Original Article
Screening for the presence of aberrantly expressed ACTR2 in osteosarcoma and analyzing its mechanism of action through an online database

Min Yi[1,2], Kai Zheng[3,4], Qian Ning[2,4], Yu Nie[5], Fuguo Huang[1,2]

[1]Department of Orthopedic Surgery, West China Hospital, Sichuan University, Chengdu 610041, Sichuan, China; [2]Trauma Center, West China Hospital, Sichuan University, Chengdu 610041, Sichuan, China; [3]Department of Operating Room, West China Hospital, Sichuan University, Chengdu 610041, Sichuan, China; [4]West China School of Nursing, Sichuan University, Chengdu 610041, Sichuan, China; [5]National Engineering Research Center for Biomaterials, Sichuan University, Chengdu 610041, Sichuan, China

Abstract: Osteosarcoma (OS) represents the most prevalent malignant bone tumor clinically, significantly impacting the health and safety of patients. The exploration of molecular pathogenic mechanisms is deemed a breakthrough for OS diagnosis and treatment. Within the GSE16088 dataset, a total of 1,948 differentially expressed genes (DEGs) were identified, comprising 1,697 down-regulated and 251 up-regulated genes. Notably, only two DEGs were associated with the response to trichostatin A: ARP2 actin-related protein 2 homolog (ACTR2) and MEF2C; ACTR2 garnered particular interest. Subsequently, 57 OS patients (research group) and 50 healthy controls from the same period (control group) were selected for analysis. The expression of ACTR2 in peripheral blood in both groups, as well as its levels in cancerous tissues and adjacent counterparts of OS patients, were evaluated, ascertaining the correlation between ACTR2 and OS. OS cases exhibited lower levels of ACTR2 compared to controls (P<0.05), with ACTR2 expression demonstrating a robust diagnostic capability for OS. Similarly, ACTR2 expression was diminished in cancer tissues (P<0.05). A three-year prognostic follow-up was conducted to assess the prognostic value of ACTR2 in OS patients. The follow-up findings revealed a significantly lower survival rate among patients with low ACTR2 expression in contrast to those with high expression (P<0.05). In vitro studies involved the construction of abnormal expression vectors for ACTR2 and miR-374a-5p, which were transfected into human OS cells (U2OS, SAOS). The outcomes indicated that elevating ACTR2 or suppressing miR-374a-5p attenuated the proliferative, invasive, and migratory capacities as well as the epithelial-mesenchymal transition (EMT) of OS cells while enhancing their apoptosis. Conversely, upregulation of miR-374a-5p yielded opposing effects (P<0.05). The dual-luciferase reporter (DLR) assay demonstrated that the fluorescence activity of ACTR2-WT was significantly inhibited by the miR-374a-5p mimic sequence (P<0.05), confirming the presence of a targeted regulatory relationship between ACTR2 and miR-374a-5p. These findings offer novel insights for future research directions in the diagnosis and treatment of OS.

Keywords: Osteosarcoma, ACTR2, miR-374a-5p, epithelial mesenchymal transition, mechanism of action

Introduction

Osteosarcoma (OS), presenting with bone and joint pain and localized masses, is the most common malignant bone tumor clinically [1]. According to the World Health Organization (WHO) statistics, the incidence of OS is about 4 to 5 out of 1 million and is the most common among teenagers, with approximately 60% of OS patients under the age of 25 [2, 3]. Recent years have witnessed the rising incidence of OS. In 2020, the global number of new OS

cases exceeded 130,000, an increase of about 3.7 times compared to 2010 [4]. As with most malignancies, radical surgery can usually lead to an ideal prognosis in patients with early OS; but for those with advanced OS, amputation is needed to save patients' lives, with the existing treatment methods being difficult to completely remove metastatic and infiltrating lesions [5]. According to statistics, the 5-year overall survival rate of advanced OS patients is below 30% [6]. At present, the pathogenesis of OS has not been fully defined. A relatively unified clinical

understanding is that a variety of carcinogens act on normal cells for a long time, which leads to the activation of proto-oncogenes and the inability to timely repair the damaged DNA during the process of replication and transcription, affecting the cell cycle and making cancer cells multiply without restriction and become cancerous [7]. Therefore, an in-depth understanding of molecular changes and cell biological behavior in the pathogenesis of OS may become the key and breakthrough to conquer OS in the future.

Genes are one of the key research areas in the medical field due to their critical roles in many life activities, such as dosage compensation, epigenetic regulation, cell cycle regulation, and cell differentiation modulation [8]. For malignancies, genes can regulate a series of physiological and pathological processes of the body and cells through interacting with Deoxyribo nucleic acids (DNAs), messenger Ribonucleic acids (mRNAs), non-coding Ribonucleic acids (ncRNAs), and proteins, and participate in multiple life activities like tumor growth, apoptosis, and invasion [9]. In OS, due to the important role they play, a variety of genes are also hailed as the breakthrough of future OS diagnosis and treatment research [10, 11]. Therefore, to explore the pathogenesis of OS through a genetic perspective, we screened differentially expressed genes (DEGs) in OS through the GEO database in an attempt to find a completely new research direction. Among the results of the analysis, ARP2 actin-related protein 2 homolog (ACTR2) caught our attention. This is because ACTR2 is not only a potential DEG for OS but also a key gene involved in trichostatin A response. It is well-known that trichostatin A is a histone deacetylase inhibitor that has been discovered in recent years to help inhibit OS cell growth and accelerate apoptosis [12, 13], and has been touted as an important direction for the clinical treatment of OS. Moreover, the regulatory effect of ACTR2 on trichostatin A also plays a potential significant role. However, no study has yet confirmed the relationship between ACTR2 and OS. Meanwhile, in the studies published so far, ACTR2 is also considered to be one of the key genes for the expression of human immune characteristics [14], while OS is a representative tumor with an imbalanced immune microenvironment. Thus, it is likely that ACTR2 have important potential significance for the development and progression of OS.

To verify our point of view, this study conducted a preliminary analysis of the clinical significance and mechanism of ACTR2 in OS, providing new reference for future OS diagnosis and treatment and laying a foundation for the subsequent research on ACTR2 and OS.

Data and methods

Data sources

The GSE16088 dataset (title: Gene expression profiles of human osteosarcoma) was selected for analysis from the PubMed GEO database (https://www.ncbi.nlm.nih.gov/). This dataset, uploaded by Davis S et al., includes 14 OS tissue samples and 6 normal tissue samples. The data platform was GPL96 [HG-U133A] Affymetrix Human Genome U133A Array.

Data processing and DEG screening

The samples in the dataset were divided into a normal tissue group and a tumor tissue group, with $P<0.05$ and $|Log^2FC|>2$ as the screening criteria. The dataset was analyzed by the GEO2R of the GEO database, after which the volcano and heat maps were drawn.

Enrichment analysis

GO annotation and KEGG pathway enrichment analyses of the integrated DEGs were performed using the DAVID (The Database for Annotation, Visualization and Integrated Discovery) online analysis tool, and the results with $P<0.05$ were included.

Patient data

From February 2019 to January 2020, 57 OS patients (research group) admitted to our hospital and 50 healthy controls (control group) were selected as the research participants. Ethical approval has been obtained from the West China Hospital's Ethics Committee, as well as informed consent from each subject.

Criteria for patient enrollment and exclusion

Research group: All patients (age >18) were diagnosed with OS by pathological biopsy and agreed to cooperate with the study, with com-

Table 1. Primer sequences

Gene	F (5'-3')	R (5'-3')
ACTR2	CACCTGTGGGACTACACATTTG	TGGTTGGGTTCATAGGAGGTTC
GAPDH	GCACCGTCAAGGCTGAGAAC	TGGTGAAGACGCCAGTGGAT
miR-374a-5p	GCGCGCTTATAATACAACCTGa	GTGCAGGGTCCGAGGT
U6	CTCGCTTCGGCAGCACA	AATCCCCTTCATGTCCTCTTTC

plete medical records; those with other tumors, cardio-cerebrovascular diseases, immunodeficiency, mental illness, organ failure or abnormality, estimated survival <3 months, and history of surgery, chemoradiotherapy before admission were excluded. Control group: Subjects (age >18) who underwent routine physical examination in our hospital, with complete medical records, normal physical examination results, and no previous major medical history were enrolled; the exclusion criteria are the same as above.

Sampling

Fasting venous blood of all participants was collected upon admission. In addition, OS patients' cancerous tissues and adjacent normal counterparts were obtained for subsequent detection with patients' consent.

Polymerase chain reaction (PCR)

We isolated total RNA from the blood and tissue samples (after grinding) as per the Trizol kit (Thermo Fisher Scientific) instructions, verified the purity using a UV spectrophotometer, and reverse transcribed 2 µg of it into complementary Deoxyribo nucleic acid (cDNA) following kit (Thermo Fisher Scientific) recommendations for the amplification reaction. Subsequently, the reaction system was prepared according to the PCR kit (Thermo Fisher Scientific): 1 µL of SYBR Green, 1 µL of each upstream and downstream primer, 1 µL of dNTP, 1 µL of Taq polymerase, 5 µL of cDNA, and 30 µL of ddH$_2$O, with a total volume of 50 µL. The reaction was carried out on a PCR instrument. The following were the reaction conditions (40 cycles): 95°C for 2 min, 95°C for 60 s, 58°C for 30 s, and 72°C for 30 s. Relative gene expression was calculated by $2^{-\Delta\Delta CT}$. Jiangsu Saisofei Biotechnology Co. was commissioned to design and construct the primer sequences (**Table 1**), Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and U6 served as the internal reference of ACTR2 and miR-374a-5p, respectively.

Prognostic follow-up

OS patients were followed up for 3 years in the form of regular hospital reviews with an interval not exceeding 3 months. The 3-year survival was counted and the survival curve was plotted.

Cell information

Human osteoblasts hFOB1.19 and human OS U2OS and SAOS cells, all supplied by Beijing BeNa Culture Collection, were cultured in supporting media. The incubator was set at 37°C with 5% CO$_2$, and passage was performed when the cell density reached 80%. PCR was responsible for the determination of ACTR2 and miR-374a-5p contents in hFOB1.19, U2OS, and SAOS.

Cell transfection

Guangzhou VectorBuilder Inc. was entrusted to design and construct the abnormal expression vectors of ACTR2 and miR-374a-5p. Subsequently, logarithmic-growth-phase U2OS and SAOS were inoculated into 6-well culture plates (2 × 10^5), and transfection was carried out with LipofectamineTM2000 when the cell fusion degree reached about 80%. RNA interfering nonsense sequence (NC-ACTR2 group), two ACTR2 overexpression vectors (we commissioned Guangzhou VectorBuilder Inc. to design two ACTR2 aberrant expression vectors, labeled el-ACTR2-1 and el-ACTR2-2, both expression vectors elevated ACTR2 expression, but the effect of el-ACTR2-1 on ACTR2 elevation was not as significant as that of el-ACTR2), miR-374a-5p mimic sequence (5'-CUUAUCAGGUUGUAUUAUAAUU-3'; miR-374a-5p-mim group), miRNA negative control (5'-UUCUCCGAACGUGUCACGUTT-3'; miR-374a-5p-NC group), and miR-374a-5p inhibitor sequence (5'-UUAUAAUACAACCUGAUAAGUG-3'; miR-374a-5p-inh group) were designed. Cells were collected 48 hours after the transfection, followed by PCR and Western blot quantification of ACTR2 and

miR-374a-5p to verify the transfection efficiency.

Western blotting

Following total protein collection from each group by using registers in probate association (RIPA) lysate (Sigma-Aldrich) and protein concentration determination by the bicinchoninic acid (BCA) (Sigma-Aldrich) method, the protein samples were denatured at 100°C for 10 minutes. Then, 40 µg of protein samples was separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE; Thermo Fisher Scientific), sealed with 5% skim milk for 2 hours immediately after membrane transfer, and immersed in ACTR2 (1:1,000, Abcam), E-cadherin (1:1,000, Abcam), N-cadherin (1:1,000, Abcam), vimentin (1:1,000, Abcam), and GAPDH (1:2,000, Abcam). A secondary antibody diluent (1:3,000, Abcam) was added to culture at 37°C for 2 hours after incubation at 4°C overnight. Finally, the protein bands were quantitatively analyzed by Quantity One software.

Target gene prediction analysis

The targeting relationship between ACTR2 and miR-374a-5p was analyzed through the online target gene prediction website Encori (URL: https://starbase.sysu.edu.cn/). Guangzhou Geneseed Biotech was entrusted to construct wild-type (WT) and mutant (MUT)-ACTR2 plasmids inserted with luciferase, which were co-transfected into U2OS and SAOS with miR-374a-5p mimic sequence and miRNA NC, respectively. Forty-eight hours later, fluorescence activities were measured as per the dual-luciferase reporter (DLR) assay kit instructions.

3-(4,5)-dimethylthiahiazo (-z-y1)-3,5-di-phenytetrazoliumromide (MTT)

5×10^3 cells were inoculated into the wells of a 96-well culture plate. Each group was provided with 4 duplicate wells. At 0, 24, 48, and 72 hours, a well from each group was added with MTT solution (20 µL) (Shanghai Qiyuan Biotechnology Co., Ltd.). After discarding the liquid in the wells, continuous culture (4 hours), and the mix of additional 150 µL of DMSO, the optical density (OD) at 570 nm was read using a microplate reader. Cell growth curves were also drawn.

Wound-healing assay

The cells (10×10^5 cells/mL) spread on a 24-well culture plate were added with 10% fetal bovine serum-supplemented Roswell Park Memorial Institute (RPMI) 1640 for 24 hours of cultivation. The monolayer cells were scratched vertically with a 10 µL pipette tip, rinsed with phosphate-buffered solution (PBS), and incubated for 24 hours. Following the sucking off of the culture solution, cells were observed and photographed with an inverted microscope, and the cell mobility was calculated. Cell mobility = (initial scratch area - final scratch area)/ initial scratch area × 100%.

Transwell

The Matrigel, diluted at a ratio of 1:8, was applied to the upper Transwell chamber (Sigma-Aldrich) and allowed to solidify at 4°C. Following adjustment of cell density to 1×10^5 cells/mL and inoculation into the lower chamber, a culture medium supplemented with 10% fetal bovine serum was added for a 24-hour incubation period. Subsequently, upon removal of the chamber, non-penetrating cells were removed, fixed, and stained with crystal violet for 20 minutes to facilitate observation, counting, and microscopic imaging.

Flow cytometry (FCM)

Cells were washed with PBS and digested by adding ethylene diamine tetraacetic acid (EDTA)-free trypsin. Then, 100 µL of cell suspension (5×10^5 cells/mL) was taken after resuspension in PBS and placed in a clean centrifuge tube. Next, 5 µL of Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) reagent (Abcam) were added sequentially, mixed well, and incubated for 15 minutes at room temperature with protection from light. The apoptosis rate was detected by a flow cytometer.

Animal information

Fifteen healthy BALB/c nude mice of SPF grade were purchased from Beijing Zhidao Biotechnology Co., Ltd. [SYXK (Beijing) 2022-0038], with a body mass of 18-20 g. The animals were fed for 1 week in an environment of (25±2)°C, humidity (50±10)%, 12 h/12 h day/night alternation, and free feeding and drinking.

Nude mice tumorigenesis experiment

The mice were randomly assigned to three groups (n=5 each). U2OS cells of the NC-ACTR2, el-ACTR2-1, and el-ACTR2-2 groups were resuspended in PBS. Subsequently, 500 μL (1 × 10^7 cells/mL) of cells were aspirated and subcutaneously injected into the right axilla of the mice, followed by ongoing feeding. After 7 days, all mice were humanely euthanized via cervical dislocation, and intact subcutaneous tumor tissue was excised. Tumor growth was monitored, and both volume and weight were measured. Volume = length × width × height × π/6.

Endpoints

ACTR2 expression in OS and its significance in OS occurrence and prognosis were discussed. In addition, the potential downstream target genes of ACTR2 and the influences of ACTR2 and miR-374a-5p abnormal expression on OS cells were observed to confirm the mechanism and action pathway of ACTR2 in OS.

Statistical analysis

This study used SPSS24.0 software (IBM) for statistical analysis. Count data, such as subjects' sex and family history, were represented by [n (%)], and comparative analysis was performed by the chi-square test. ACTR2 and miR-374a-5p levels and other quantitative data were represented by ($\bar{x}$±s); the comparisons between two groups were carried out using independent samples t-tests, and comparisons between consecutive time points and multiple groups were analyzed using analysis of variance plus Least-significant difference (LSD) intra-group test. Diagnostic value was analyzed by receiver operating characteristic (ROC) curves. The Kaplan-Meier method and Log-rank test were adopted for the calculation and comparison of the survival rate, respectively. The prognostic impact of ACTR2 on OS patients was analyzed using COX regression. Correlations were analyzed by Pearson correlation coefficients. A significance level of P<0.05 was set for all analyses.

Results

DEG screening

In the GSE16088 dataset, we identified a total of 1,948 DEGs, of which 1,697 were down-regulated and 251 were up-regulated (**Figure 1A**). The expression of DEGs is shown in **Figure 1B** (due to the large number of DEGs, we only chose the top 50 genes for display; the complete DEG heatmap is available in Supplementary Material). In the GO analysis, there were 2,666 keywords with FDR<1 and P<0.05, among which the keywords involving the most differential genes were RNA biosynthetic process regulation of transcription, DNA-templated bounding membrane of organelle, etc. (**Figure 1C**). In contrast, KEGG analysis showed that the keywords involving the most differential genes included Proteasome, Protein processing in endoplasmic reticulum, RNA transport, etc. (**Figure 1D**). Among them, there were only 2 DEGs involving response to trichostatin A, ACTR2, and MEF2C.

Comparison of clinical baseline data between research group and control group

No marked differences were identified between the research and control groups when comparing their age, sex, family history, and other baseline data (P>0.05, **Table 2**), which indicates that the two groups are comparable.

Expression and clinical significance of ACTR2 in OS

OS patients had lower ACTR2 mRNA levels than healthy controls (P<0.05, **Figure 2A**). According to ROC results, when peripheral blood ACTR2 mRNA was less than 2.17, with sensitivity and specificity for diagnosing OS being 78.95% and 78.00%, respectively (P<0.05, **Figure 2B**). Similarly, we identified lower ACTR2 mRNA in cancer tissues of OS patients versus adjacent tissues (P<0.05, **Figure 2C**). Moreover, Pearson correlation coefficients showed a significant positive connection between ACTR2 mRNA in cancer tissues with that in peripheral blood in OS patients (P<0.05, **Figure 2D**).

*Correlation of ACTR2 with OS patients' clinico-pathological features*

Peripheral blood ACTR2 mRNA had no obvious relationship with the sex and age of OS patients (P>0.05); however, ACTR2 mRNA expression was significantly lower in stage III patients and those with a tumor diameter ≥5 cm (P<0.05), indicating that ACTR2 mRNA expression is closely related to the malignant progression of OS (**Table 3**).
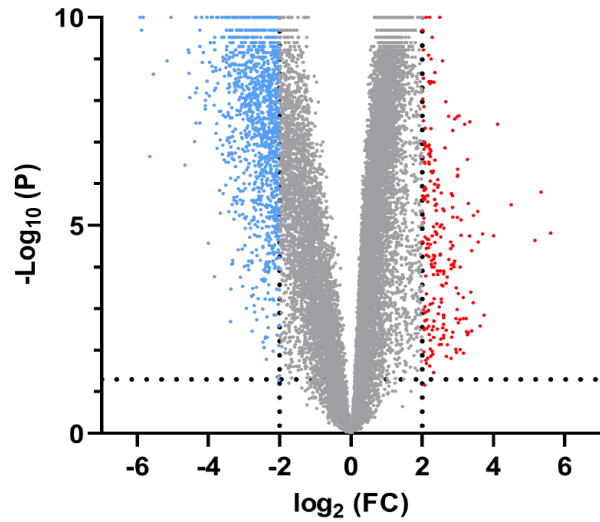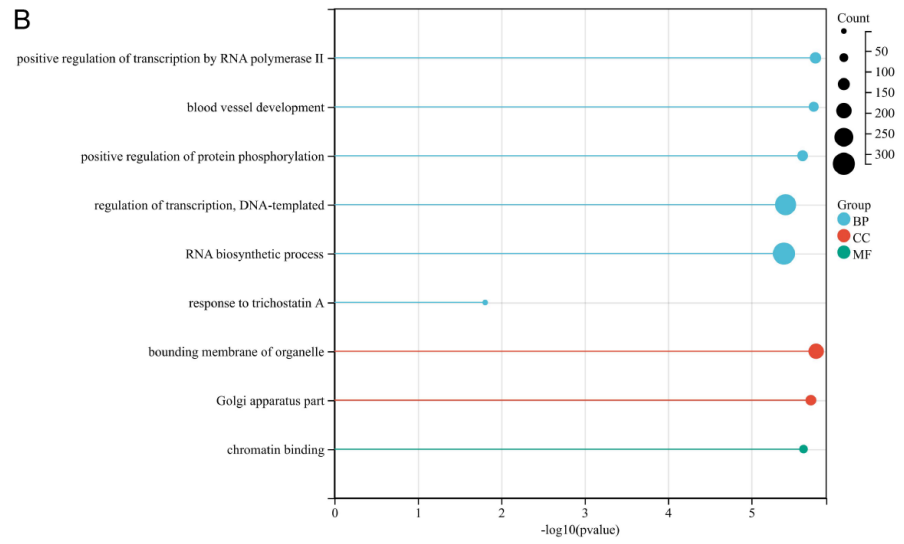
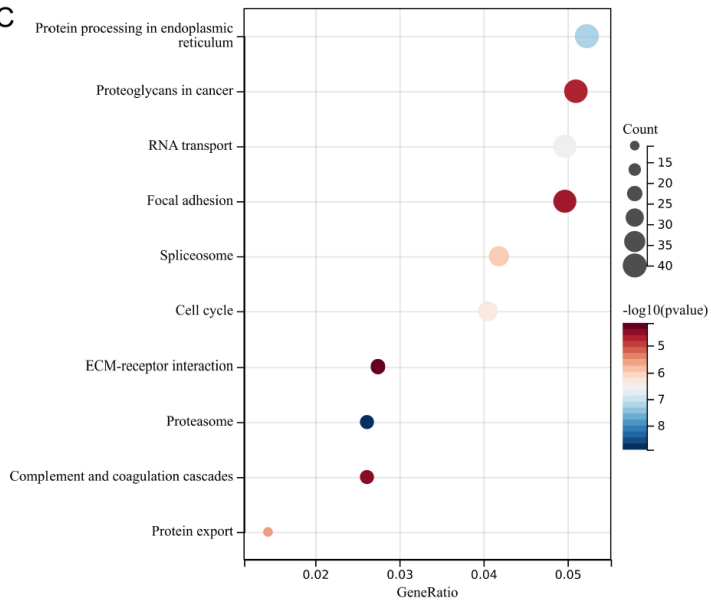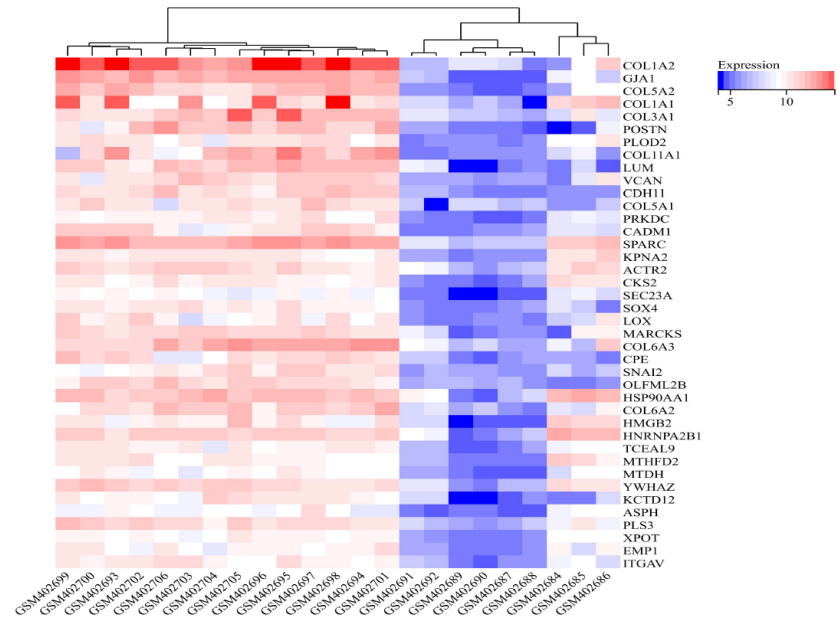Figure 1. Results of DEG screening. A: DEGs in the GSE16088 dataset. B: Heatmap of the expression of DEGs (full heatmap in Supplementary Material). C: Results of the GO analysis of DEGs. D: Results of the KEGG analysis of DEGs.

Table 2. Clinical baseline data

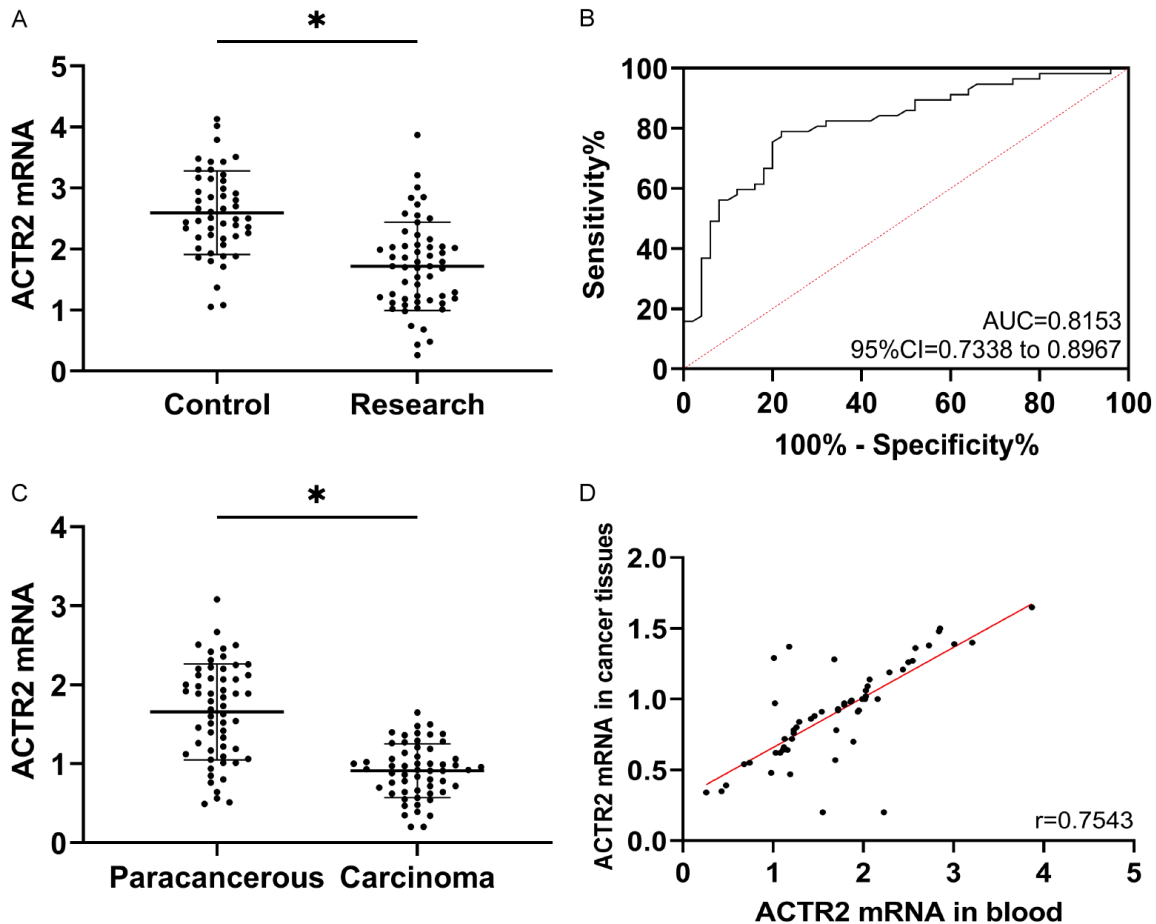| Group	Control group (n=50)	Research group (n=57)	t (or χ^2)	P
Age	63.66±4.04	64.07±6.31	0.394	0.695
Male/Female	34/16	35/22	0.506	0.477
Family history of OS			0.992	0.319
Yes/No	2/48	5/52		
Smoking			0.794	0.373
Yes/No	22/28	30/27		
Pathological Staging			-	-
I/II/III	-	22/21/14		
Diameter of the tumor			-	-
<5 cm/≥5 cm	-	39/18		

Figure 2. Expression and clinical significance of ACTR2 in OS. A: Comparison of ACTR2 mRNA expression levels in peripheral blood between research and control groups. *P<0.05. B: ROC curve of ACTR2 mRNA for diagnosis of OS. C: Comparison of ACTR2 mRNA expression levels between cancer and paraneoplastic tissues. *P<0.05. D: Correlation of ACTR2 mRNA expression levels in peripheral blood and in cancer tissues in the research group.

Table 3. Correlation of ACTR2 with OS patients' clinicopathological features

Clinicopathological features		n	ACTR2 mRNA	t (or F)	P
Age	≤64	30	1.68±0.78	0.457	0.650
	>64	27	1.76±0.67		
Sex	Male	35	1.68±0.71	0.508	0.614
	Female	22	1.78±0.77		
Family history of OS	Yes	5	1.60±0.35	0.363	0.718
	No	52	1.73±0.75		
Smoking	Yes	30	1.82±0.63	1.098	0.277
	No	27	1.61±0.82		
Pathological Staging	I	22	2.31±0.63	28.900	<0.001
	II	21	1.57±0.40*		
	III	14	1.01±0.45*,#		
Diameter of the tumor	<5 cm	39	1.92±0.71	3.484	0.001
	≥5 cm	18	1.27±0.53		

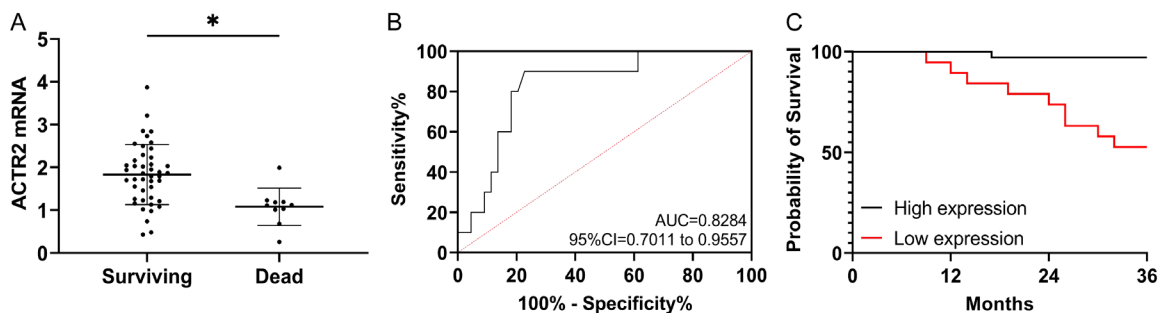Note: *$P<0.05$ compared with stage I, #$P<0.05$ compared with stage II.

Figure 3. Prognostic significance of ACTR2 in OS. A: Comparison of the expression levels of ACTR2 mRNA in dead and surviving patients. B: ROC curves of ACTR2 mRNA for prognostic death of OS patients. C: Prognostic 3-year survival curves for the ACTR2 mRNA high expression group and the ACTR2 mRNA low expression group. *$P<0.05$.

Table 4. COX analysis of ACTR2 affecting prognostic mortality in OS patients

	B	S.E.	Wald χ²	P	OR	95% CI
ACTR2 mRNA	1.642	-0.672	12.642	<0.001	0.672	0.471-1.662

Note: B, regression coefficient; S.E., standard error; OR, odds ratio; 95% CI, 95% confidence interval.

Prognostic significance of ACTR2 in OS

In the follow-up, 54 OS patients were successfully tracked, of which 10 patients died, with an overall 3-year mortality of 18.52%. According to the statistical analysis, the dead patients had lower ACTR2 mRNA levels than the surviving patients (P<0.05, **Figure 3A**). As indicated by ROC analysis, the sensitivity and specificity of ACTR2 mRNA in predicting the 3-year death of patients were 90.00% and 77.27%, respectively, when its peripheral blood level was less than 1.25 (P<0.05, **Figure 3B**). We further grouped OS patients as high (n=35) and lower (n=19) expression groups based on ACTR2 mRNA cut-off. The survival rate was found to be statistically lower in the low versus the high expression group after analyzing the prognostic survival curves (P<0.05, **Figure 3C**). Using COX analysis, we found that ACTR2 mRNA was a relevant factor influencing the prognostic mortality in OS patients (P<0.001, OR=0.672). That is, the lower the ACTR2 mRNA, the higher the risk of prognostic death in OS patients (**Table 4**).

ACTR2 in cells

U2OS and SAOS also had lower ACTR2 mRNA levels than hFOB1.19 (P<0.05, **Figure 4A**).

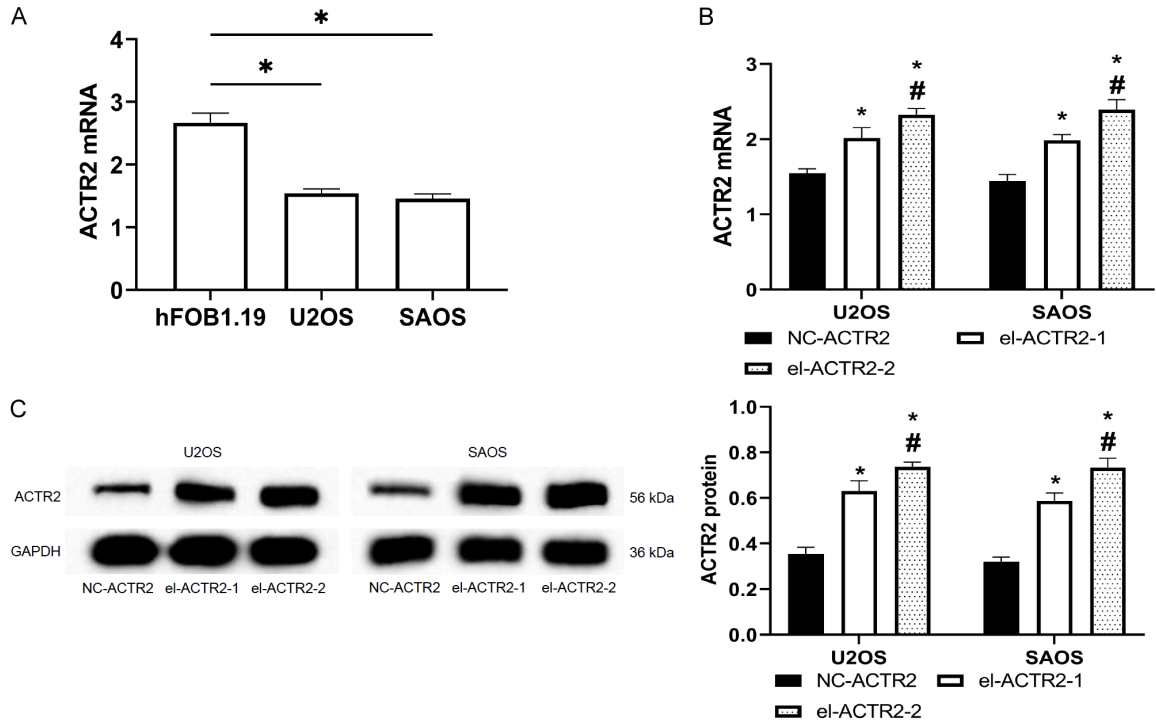**Figure 4.** ACTR2 in cells. A: Comparison of ACTR2 mRNA expression in U2OS, SAOS, and hFOB1.19. *P<0.05. B: Comparison of ACTR2 mRNA expression levels after transfection with ACTR2 aberrant expression vectors (by PCR). C: Comparison of ACTR2 protein expression levels after transfection with ACTR2 aberrant expression vectors (by Western blot). *vs.* NC-ACTR2 group, *P<0.05, *vs.* el-ACTR2-1 group, #P<0.05.

After transfection, ACTR2 mRNA was found to be higher in the el-ACTR2-1 and el-ACTR2-2 groups than in the NC-ACTR2 group, confirming successful transfection. Among them, the ACTR2 mRNA of the el-ACTR2-2 group was higher than that of the el-ACTR2-1 group (P<0.05, **Figure 4B**). The results of Western blot were consistent with PCR, i.e., ACTR2 protein expression was highest in the el-ACTR2-2 group, followed by the el-ACTR2-1 group, and lowest in the nc-ACTR2 group (P<0.05, **Figure 4C**), indicating that the effect of elevating ACTR2 mRNA expression was more significant in the el-ACTR2-2 group.

Influence of ACTR2 on the biological behavior of U2OS and SAOS

In the MTT assay, the cell growth ability of the el-ACTR2-1 and el-ACTR2-2 groups was significantly weakened compared with the NC-ACTR2 group (P<0.05, **Figure 5A**). The results of the wound healing assay, on the other hand, showed that the migration rate of the NC-ACTR2 group was the highest among the three groups (P<0.05). In U2OS, the migration rate of the el-

ACTR2-1 group was higher than that of the el-ACTR2-2 group (P<0.05); in SAOS, there was no difference in the migration rate between the el-ACTR2-1 and el-ACTR2-2 groups (P>0.05, **Figure 5B**). Similarly, the number of invading cells in the NC-ACTR2 group was higher compared with the other two groups, while that in the el-ACTR2-2 group was lower versus the el-ACTR2-1 group (P<0.05, **Figure 5C**). Finally, the detection results of the apoptosis rate showed that the el-ACTR2-2 group had the highest apoptosis rate, while the NC-ACTR2 group had the lowest (P<0.05, **Figure 5D**).

Prediction of ACTR2's target genes

In ENCORI, ACTR2 had 323 potential downstream target genes. See **Figure 6A** for the binding complementary sites of miR-374a-5p and ACTR2. Similarly, the detection of miR-374a-5p expression in cells showed significantly increased miR-374a-5p in U2OS and SAOS (P<0.05, **Figure 6B**). After transfection with miR-374a-5p abnormal expression sequences, the miR-374a-5p-mim group had higher miR-374a-5p levels than the other two groups, while
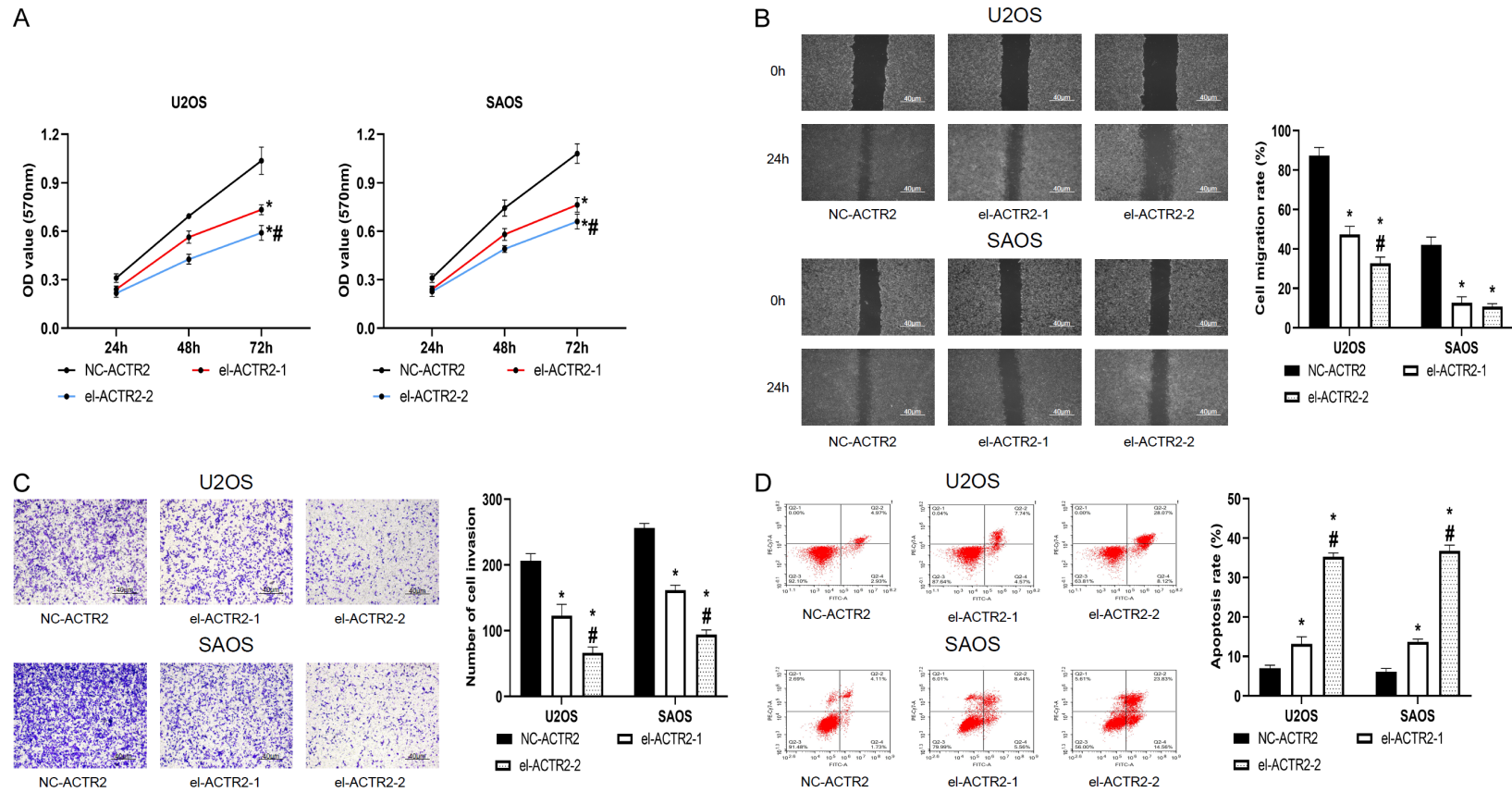
Figure 5. Influence of ACTR2 on the biological behavior of U2OS and SAOS. A: Growth curves of U2OS and SAOS. B: Effect of ACTR2 on the migration ability of U2OS and SAOS (200×). C: Effect of ACTR2 on the invasion ability of U2OS and SAOS (200×). D: Effect of ACTR2 on the apoptosis rate of U2OS and SAOS. *vs.* NC-ACTR2 group, *P<0.05, *vs.* el-ACTR2-1 group, #P<0.05.
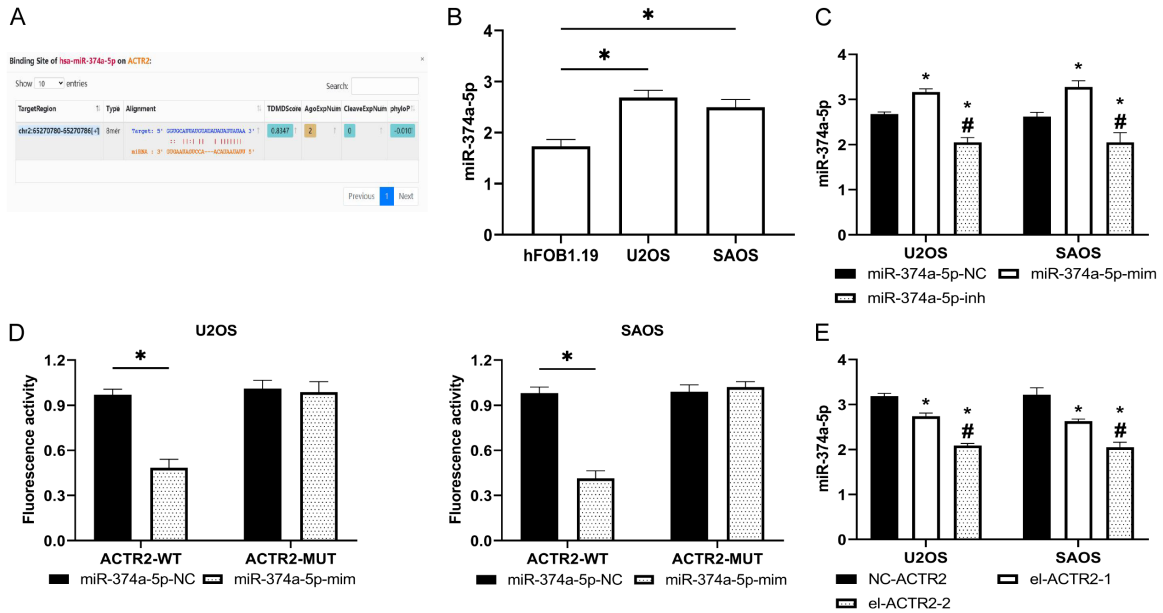
Figure 6. Prediction of ACTR2's target genes. A: Binding complementary sites of ACTR2 and miR-374a-5p (from ENCORI database). B: Comparison of expression levels of miR-374a-5p in U2OS, SAOS, and hFOB1.19. *P<0.05. C: Comparison of miR-374a-5p expression levels after transfection with miR-374a-5p aberrant expression vectors. *vs.* miR-374a-5p-NC group, *P<0.05, *vs.* miR-374a-5p-mim group, #P<0.05. D: Results of DLR assay. *P<0.05. E: Effect of ACTR2 on miR-374a-5p expression levels. *vs.* NC-ACTR2 group, *P<0.05, *vs.* el-ACTR2-1 group, #P<0.05.

the miR-374a-5p-inh group had lower miR-374a-5p expression than the miR-374a-5p-NC group (P<0.05, **Figure 6C**). As shown by the DLR assay, the fluorescence activity of ACTR2-WT was significantly decreased after transfection with miR-374a-5p-mim compared with miR-374a-5p-NC transfection (P<0.05), while that of ACTR2-MUT remained unchanged (P>0.05, **Figure 6D**). Finally, after transfection with ACTR2 abnormal expression vectors, it was seen that miR-374a-5p was lower in the el-ACTR2-1 and el-ACTR2-2 groups than in the NC-ACTR2 group (P<0.05, **Figure 6E**).

Impacts of miR-374a-5p on the biological behaviors of U2OS and SAOS

After transfection with miR-374a-5p abnormal expression vectors, the cell growth ability was significantly increased in the miR-374a-5p-mim group, but it was decreased in the miR-374a-5p-inh group (P<0.05, **Figure 7A**). Similarly, cell mobility and invasion counts were markedly higher in the miR-374a-5p-mim group versus the miR-374a-5p-NC and miR-374a-5p-inh groups, while those in the miR-374a-5p-inh group were lower compared with the miR-374a-5p-NC group (**Figure 7B**, **7C**). Finally, the apop-tosis rate was the highest in the miR-374a-5p-inh group and the lowest in the miR-374a-5p-mim group, with statistical significance (P<0.05, **Figure 7D**).

Impacts of ACTR2 and miR-374a-5p on epithelial-mesenchymal transition (EMT) in OS cells

The detection results of EMT marker proteins showed that N-cadherin and vimentin protein levels were reduced, while E-cadherin protein increased in the ACTR2-el-1 and ACTR2-el-2 groups (P<0.05, **Figure 8A**). The miR-374a-5p-mim group showed higher N-cadherin and vimentin and lower E-cadherin protein levels than the miR-374a-5p-inh and miR-374a-5p-NC groups (P<0.05); compared with those in the miR-374a-5p-NC group, N-cadherin and vimentin were lower and E-cadherin was higher in the miR-374a-5p-inh group (P<0.05, **Figure 8B**).

Effect of ACTR2 on tumor growth

Finally, observation of the growth of subcutaneous tumors in the three groups of mice showed that the tumor volume and weight of the mice in the NC-ACTR2 group were the highest among
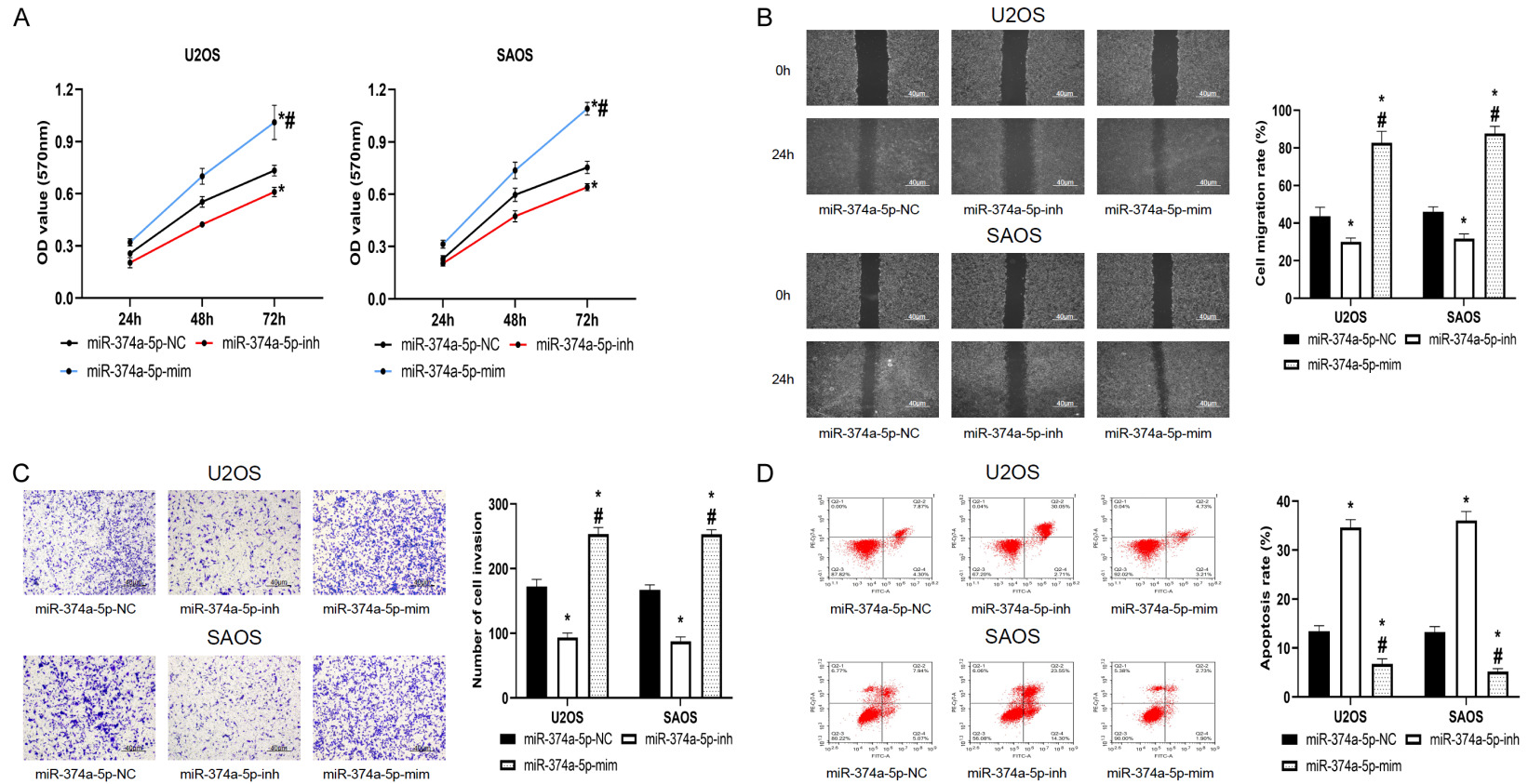
Figure 7. Impacts of miR-374a-5p on the biological behaviors of U2OS and SAOS. A: Growth curves of U2OS and SAOS. B: Effect of miR-374a-5p on the migration ability of U2OS and SAOS (200×). C: Effect of miR-374a-5p on the invasion ability of U2OS and SAOS (200×). D: Effect of miR-374a-5p on the apoptosis rate of U2OS and SAOS. *vs.* miR-374a-5p-NC group, *P<0.05, *vs.* miR-374a-5p-mim group, #P<0.05.
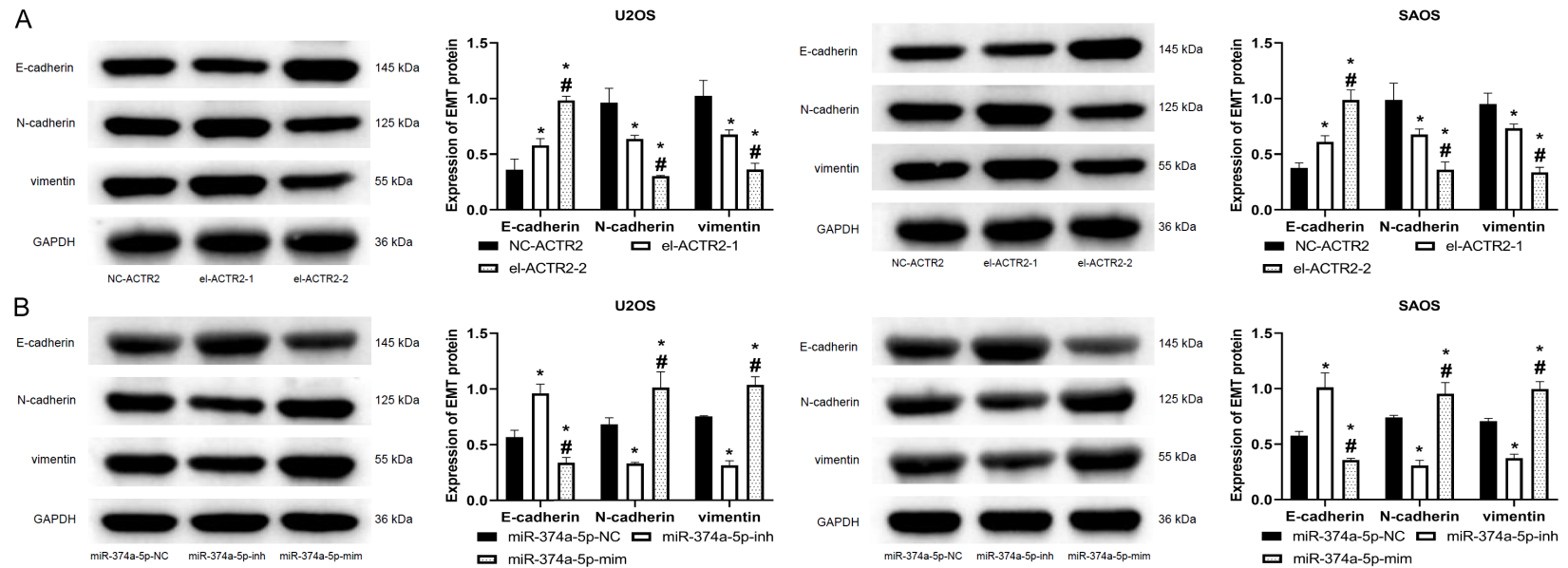
Figure 8. Impacts of ACTR2 and miR-374a-5p on EMT in OS cells. A: Effect of ACTR2 on EMT proteins in U2OS and SAOS. *vs.* NC-ACTR2 group, *P<0.05, *vs.* el-ACTR2-1 group, #P<0.05. B: Effect of miR-374a-5p on EMT proteins in U2OS and SAOS. *vs.* miR-374a-5p-NC group, *P<0.05, *vs.* miR-374a-5p-mim group, #P<0.05.
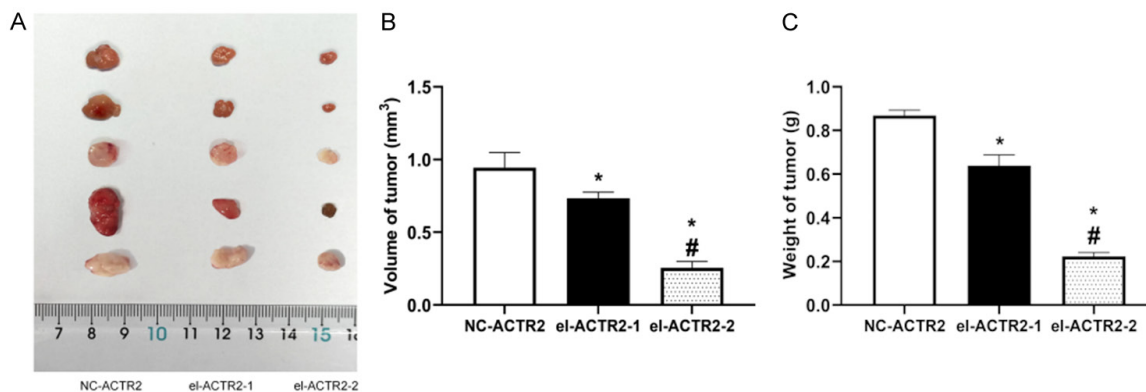
Figure 9. Effect of ACTR2 on tumor growth. A: Growth of subcutaneous tumor. B: Tumor volume. C: Tumor weight. *vs.* NC-ACTR2 group, *P<0.05, *vs.* el-ACTR2-1 group, #P<0.05.

the three groups, while those in the el-ACTR2-2 group were lower compared with the el-ACTR2-1 group (P<0.05, **Figure 9A-C**).

Discussion

OS, as a high-risk malignant tumor, has the characteristics of strong concealment, rapid development, and poor prognosis, causing a great negative impact on patients' health and life safety [15]. In recent years, the role of genes in malignancies has gradually received growing clinical attention, and they are also hailed as a breakthrough in future diagnosis and treatment of tumor diseases [16]. This study found that ACTR2 had obvious abnormal expression in OS and participated in its malignant progression, which has important reference significance for OS diagnosis and treatment.

First, through the raw data analysis, we identified a total of 1,948 DEGs in OS (in the pre-preparation of the study, we tried to use |Log^{2}FC|>1 as the screening criterion, and obtained more than 10,000 DEGs. To obtain DEGs with a more significant difference among them, we set the screening criterion to |Log^{2}FC|>2 in the current study). Through enrichment analysis, it was found that the main biological behaviors involved in these DEGs included RNA biosynthetic process regulation of transcription, DNA-templated bounding membrane of organelle, Proteasome, Protein processing in endoplasmic reticulum, RNA transport, and so on. Among them, there were only two DEGs involving response to trichostatin A, ACTR2, and MEF2C, which drew

our attention. As mentioned above, trichostatin A is a recently discovered novel potential therapeutic option for OS [17]. MEF2C has been shown to have an effect on OS in previous studies [18, 19]. In these studies, they found that lncRNA KLF3-AS1 inhibited OS growth by silencing the miR-338-3p/MEF2C axis, so we focused our study on ACTR2. In our analysis, ACTR2 was downregulated in OS, but there are no clinical studies to confirm this. Therefore, our first task is to confirm the expression profile of ACTR2 in OS patients. In OS patients, we observed a significant reduction in ACTR2 expression, which is also consistent with the results of the raw data analysis described above. In addition, ACTR2 was significantly under-expressed in OS tissues, consistent with its expression in the peripheral blood of OS patients, supporting the above experimental results. Considering that the collection and preservation of blood samples are convenient and have higher clinical application value, we conducted a follow-up analysis based on peripheral blood ACTR2 levels. The results indicated that ACTR2 not only showed excellent diagnostic effects on OS occurrence, but also had a close relationship with the pathological staging of OS. Therefore, ACTR2 also has the potential to be an index for evaluating the condition of OS. This is of great clinical significance for OS, which still lacks reliable early markers and objective quantitative evaluation indicators. We believe that in the future, the detection of ACTR2 expression may effectively improve the early diagnosis rate of OS and provide a more reliable assessment for the prognosis of patients. Furthermore, in the prognosis analysis, we identified a close con-

nection between ACTR2 and patient prognosis, as well as the excellent predictive value of ACTR2 for the prognostic death of OS, which further validates our idea and demonstrates the important potential significance of ACTR2 in OS. However, in the study of Chen et al., ACTR2 was highly expressed in lymphomas [20], which is inconsistent with our findings, suggesting that ACTR2 may exert different biological effects in different diseases.

Therefore, to further characterize the mechanism of ACTR2's effect on OS, we validated the role of ACTR2 in OS by in vitro experiments. First of all, ACTR2 showed obvious low expression in U2OS and SAOS cells in OS, which is in line with the above experimental results. Subsequently, we intervened in OS cells using two vectors that elevated the expression of ACTR2, with the aim of observing whether there were differences in the biological behavior of OS under different expressions of ACTR2. The biological behavior tests revealed that the proliferative, migrating, and invasive capacities of OS cells decreased significantly after increasing ACTR2. This result is also consistent with the previous research results [21]. It suggests that elevating ACTR2 expression can reverse the progression of OS, and this reversal effect becomes more pronounced with higher ACTR2 expression. Based on the above, we can initially understand the mechanism of ACTR2 in OS, which can lay a reliable foundation for the subsequent molecular targeted therapy for ACTR2.

Genes can affect cell biological behavior by binding with DNAs, mRNAs, ncRNAs, etc., among which microRNAs are one of the most concerned pathways in the current clinical practice [22]. To further understand the influence of ACTR2 on OS, we screened its potential downstream microRNAs in the online target gene database, among which miR-374a-5p caught our attention. In a previous study, Lian et al. found that miR-374a-5p was closely related to the histological subtypes of OS, which was expected to be a biomarker of OS [23]. Hence, we suspect that the influence of ACTR2 on OS may be carried out through miR-374a-5p. Therefore, we also detected miR-374a-5p in cells and found that it was highly expressed. Furthermore, in the DLR assay, the fluorescence activity of ACTR2-WT was obviously inhibited by the miR-374a-5p mimic sequence.

After increasing ACTR2 levels, miR-374a-5p expression in OS cells decreased, indicating the presence of an obvious targeting relationship between them. Moreover, in vitro, silencing miR-374a-5p contributed to decreased OS activity, while increasing its expression promoted OS growth, which confirms our conjecture. It shows that the highly expressed miR-374a-5p also promotes the malignant development of OS and that miR-374a-5p is targeted and regulated by ACTR2. Previous studies have also shown the influence of miR-374a-5p on non-small cell lung cancer, gastric cancer, and other malignant tumors [24, 25], so miR-374a-5p may only be one of the action pathways of ACTR2, rather than the only one.

OS is a malignant and invasive tumor, and the EMT of OS cells is also one of the key points worthies of our attention because EMT directly determines the invasion and metastasis ability of cells [26, 27]. The detection results of EMT marker proteins revealed that increasing ACTR2 or silencing miR-374a-5p inhibited the EMT progress, while the reverse was true when miR-374a-5p was increased. This is also consistent with the results of the above-mentioned Transwell and wound-healing assays, which further help us to understand the influence mechanism of ACTR2 on OS, that is, the regulation of the EMT process.

Finally, in the tumorigenic assay in nude mice, we also saw significantly enhanced tumor growth in the NC-ACTR2 group and the lowest in the el-ACTR2-2 group, which validated the inhibitory effect of elevated ACTR2 expression on OS, suggesting that future molecular therapeutic pathways by targeting elevated ACTR2 expression may be a new direction for OS treatment.

All in all, ACTR2 is lowly expressed in OS and shows an excellent evaluation effect on the occurrence of OS and the prognostic death of patients. As ACTR2 expression elevates, the activity of OS cells are significantly inhibited, and its effect is related to the mediation of the effect via targeted regulation of miR-374a-5p. Understanding the role of ACTR2 in OS may provide a new research direction and reference for future diagnosis and treatment of OS.

However, this study still has some shortcomings. For instance, the limited clinical cases

may lead to a low representativeness of the test results. Besides, the included OS patients were of different stages, which may also affect the reliability of the experimental results regarding the influence of ACTR2 on the early evaluation of OS. Therefore, we need to conduct more experiments for further research. For example, rescue experiments can be carried out to validate the targeted regulation of ACTR2 on miR-374a-5p. In the follow-up, we will further study the role of ACTR2 in OS to provide a more reliable reference for clinical practice.

Disclosure of conflict of interest

None.

Address correspondence to: Fuguo Huang, Department of Orthopedic Surgery, West China Hospital, Sichuan University, No. 37 Guoxuexiang Street, Chengdu 610041, Sichuan, China. E-mail: huang-guofu8520@163.com

References

[1] Chen C, Xie L, Ren T, Huang Y, Xu J and Guo W. Immunotherapy for osteosarcoma: fundamental mechanism, rationale, and recent breakthroughs. Cancer Lett 2021; 500: 1-10.

[2] Eaton BR, Schwarz R, Vatner R, Yeh B, Claude L, Indelicato DJ and Laack N. Osteosarcoma. Pediatr Blood Cancer 2021; 68 Suppl 2: e28352.

[3] Gill J and Gorlick R. Advancing therapy for osteosarcoma. Nat Rev Clin Oncol 2021; 18: 609-624.

[4] Belayneh R, Fourman MS, Bhogal S and Weiss KR. Update on osteosarcoma. Curr Oncol Rep 2021; 23: 71.

[5] Corre I, Verrecchia F, Crenn V, Redini F and Trichet V. The osteosarcoma microenvironment: a complex but targetable ecosystem. Cells 2020; 9: 976.

[6] Shoaib Z, Fan TM and Irudayaraj JMK. Osteosarcoma mechanobiology and therapeutic targets. Br J Pharmacol 2022; 179: 201-217.

[7] Ferri A, Bianchi B and Ferrari S. Mandibular osteosarcoma: diagnosis and treatment. Curr Opin Otolaryngol Head Neck Surg 2022; 30: 154-160.

[8] Bridges MC, Daulagala AC and Kourtidis A. LNCcation: lncRNA localization and function. J Cell Biol 2021; 220: e202009045.

[9] Ali T and Grote P. Beyond the RNA-dependent function of LncRNA genes. Elife 2020; 9: e60583.

[10] Wang JY, Yang Y, Ma Y, Wang F, Xue A, Zhu J, Yang H, Chen Q, Chen M, Ye L, Wu H and Zhang Q. Potential regulatory role of lncRNA-miRNA-mRNA axis in osteosarcoma. Biomed Pharmacother 2020; 121: 109627.

[11] Huang X, Wu W, Jing D, Yang L, Guo H, Wang L, Zhang W, Pu F and Shao Z. Engineered exosome as targeted lncRNA MEG3 delivery vehicles for osteosarcoma therapy. J Control Release 2022; 343: 107-117.

[12] Bai Y, Chen Y, Chen X, Jiang J, Wang X, Wang L, Wang J, Zhang J and Gao L. Trichostatin A activates FOXO1 and induces autophagy in osteosarcoma. Arch Med Sci 2019; 15: 204-213.

[13] Deng Z, Liu X, Jin J, Xu H, Gao Q, Wang Y and Zhao J. Histone deacetylase inhibitor trichostatin a promotes the apoptosis of osteosarcoma cells through p53 signaling pathway activation. Int J Biol Sci 2016; 12: 1298-1308.

[14] He X, Fang J, Gong M, Zhang J, Xie R, Zhao D, Gu Y, Ma L, Pang X and Cui Y. Identification of immune-associated signatures and potential therapeutic targets for pulmonary arterial hypertension. J Cell Mol Med 2023; 27: 3864-3877.

[15] Cersosimo F, Lonardi S, Bernardini G, Telfer B, Mandelli GE, Santucci A, Vermi W and Giurisato E. Tumor-associated macrophages in osteosarcoma: from mechanisms to therapy. Int J Mol Sci 2020; 21: 5207.

[16] Ghafouri-Fard S, Shirvani-Farsani Z, Hussen BM and Taheri M. The critical roles of lncRNAs in the development of osteosarcoma. Biomed Pharmacother 2021; 135: 111217.

[17] Cheng DD, Yang QC, Zhang ZC, Yang CX and Liu YW. Antitumor activity of histone deacetylase inhibitor trichostatin A in osteosarcoma cells. Asian Pac J Cancer Prev 2012; 13: 1395-1399.

[18] Chen C and Liu L. Silencing of lncRNA KLF3-AS1 represses cell growth in osteosarcoma via miR-338-3p/MEF2C axis. J Clin Lab Anal 2022; 36: e24698.

[19] Tan J, Liang H, Yang B, Zhu S, Wu G, Li L, Liu Z, Li L, Qi W, Li S and Lin L. Identification and analysis of three hub prognostic genes related to osteosarcoma metastasis. J Oncol 2021; 2021: 6646459.

[20] Chen D and Jiang L. Upregulation of actin-related protein 2 (ACTR2) exacerbated the malignancy of diffuse large B-cell lymphoma through activating wnt signaling. Comput Math Methods Med 2022; 2022: 9351921.

[21] Li N and Zhan X. Identification of clinical trait-related lncRNA and mRNA biomarkers with weighted gene co-expression network analysis as useful tool for personalized medicine in ovarian cancer. EPMA J 2019; 10: 273-290.

[22] Zhao Z, Sun W, Guo Z, Zhang J, Yu H and Liu B. Mechanisms of lncRNA/microRNA interactions in angiogenesis. Life Sci 2020; 254: 116900.

[23] Lian F, Cui Y, Zhou C, Gao K and Wu L. Identification of a plasma four-microRNA panel as potential noninvasive biomarker for osteosarcoma. PLoS One 2015; 10: e0121499.

[24] Guo Q, Wang H, Xu Y, Wang M and Tian Z. miR-374a-5p inhibits non-small cell lung cancer cell proliferation and migration via targeting NCK1. Exp Ther Med 2021; 22: 943.

[25] Ji R, Zhang X, Gu H, Ma J, Wen X, Zhou J, Qian H, Xu W, Qian J and Lin J. miR-374a-5p: a new target for diagnosis and drug resistance therapy in gastric cancer. Mol Ther Nucleic Acids 2019; 18: 320-331.

[26] Lamouille S, Xu J and Derynck R. Molecular mechanisms of epithelial-mesenchymal transition. Nat Rev Mol Cell Biol 2014; 15: 178-196.

[27] Dongre A and Weinberg RA. New insights into the mechanisms of epithelial-mesenchymal transition and implications for cancer. Nat Rev Mol Cell Biol 2019; 20: 69-84.