7174 or not for 24h,  $1 \times 10^5$  cells were collected in 200  $\mu$ L medium without FBS and added to upper chamber, meanwhile 800  $\mu$ L medium with 20% FBS was added to the lower chamber. After 24h, cells on the upper surface were wiped away and those on the lower surface were stained with 0.1% crystal violet. Photomicrographs were taken and migrated cells was counted in three random microscope fields.

#### Western blot

Cells were treated with 20 $\mu$ M K-7174 or not for 24h in advance. The total protein extraction was performed using RIPA Buffer. BCA protein assay was used to measure protein concentrations. Premixed with 5  $\times$  loading buffer, proteins were separated by

10% SDS-PAGE and transferred to PVDF membranes. After blocked with FBS, the membranes were incubated with the respective primary antibodies overnight. After washed by TBS-T, HRP conjugated secondary antibody was then used to chemiluminescence.

#### Statistical analysis

GraphPad Prism 8.0 was used to make statistical analysis and draw statistical chart. The data were analyzed using the GraphPad Prism 8.0 program (GraphPad Software, Inc., San Diego, CA). An independent-samples T test was selected to analyze data. Data are expressed as the mean  $\pm$  standard deviation of three repeated experiments. P-values just below 0.05 was considered to be statistically significant.

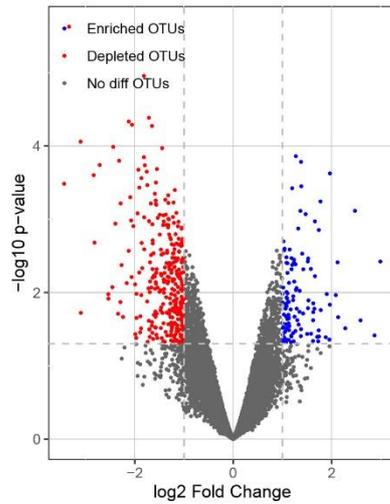
### Results

#### **Differential expression analysis for gene expression.**

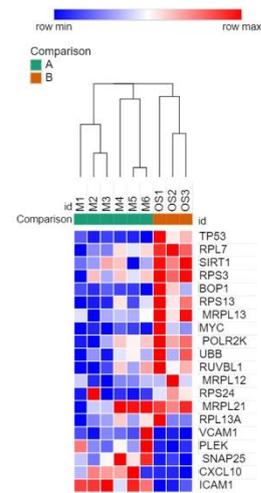
A total of 1069 differential expression genes or DEGs were identified, of which 375 genes were upregulated and 694 genes were downregulated. The volcano map of differential genes is shown in **Figure 1A**

**Figure 1. A** The volcano map of DEGs. **B** The heat map of top 20 ranked hub genes. **C** and **D** Gene Set Enrichment Analysis of DEGs.

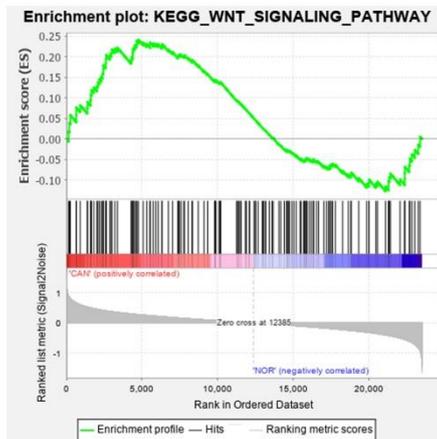
A



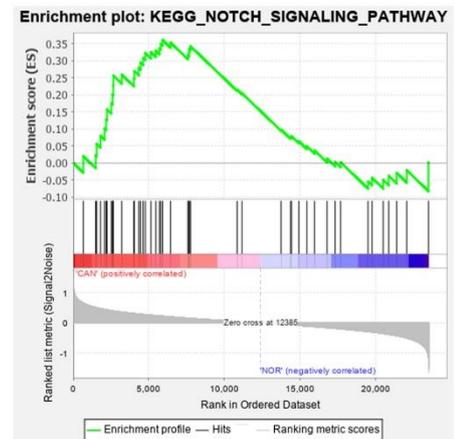
B



C



D



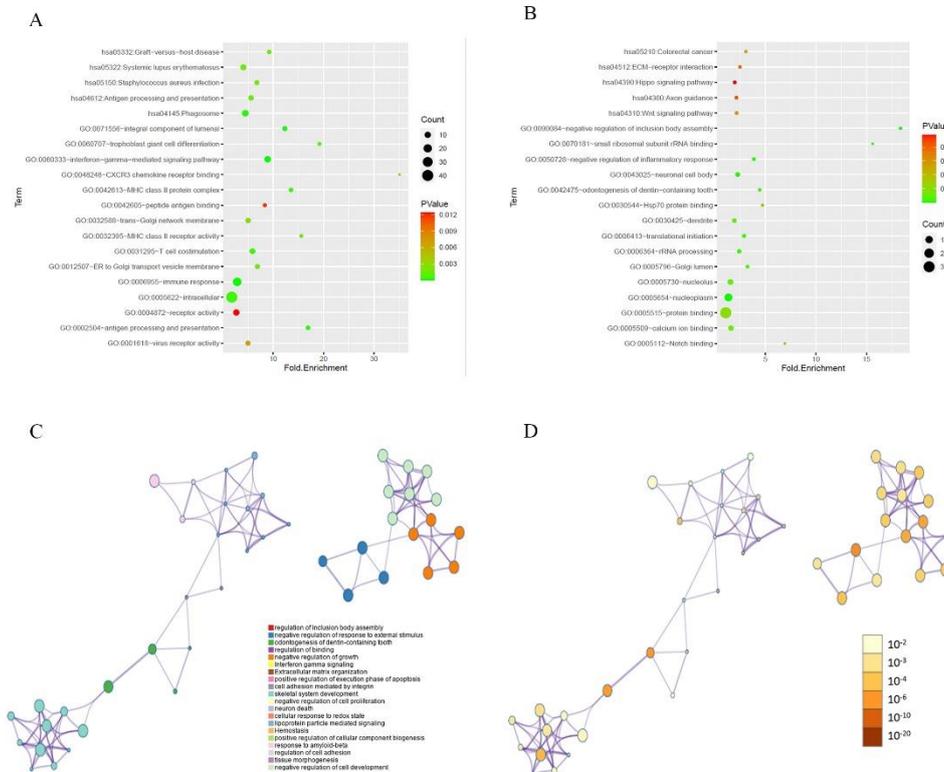
**Table 1.** Detailed information of the top 20 ranked hub genes

Gene symbol	Degree	Betweenness Centrality	Gene symbol	Degree	Betweenness Centrality
TP53	116	0.2570837	RPL13A	31	0.00904668
MYC	79	0.1011128	RPS24	31	0.00714065
VCAM1	70	0.08934061	PLEK	31	0.0342334
UBB	45	0.0333143	RPL7	30	0.00603982
ICAM1	43	0.0362967	RUVBL1	30	0.016056
RPS3	41	0.02851175	RPL21	29	0.00959651
RPS13	37	0.01183977	RPL12	29	0.00290821
CXCL10	35	0.01336454	POLR2K	28	0.01309739
SIRT1	34	0.03668602	SNAP25	28	0.03682958
BOP1	33	0.01723852	RPL13	27	0.00243803

## Functional and pathway enrichment analysis of DEGs

All DEGs were uploaded to the DAVID online tool for further GO functional analysis and KEGG pathway enrichment analysis. As shown in **Table 2**, **Figure 1C** and **D**, **Figure 2A**, **B**, **C** and **D**, the results showed that upregulated DEGs were mainly associated with cellular immune function, such as interferon-gamma-mediated signaling pathway and antigen processing and presentation of peptide. Furthermore, the down-regulated genes were also associated inflammation reaction and immune response, including ECM-receptor interaction and regulation of inflammatory response. GSEA results also reveal DEGs mainly enriched in Wnt and Notch signal pathway.

**Figure 2.** **A** and **B** Functional and pathway enrichment analysis of upregulated genes(**A**) and downregulated Genes(**B**). The size of node is proportional to the number of input genes fall into that term, and the color of node represent its cluster identity. **C** and **D** Network of enriched term, colored by cluster identity or p-value.



**Table 2.** Functional and pathway enrichment analysis of upregulated and downregulated Genes

	Term	Count	%	P Value
Up-regulation	GO:0060333~interferon-gamma-mediated signaling pathway	11	2.997275 2	3.80E-07
	GO:0006955~immune response	21	5.722070 84	4.80E-05
	GO:0002504~antigen processing and presentation of peptide or polysaccharide antigen via MHC class II	5	1.362397 82	1.76E-04
	GO:0031295~T cell costimulation	8	2.179836 51	4.03E-04
	GO:0060707~trophoblast giant cell differentiation	4	1.089918 26	0.001009 11
	GO:0071556~integral component of luminal side of endoplasmic reticulum membrane	6	1.634877 38	1.09E-04
	GO:0042613~MHC class II protein complex	5	1.362397 82	4.43E-04
	GO:0005622~intracellular	40	10.89918 26	4.53E-04
	GO:0012507~ER to Golgi transport vesicle membrane	6	1.634877 38	0.001735 36
	GO:0032588~trans-Golgi network membrane	7	1.907356 95	0.002684 21
	GO:0032395~MHC class II receptor activity	4	1.089918 26	0.001920 49
	GO:0048248~CXCR3 chemokine receptor binding	3	0.817438 69	0.002803 65
	GO:0001618~virus receptor activity	6	1.634877 38	0.006848 22
	GO:0042605~peptide antigen binding	4	1.089918 26	0.011745 47
	GO:0004872~receptor activity	10	2.724795 64	0.012353 95
	hsa04145:Phagosome	11	2.997275 2	1.52E-04
	hsa04612:Antigen processing and presentation	7	1.907356 95	0.001410 95
	hsa05322:Systemic lupus erythematosus	9	2.452316 08	0.001461
	hsa05150:Staphylococcus aureus infection	6	1.634877 38	0.001753 03
	hsa05332:Graft-versus-host disease	5	1.362397 82	0.001893 42

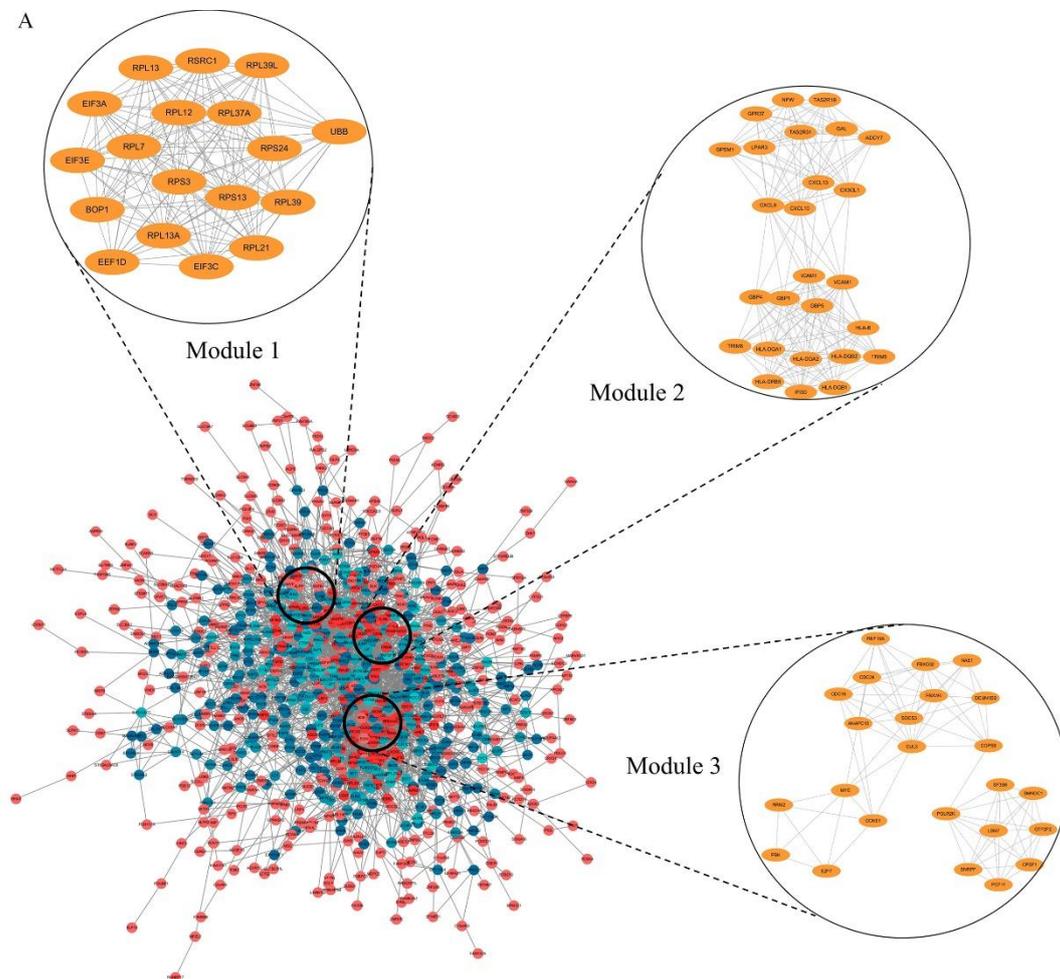
Down-regulation	GO:0090084~negative regulation of inclusion body assembly	6	0.878477 31	8.00E-06
	GO:0050728~negative regulation of inflammatory response	10	1.464128 84	0.001061 59
	GO:0006413~translational initiation	13	1.903367 5	0.001777 31
	GO:0006364~rRNA processing	17	2.489019 03	0.001815 3
	GO:0042475~odontogenesis of dentin-containing tooth	8	1.171303 07	0.001981 17
	GO:0005654~nucleoplasm	122	17.86237 19	2.15E-04
	GO:0043025~neuronal cell body	23	3.367496 34	5.55E-04
	GO:0005796~Golgi lumen	10	1.464128 84	0.003659 02
	GO:0005730~nucleolus	43	6.295754 03	0.004142 08
	GO:0030425~dendrite	21	3.074670 57	0.005990 36
	GO:0070181~small ribosomal subunit rRNA binding	4	0.585651 54	0.001616 44
	GO:0005509~calcium ion binding	37	5.417276 72	0.005152 13
	GO:0005515~protein binding	309	45.24158 13	0.010109 94
	GO:0005112~Notch binding	4	0.585651 54	0.018569 97
	GO:0030544~Hsp70 protein binding	5	0.732064 42	0.020330 07
	hsa05210:Colorectal cancer	7	1.024890 19	0.024808 78
	hsa04310:Wnt signaling pathway	11	1.610541 73	0.029003 73
	hsa04512:ECM-receptor interaction	8	1.171303 07	0.038955 83
	hsa04360:Axon guidance	10	1.464128 84	0.042062 39
	hsa04390:Hippo signaling pathway	11	1.610541 73	0.048956 95

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## Module Screening from the PPI Network

the previous 1069 DEGs were further analyzed with the PPI network, and hub genes with top 20 ranked degree in the PPI network were screened based on the STRING database. As listed in **Table 1**, hub genes including TP53, MYC, VCAM1, ICAM1, RPS3, UBB, RPS13, CXCL10, SIRT1, BOP1 etc. And the heat map of hub genes expression was drawn as shown in **Figure 1B**. MCODE analysis were further performed, top 3 significant modules were selected as shown in **Figure 3 and Table 3**.

**Figure 3.** Top 3 modules from the protein interaction network.



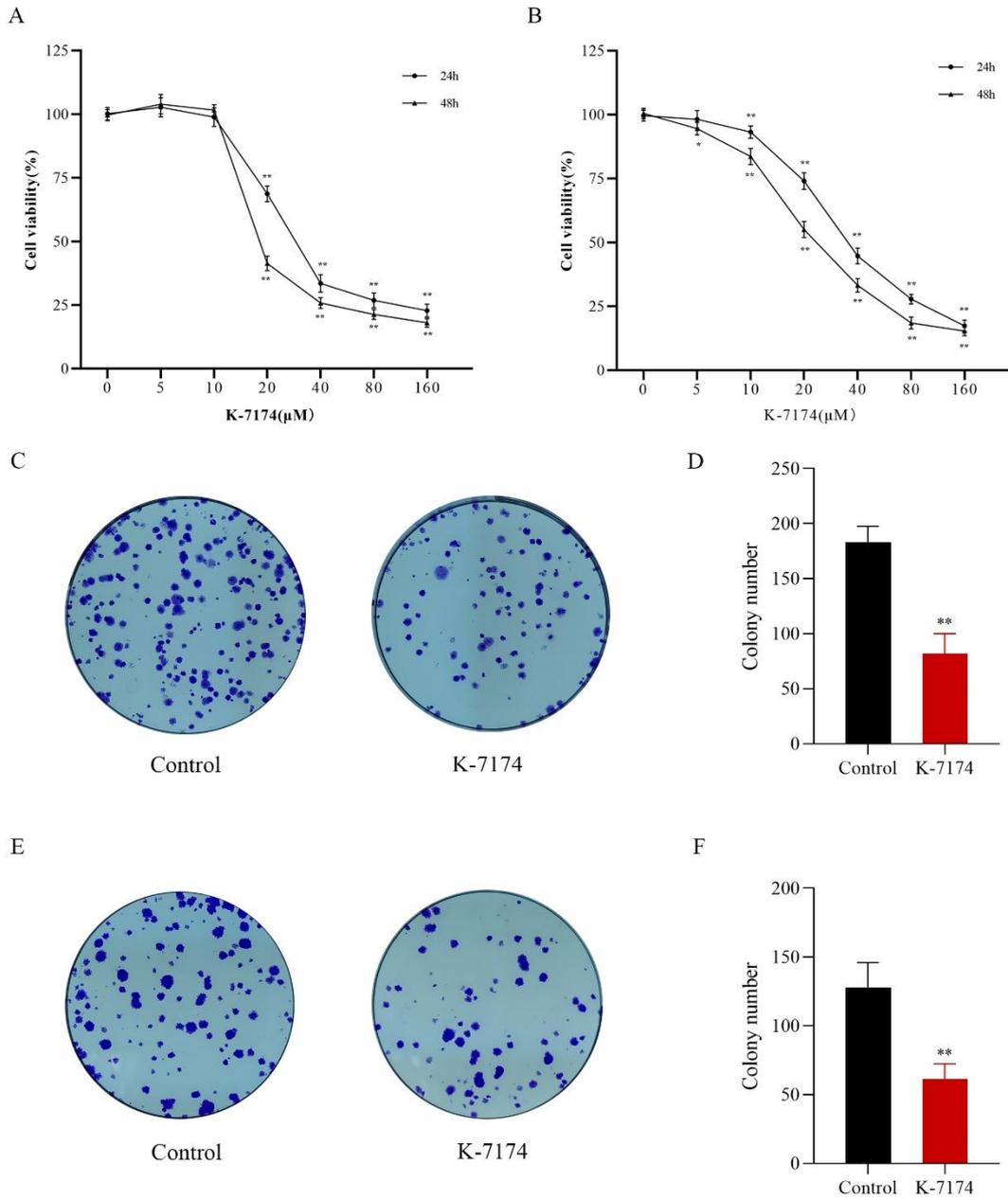
**Table 3.** Functional and enrichment analysis of the module genes

Modul es	Term	Cou nt	PVal ue	FDR	Genes
	GO:0006413~translational initiation(BP)	13	3.17 E-22	3.78E- 19	RPL13, RPL39, RPS3, EIF3C, EIF3A, RPL7, RPL13A, RPL21, EIF3E, RPS13, RPL37A, RPL12, RPS24
Modul e 1	GO:0000184~nuclear-transcribed mRNA catabolic process, nonsense-mediated decay(BP)	11	4.04 E-18	4.81E- 15	RPL7, RPL13A, RPL13, EIF3E, RPL21, RPS13, RPL37A, RPL12, RPL39, RPS3, RPS24
	GO:0006614~SRP-dependent cotranslational protein targeting to membrane(BP)	10	8.51 E-17	1.33E- 13	RPL7, RPL13A, RPL13, RPL21, RPS13, RPL37A, RPL12, RPL39, RPS3, RPS24
	GO:0060333~interferon-gamma-mediated signaling pathway(BP)	12	8.16 E-21	1.06E- 17	HLA-DQB1, VCAM1, ICAM1, HLA-DQB2, TRIM5, TRIM8, HLA- DRB5, IFI30, HLA-B, HLA-DQA2, HLA-DQA1, GBP1
Modul e 2	GO:0071556~integral component of luminal side of endoplasmic reticulum membrane(CC)	6	3.69 E-10	3.64E- 07	HLA-DQB1, HLA-DQB2, HLA- DRB5, HLA-B, HLA-DQA2, HLA- DQA1
	GO:0006955~immune response(BP)	10	3.37 E-09	4.36E- 06	HLA-DQB1, HLA-DQB2, CXCL13, CXCL9, HLA-DRB5, HLA-B, CX3CL1, HLA-DQA2, HLA-DQA1, CXCL10
	GO:0005654~nucleoplasm(CC)	17	7.07 E-09	7.01E- 06	POLR2K, E2F7, LSM7, ANAPC10, CDC16, CDC34, SF3B6, COPS8, CUL3, CCNE1, PCF11, RRM2, GTF2F2, FBXO32, SNRPF, MYC, CPSF1
Modul e 3	GO:0000398~mRNA splicing, via spliceosome(BP)	7	4.17 E-07	5.37E- 04	PCF11, POLR2K, GTF2F2, LSM7, SF3B6, SNRPF, CPSF1
	hsa04120:Ubiquitin mediated proteolysis(PATHWAY)	5	2.93 E-04	0.2888 87	CUL3, SOCS3, ANAPC10, CDC16, CDC34

### The anti-proliferation effect of K-7174 in osteosarcoma cell lines

Among these hub genes, we noticed VCAM1 which were frequently associated with tumor progression and metastasis were high in degree. Thus, we want to demonstrate if VCAM1 could be an anti-cancer drug target for Osteosarcoma. So, K-7174, one VCAM1 inhibitor was selected to perform following experiments. To investigate the effect of k-7174 on the proliferation of human osteosarcoma cells U2OS and MG63, the cell lines were treated with k-7174 in gradient concentration for 24 or 48 hours in vitro, and CCK8 method was used to detect cell viability. As shown in **Figure 4 A and B**, when the concentration of K-7174 were 20  $\mu\text{M}$  or higher, the U2OS cell viability were markedly decreased,  $68.7\% \pm 3.1\%$  (mean $\pm$ SD) for 24h or  $41.4\% \pm 2.9\%$  for 48h. However, in MG63 cell lines, 10  $\mu\text{M}$  or 5  $\mu\text{M}$  K-7174 could represent obvious inhibitory effect,  $93.2\% \pm 2.4\%$  for 24h or  $94.6\% \pm 2.5\%$  for 48h in cell viability respectively. Thus, 20  $\mu\text{M}$  of K-7174 were selected for following experiments. Furthermore, we further analyzed the effect of k-7174 on the proliferation of U2OS and MG63 by colony formation assay. As shown in **Figure 4C, D, E and F**, compared with the control group, the number of colonies formed by U2OS and MG63 cells were obviously decreased after treated K-7174. These results further demonstrated that K-7174 did have the ability to inhibit the proliferation of osteosarcoma cells.

**Figure 4. A and B** Cell proliferation ability was assessed after U2Os or MG63 cell were treated with K-7174 after 24h or 48h using CCK-8 assay. **C, D, E and F** The inhibiting effects on colony formation of U2OS or MG63 by treated 20 $\mu\text{M}$  K-7174 the colony numbers were calculated.

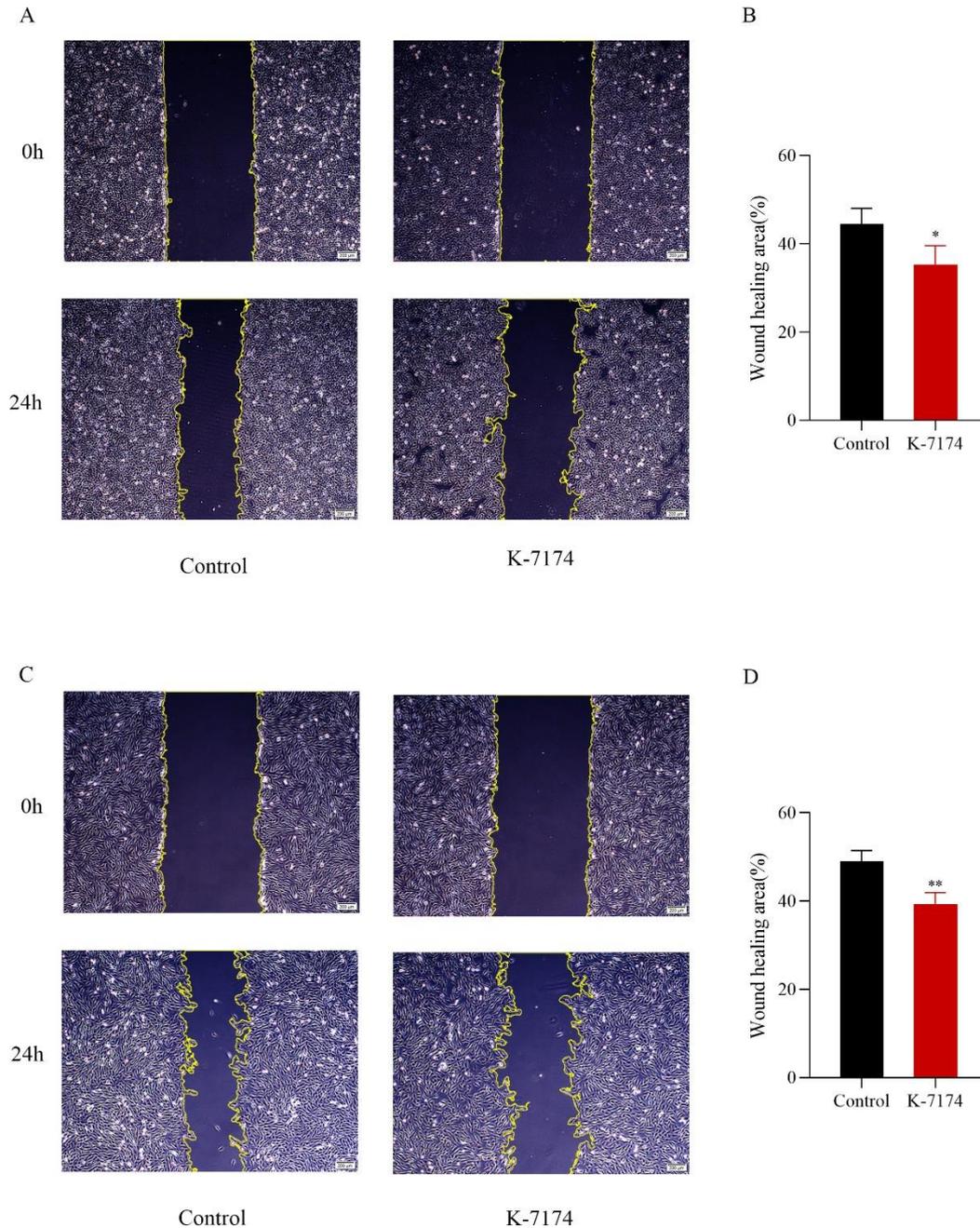


### The anti-migration effect of K-7174 in osteosarcoma cell lines

Tumor cells still have migration ability when cultured in vitro. Scratch assay is based on the wound healing model to identify the migration and movement characteristics of tumor cells in vitro. We performed Scratch assay to investigate the effect of K-7174 on migration ability of osteosarcoma cells U2OS and MG63. As shown in **Figure 5A, B, C and D**. After treatment with K-7174 for 24h, the percentage repair area of scratch in U2OS cell lines was decreased from  $44.5\% \pm 3.5\%$  to  $44.5\% \pm 3.5\%$  compare with control group. Similar results also found in MG63 cell lines treated with 24h K-7174. Thus, 20

$\mu\text{M}$  K-7174 had the ability to decrease the migration of osteosarcoma cell lines.

**Figure 5.** **A** and **C** Representative image of healing area of U2OS or MG63 cells at 0 and 24h after wound scratch when exposed to 20  $\mu\text{M}$  K-7174. **B** and **D** The results of wound repair inhibition under different treatment conditions were compared.

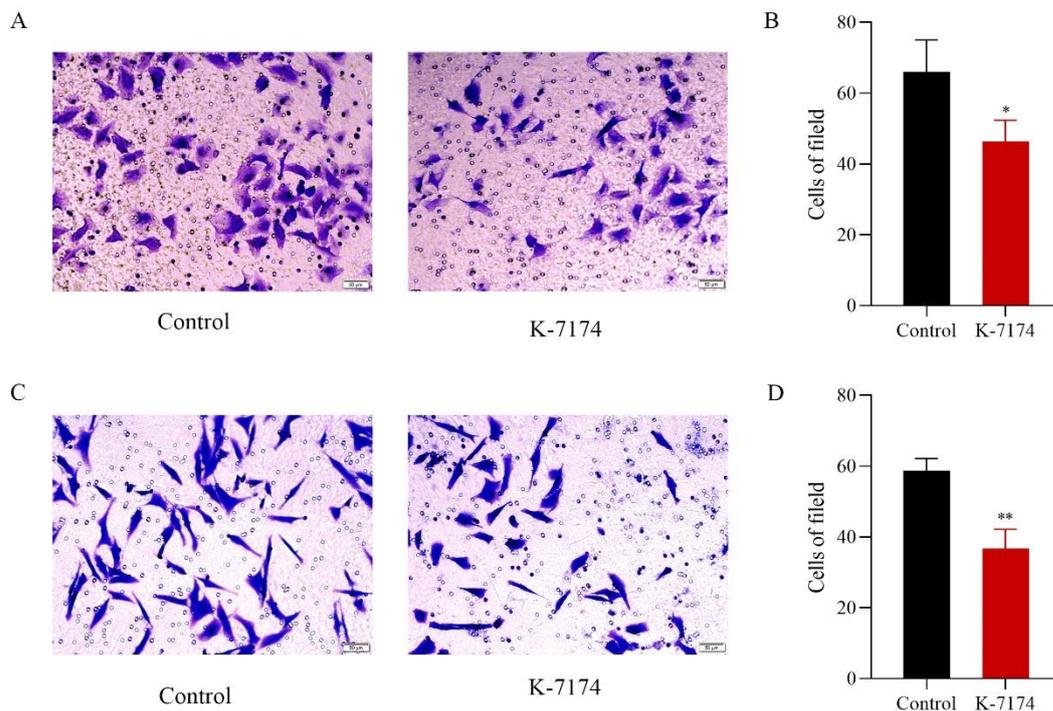


### The anti-invasion effect of K-7174 in osteosarcoma cell lines

Tumor cells still have high invasive property in vitro, The matrix glue in Transwell

chamber can simulate biomembrane, and the invasion ability of tumor cells can be determined by the number of cells that passing through chamber coated by matrix glue. We analyzed the effect of K-7174 on the invasion ability of U2OS and MG63 cell lines by Transwell assay. As shown in **Figure 6A, B, C and D** Compared with control group, 24h treatment with K-7174 obviously decrease number of cells passing through the chamber, from  $66.3\pm 8.5$  cells of field to  $46.3\pm 6.0$  cells of field in U2OS cell lines and from  $58.7\pm 3.5$  cells of field to  $36.6\pm 5.5$  cells of field in MG63 cell lines. Above results also demonstrated that  $20\ \mu\text{M}$  of K-7174 could effectively inhibit the invasion of osteosarcoma cell lines.

**Figure 6.** **A and C** Representative images of Transwell assay for invasion using Transwell chambers with Matrigel coating **B and D** The results of invasion inhibition under different treatment conditions were compared.

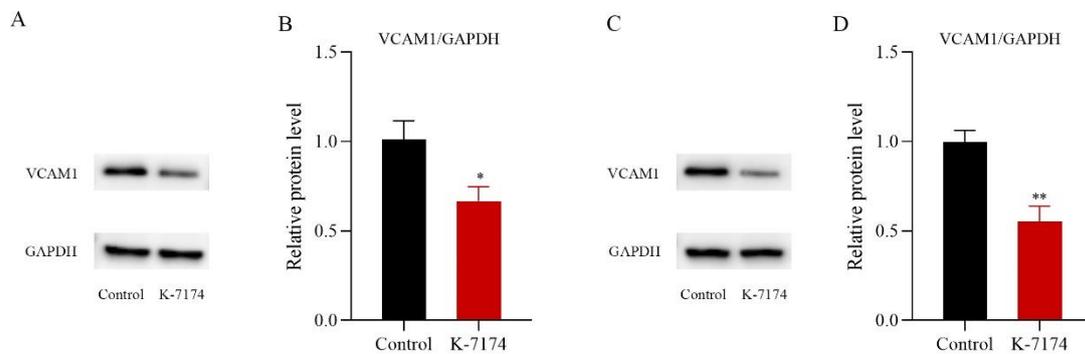


### **The expression of VCAM1 was inhibited by K-7174 in osteosarcoma cell lines**

As K-7174 was reported to be an inhibitor of VCAM1, but it was still unknown whether the expression of VCAM1 in osteosarcoma cell lines could be decreased by K-7174. Western blot assay was performed to gain further insight. As shown in **Figure 7A, B**,

**C** and **D**, the relative expression of VCAM1 in protein level was apparently inhibited by K-7174 in both U2OS and MG63,  $0.67\pm 0.08$  and  $0.55\pm 0.09$  respectively. Change in protein expression level indicated that K-7174 could inhibit expression of VCAM1 in osteosarcoma cell lines.

**Figure 7. A** and **C** Western blot results of VCAM1 proteins expression after 24 h treatment with K-7174 in U2OS and MG63 cells. **B** and **D** Relative optical density of proteins were analyzed and compared based on gray value of bands. Full-length gels and blots were included in supplement materials.



## Discussion

As a highly malignant primary bone tumor, osteosarcoma is characterized by immature osteoid tissue formed from tumor cells, osteosarcoma may occur in any bone of the body, although the femur and tibia are the most common location [8]. The poor prognosis and high mortality of osteosarcoma mainly due to early local diffusion and distant metastases of tumor. Local diffusion lead amputation to the only curative modality for surgery and cause disability and lung is the main target organ of osteosarcoma metastasis, 80% of patients with advanced osteosarcoma have lung metastasis and respiratory failure caused by that is the main cause of death of osteosarcoma patients [9]. Although the diagnosis and treatment of osteosarcoma have improved significantly in the past few decades, new treatment options like neoadjuvant chemotherapy and radiotherapy are constantly being explored in clinical practice, more than half of osteosarcoma patients still do not benefit from current treatments [10]. Therefore, the development of new target for therapy and effective drugs is urgent for

both patients and medical researchers.

The exact etiology of osteosarcoma remains unclear, both genetic and environmental factors reported to contribute osteosarcoma development. Long term exposure to radiation and alkylating agents can also promote the development of osteosarcoma [11]. Studies have shown that the pathogenesis of osteosarcoma is closely related to genes and genetic factors [12]. Therefore, clarifying the pathogenesis of OS from the molecular level is a challenge but also an opportunity. With its rapid development, gene chip is widely used in the diagnosis, treatment and prognosis evaluation of cancers[13]. As a new interdisciplinary subject in the field of life science, bioinformatics could deeply mine the microarray data of gene expression profile. On the one hand, it provides the possible molecular mechanism basis for the occurrence and development of diseases, on the other hand, it also provides feasible ideas for experimental research. Thus, based on the intersection of biology and information technology, we designed and implemented this study.

After tumor tissue from non-metastatic metastatic group compared with tumor tissue from metastatic group, A total of 1069 differential expression genes were identified, including 375 upregulated genes and 694 downregulated genes. The subsequent GO analysis revealed that DEGs mainly involved inflammatory- related and immune-related biological processes, such as interferon-gamma-mediated signaling pathway, antigen processing and presentation of peptide, and regulation of inflammatory response. Both immunity and inflammation process constitute the basic characteristic of tumor microenvironment, although their relationship is still vague [14]. It is speculated immune response is stronger in the early stage of tumor development, while inflammation response is stronger in the later stage. In the osteosarcoma, tumor cells not only control the recruitment of immune cells such as T-lymphocytes, mast cell and macrophages, but also regulate aggregation of inflammatory related factors like interleukin, interferon and transforming growth factor [15]. The dynamic state of both cell and molecules eventually establish a tolerant environment available for

development, drug resistant and metastasis of osteosarcoma. In previous study, some agents which affect immune function has been used in osteosarcoma patients such as Anti-GD2 antibody and Anti-IGF-1R, some clinical effects were also observed [16,17]. Furthermore, KEGG and GSEA analysis were performed, the result showed DEGs were highly enriched in phagosome, antigen processing and presentation, wnt signaling pathway and ECM-receptor interaction. Both the processing and presentation of antigen and formation of phagosome are the process of cellular immunity [18]. Wnt signaling pathway and ECM-receptor interaction which was considered to be related to epithelial mesenchymal transition and tumor metastasis, has also been observed that the enrichment in various tumors like breast cancer and glioma [19,20].

For the purpose of screening hub genes among DEGs, PPI network was used to analyze based on the STRING database. Top 20-ranked degree gene were pick out, TP53,MYC and VCAM1 were at the front end. There are many studies about TP53 and MYC in progression and development of osteosarcoma. TP53 is an important tumor suppressor gene linked to cell apoptosis and cell cycle. structural variations of TP53 gene are frequent in osteosarcoma, translocations within the first intron was believed to result the loss expression of TP53 and eventually lead to occurrence of osteosarcoma [21]. Animal models with a mutation in TP53 developing osteosarcoma also demonstrate that Tp53 essentiality [22]. Myc was reported to be related in OS development and metastasis by activating ERK pathways[23]. High expression of Myc increase cell proliferation, migration and clonogenicity in in osteosarcoma cell lines, and mutation of Myc gene were observed in more than 10% clinical cases of osteosarcoma[24]. While only only a few studies focused VCAM1 in osteosarcoma. VCAM1 is a 90-kDa glycoprotein which mainly expressed in the surface of endothelial cells[25]. Some cytokines, such as IFN and TNF could stimulate the expression of VCAM1 in other cells, fibroblasts, myoblasts and cancer cells were found express VCAM1 under inflammation and chronic conditions[26]. Six immunoglobulin (Ig)-like domains, one transmembrane domain and one cytoplasmic domain comprised the human VCAM1,and  $\alpha 4\beta 1$  integrin play mainly role in ligand binding with VCAM1. During

inflammatory responses, ligands binding to VCAM-1 and stimulating a series of inner cell affair, subsequently rolling and firm adhesion between endothelium and leukocyte are regulated[27]. Besides inflammation, VCAM-1 has also reported to be involved in tumor angiogenesis and metastasis. In breast cancer, micro-vessel density are relevant to serum VCAM-1[28]. In another study, VCAM-1 knockdown helped reduce tube formation ability of HUVEC that could be a potential angiogenic target[29]. Meanwhile, high VCAM-1 expression was also considered to be a hint for metastasis in various cancer cells. A comparative gene expression profile analysis of breast cancer cell suggest a lung metastatic activity with VCAM1 expression, and patients with early lymph node metastasis exhibited overexpression of VCAM1 in a study of colorectal cancer[30,31]. These related studies suggest that VCAM1 might be target of tumor metastasis.

In previous study, upregulation of VCAM-1 was observed to increase the migration of osteosarcoma cell[32]. Further study showed that CXCL13/CXCR5 axis activation facilitates the production of VCAM-1 via NF-kb pathway[33]. Thus, VCAM1 might be target of osteosarcoma progression and metastasis. We combined with relevant literature, and assumed that a VCAM1 inhibitor K-7174 had anti-tumor effect in osteosarcoma progression and metastasis. To verify our thoughts, a series experiments were performed. First of all, CCK-8 assay was used to detect the cytotoxicity of k-7174 on osteosarcoma cell line. For MG63, 5  $\mu$ M for 48h or 10  $\mu$ M for 24h could significantly inhibited the proliferation of tumor cell. And inhibitory effect exhibited a time and dose-dependent manner. While the inhibitory effect could only be observed at a relatively high dose in U2OS. The results of clone formation also confirmed the proliferation inhibition effect of k-7174 in osteosarcoma. K-7174 was first found have an inhibitory effect on binding to the GATA motifs in the VCAM-1 gene promoter region, and was used as a potential anti-inflammatory drug[34]. In another study, K-7174 was also found to help recover the production of erythropoietin inhibited by IL-1 in animal model[35]. So far, the only research related the application of K-7174 in

cancer treatment was from myeloma. K-7174 exhibits anti-myeloma effects by inhibiting class I histone deacetylases transcriptional activities, and the IC<sub>50</sub> of K-7174 varied within 5 $\mu$ M to 20  $\mu$ M in different myeloma cell lines [36]. Besides anti-proliferation effect, anti-migration and anti-invasion effect of K-7174 were detected by us. Results of scratch assay and transwell showed both migration ability and invasive capacity of osteosarcoma cells were markedly suppressed by K-7174. Further more treatment of deletion K-7174 did reduce the protein expression level of VCAM-1 in osteosarcoma cells. Although, K-7174 has not been reported used to inhibit the osteosarcoma metastasis, inhibition of VCAM-1 by a natural compound called DHTI had been proved effective in anti- metastasis of osteosarcoma cell lines[37]. AS a VCAM1 inhibitor, K-7174 does exist potential for preventing tumor metastasis.

## **Conclusion**

In this study, bioinformatics method was used to mine the osteosarcoma microarray data, and relevant DEGs was identified. Biological process, molecular function and signal pathway involved in the DEGs were also analyzed. The hub gene was obtained by functional enrichment. The gene of interest, VCAM1 was picked out from top 20-ranked degree hub gene. In vitro experiments on osteosarcoma cell lines showed that an inhibitor of VCAM1, k-7174 could inhibit the proliferation, migration and invasion of tumor cells, reducing the protein expression of VCAM1 as well. Our study suggested that VCAM1 could be used as a target in treatment of osteosarcoma, and k-7174 was a potential drug. Of course, more detailed experiments including animal studies and clinical trials need to be performed to further validate our idea.

## **Declaration**

### **Authors' contributions**

Conceived and designed the experiments: Zhaoyu Fu, Dong Zhu. Performed the Bioinformatic analysis: Zhaoyu Fu, Jing Yu, Bo Wu. Performed in vitro experiments: Zhaoyu Fu, Yan Liu, Long Cheng, Pengcheng Zhou. Wrote the paper: Zhaoyu Fu, Dong Zhu. All authors read and approved the final manuscript.

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**Availability of data and materials**

All the data in this research are accessible by connecting with the corresponding author.

**Ethics approval and consent to participate**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

**Consent for publication**

Not applicable.

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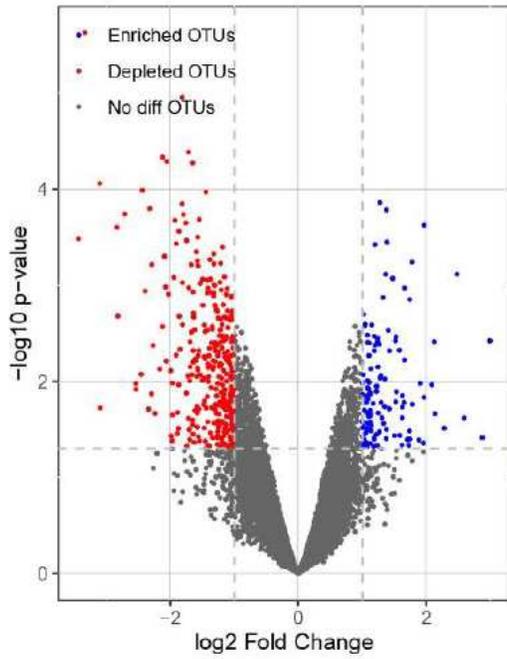
inhibits GATA binding activity, enhances hypoxia-inducible factor 1 binding activity, and restores indicators in an in vivo mouse model of anemia of chronic disease. *Blood*. 2004;104(13):4300-4307. doi:10.1182/blood-2004-04-1631

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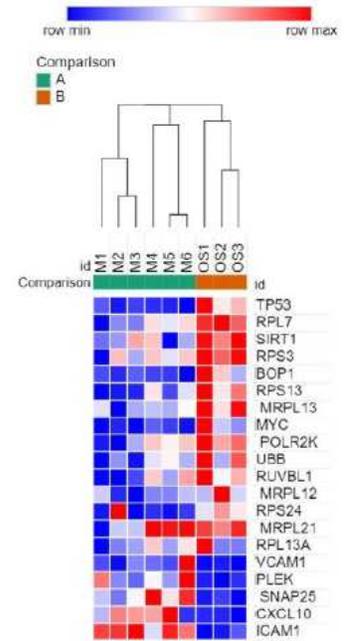
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# Figures

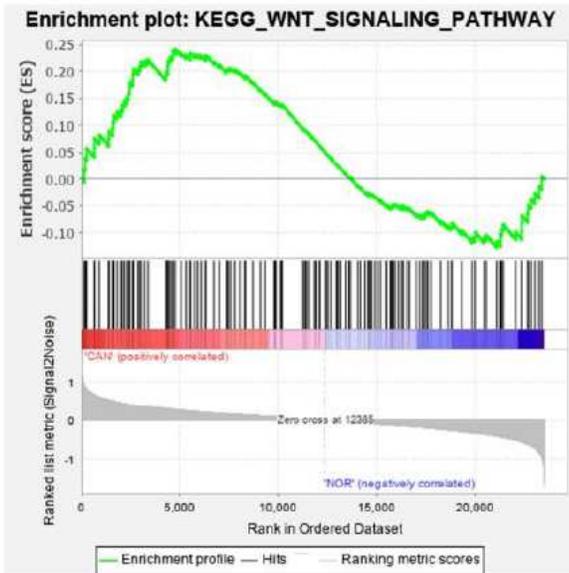
A



B



C



D

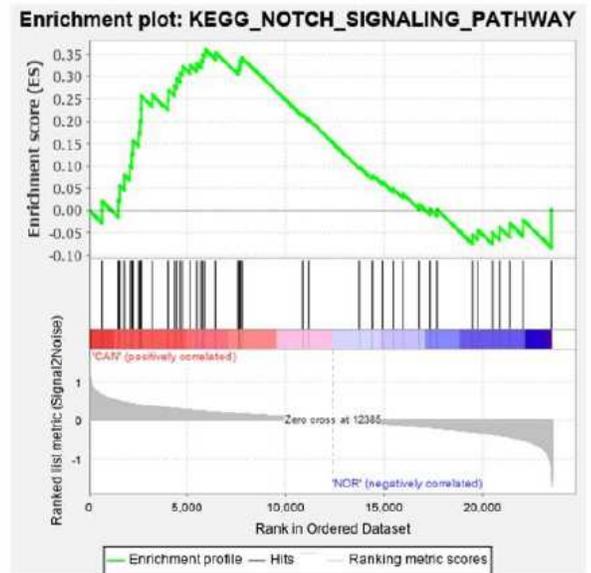


Figure 1

A The volcano map of DEGs. B The heat map of top 20 ranked hub genes. C and D Gene Set Enrichment Analysis of DEGs.



A

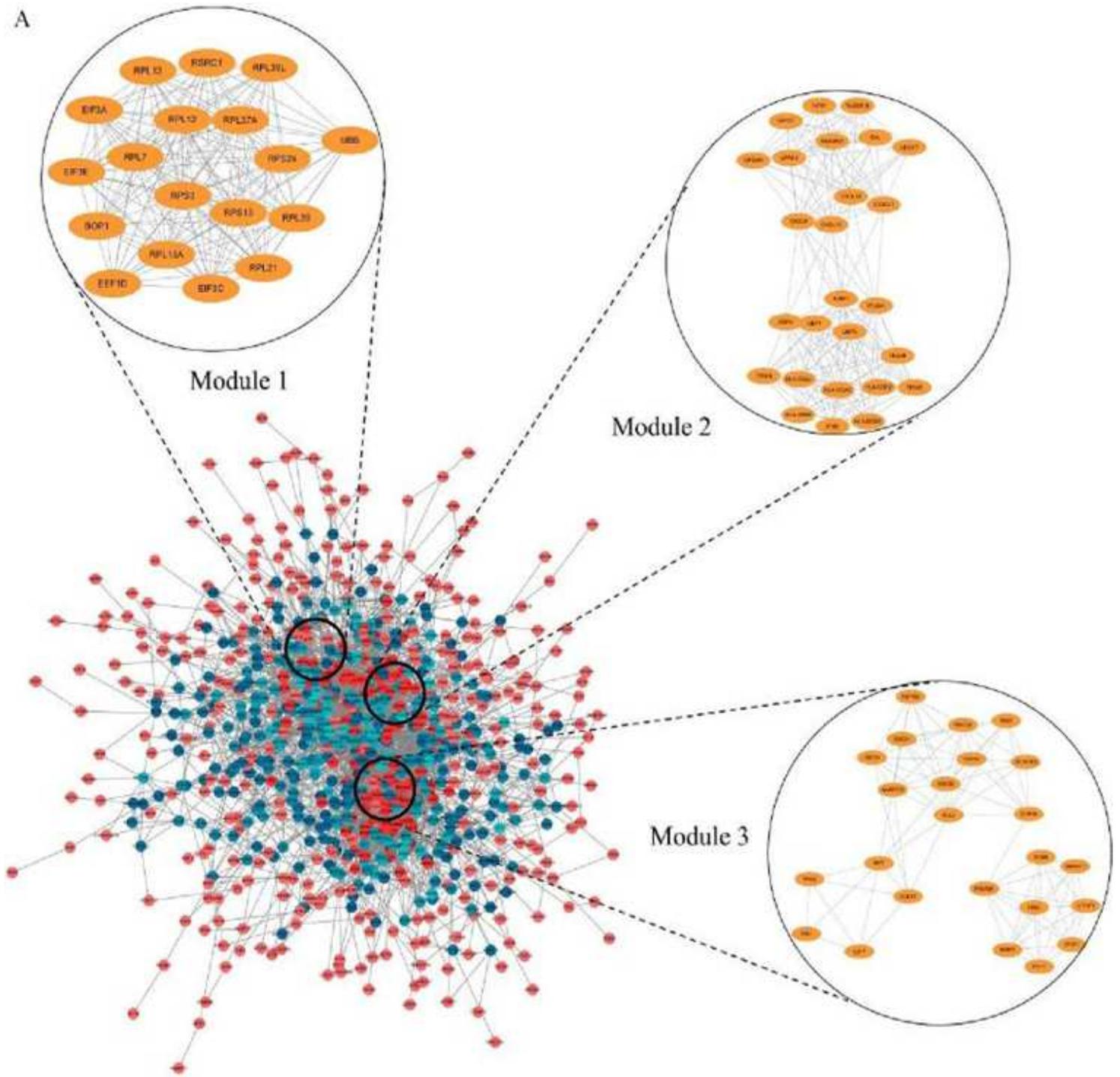
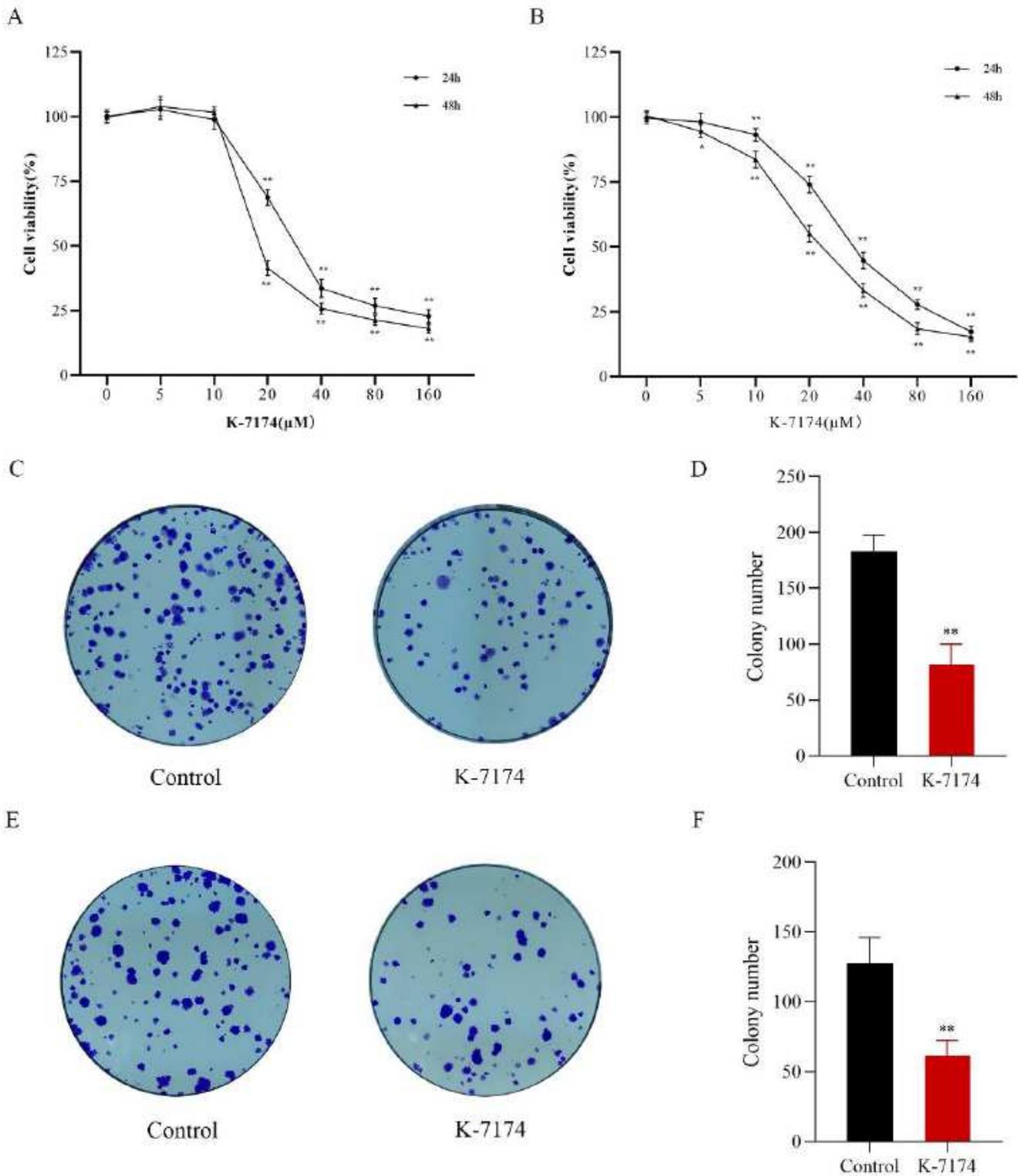


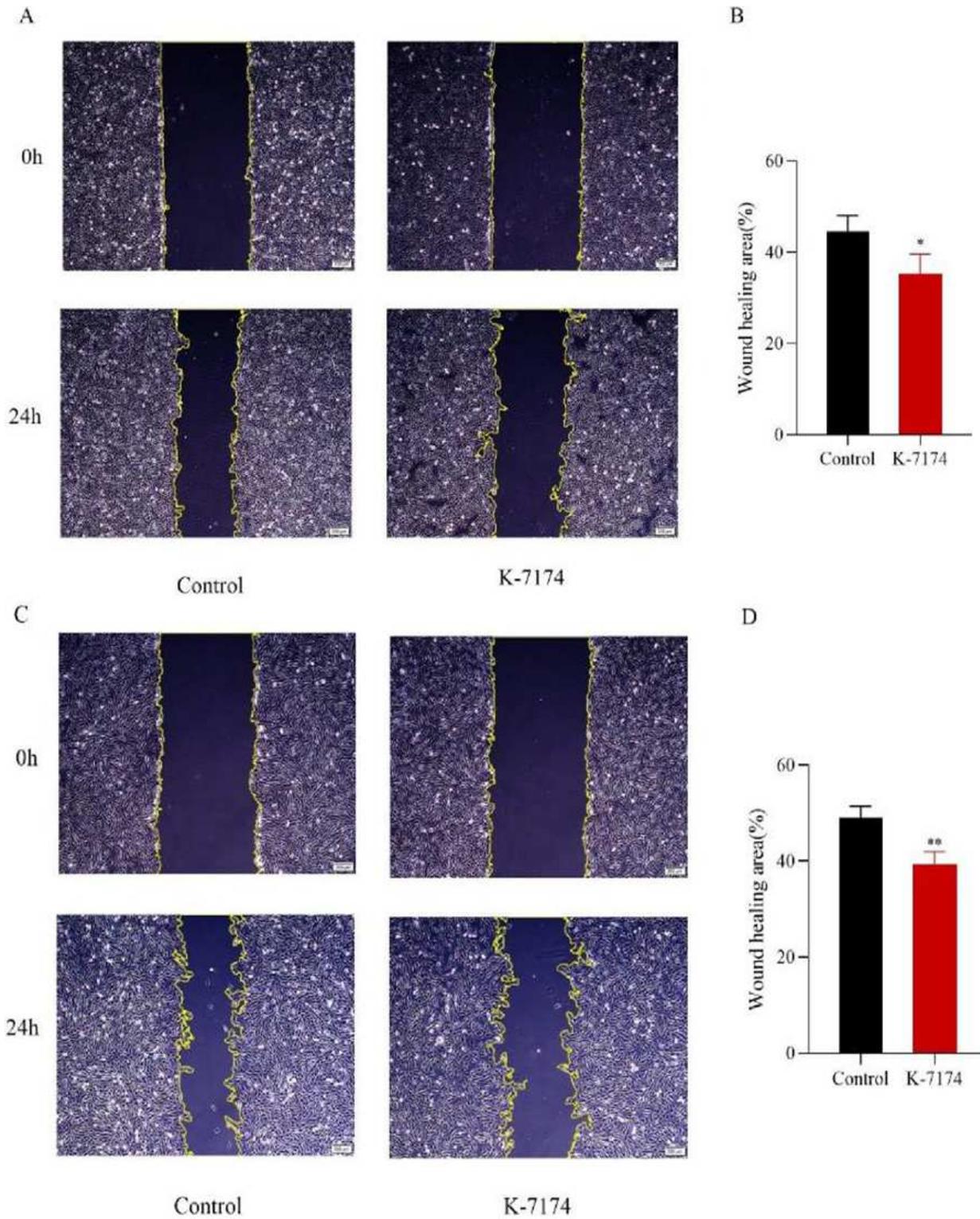
Figure 3

Top 3 modules from the protein interaction network.



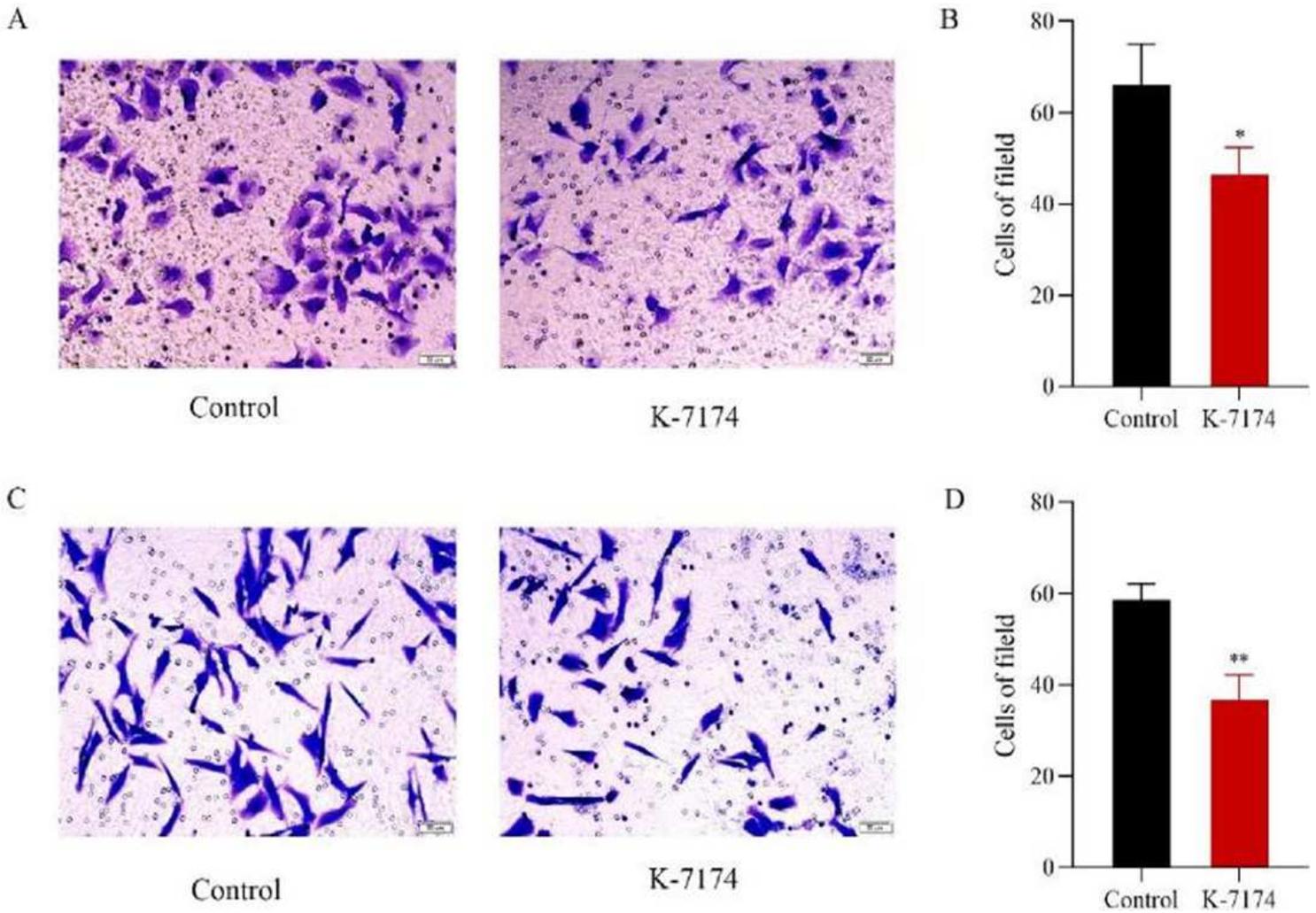
**Figure 4**

A and B Cell proliferation ability was assessed after U2Os or MG63 cell were treated with K-7174 after 24h or 48h using CCK-8 assay. C, D, E and F The inhibiting effects on colony formation of U2OS or MG63 by treated 20μM K-7174 the colony numbers were calculated.



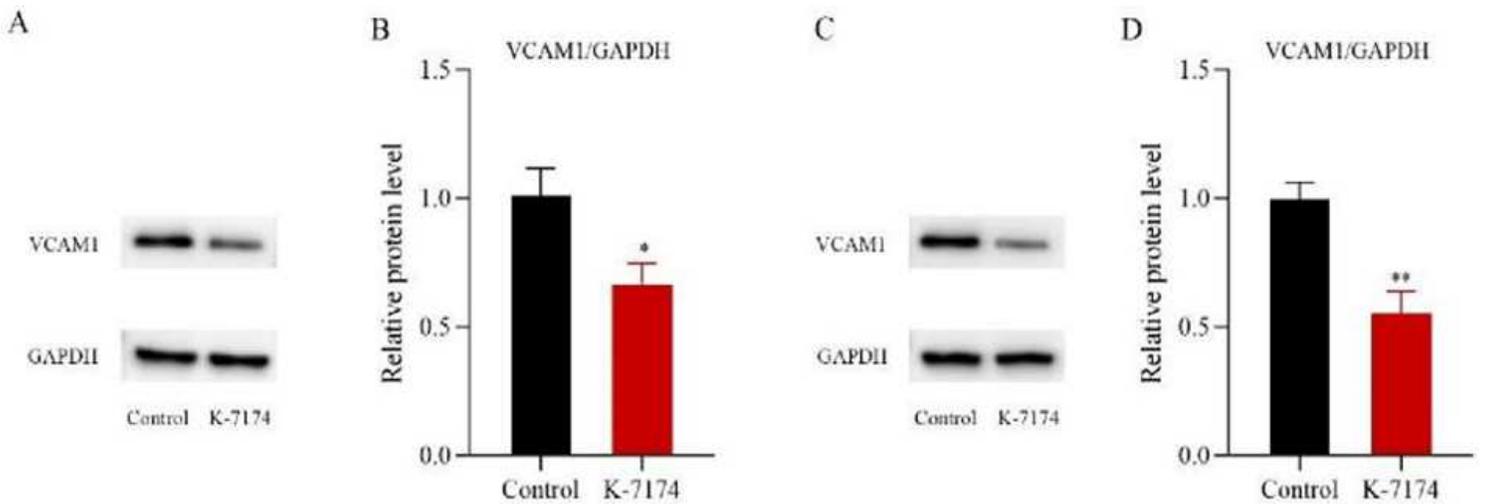
**Figure 5**

A and C Representative image of healing area of U2OS or MG63 cells at 0 and 24h after wound scratch when exposed to 20  $\mu$ M K-7174. B and D The results of wound repair inhibition under different treatment conditions were compared.



**Figure 6**

A and C Representative images of Transwell assay for invasion using Transwell chambers with Matrigel coating B and D The results of invasion inhibition under different treatment conditions were compared.



**Figure 7**

A and C Western blot results of VCAM1 proteins expression after 24 h treatment with K-7174 in U2OS and MG63 cells. B and D Relative optical density of proteins were analyzed and compared based on gray value of bands. Full-length gels and blots were included in supplement materials.

## Supplementary Files

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

- [RawdatawesternblotbandofU2OScells.png](#)
- [RawdatawesternblotbandofMG63cells.png](#)