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MicroRNA-1269a promotes the occurrence and progression of osteosarcoma by inhibiting TGF-β1 expression

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Abstract. – OBJECTIVE: MicroRNAs are endogenous, non-coding small RNAs that are capable of regulating biological and pathological processes. Previous studies have shown that microRNA-1269a serves as an oncogene. However, the role of microRNA-1269a in the pathogenesis of osteosarcoma (OS) has not been reported. The aim of this work was to investigate the expression characteristics of microRNA-1269a in OS and to further study its regulatory effects on the malignant progression of OS.

PATIENTS AND METHODS: The expres[illegible] microRNA-1269a in 61 pairs of OS tiss[illegible] and para-cancerous tissues was detected by [illegible]ntitative Real Time-polymerase Chain Rea[illegible]n (qRT-PCR). Chi-square test was used to ana[illegible] the relationship between micr[illegible]-1269a [illegible]pression and the characterist[illegible] patients including age, sex, clinic[illegible]tage [illegible] distant metastasis. Subsequent[illegible]icroRN[illegible]269a expression in OS cell lines [illegible] dete[illegible]. After knockdown of [illegible]icro[illegible]structing relevant [illegible]all inter[illegible]e RNA, biological performa[illegible] of MG63 a[illegible]2OS cells were accessed [illegible] counting k[illegible] (CCK-8), colony forma[illegible]n a[illegible]nswell ass[illegible]y. Meanwhile, the [illegible]rotein expr[illegible]ns of key genes in the EMT/[illegible]ad pathway w[illegible]etected by Western bl[illegible]inally, si-TGF-β1 (t[illegible]forming growth fact[illegible]1) was transfected into OS cells, and cell mi[illegible]n an[illegible]vasion were detected by transwell [illegible].

RESU[illegible]: MicroR[illegible]-1269a was highly ex[illegible]ed in [illegible] compared with para-can[illegible]c[illegible]s tissu[illegible]gh expression of microRNA[illegible]69a was [illegible]sitively correlated with young OS [illegible]tients and high rate of distant metastasis, [illegible] not correlated with age, sex and [illegible]neking [illegible]age. Kaplan-Meier survival curves [illegible]wed that high expression of microRNA-1269a [illegible]ignificantly associated with poor prognosis [illegible] OS. The knockdown of microRNA-1269a in MG63 and U2OS cells significantly inhibited cell proliferation, migration and invasion. Meanwhile, micr[illegible]69a knockd[illegible]n OS cells markedly [illegible]own[illegible]ted the expressions of TGF-β1, p-Smad2, [illegible]ad3, N-cad, Vimentin and [illegible]. Furthermo[illegible]GF-β1 knockdown rema[illegible]ecreased mig[illegible]y and invasive abilit[illegible] of OS cells.

CONCLUSION[illegible] MicroRNA-1269a is highly exp[illegible]sed in OS, [illegible]ich is remarkably correlated w[illegible]umor stag[illegible] distant metastasis and poor pro[illegible]is of O[illegible] In addition, microRNA-1269a prom[illegible]alignant progression of OS by regulating [illegible]GF-β1 expression.

[illegible]
Mic[illegible]RNA-1269a, TGF-β1, OS, Malignant progression.

Introduction

Osteosarcoma (OS) is the most common primary malignancy. In recent years, the incidence of OS has gradually increased, accounting for 20% of all primary malignancies[1-3]. OS is particularly common among adolescents and children, especially in those with 15-19 years old, with a prevalence of 5% of childhood tumors[4]. OS originates from immature tissues, such as osteoid tissue and mesenchymal tissue. Due to the rapid differentiation, proliferation, growth and invasion of OS cells, normal skeletal tissues are often affected and destroyed by malignant OS tissues[5]. As a bone tumor, OS frequently occurs in the proximal end of the long bone and the active end of the epiphysis. Meanwhile, it seriously affects the life quality of OS patients[1,6]. Although improved surgical resection, adjuvant radiotherapy and chemotherapy techniques can achieve extensive removal of OS tissues and affected extremities,

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the recurrence rate and mortality of OS remain high[2,6]. High malignancy and metastatic rate result in the poor prognosis of OS patients[6,7]. It is estimated that the 5-year survival of postoperative OS patients is only 10%. About 40% of OS patients die from lung metastasis. Hence, it is of great significance to search for more efficient therapeutic targets for OS, thereby improving its clinical outcomes[8-10].

The incidence of OS results from a complex network instead of a single mutation or deletion. The interaction of multiple unstable genome and proteins at the genetic level altogether leads to the pathogenesis of OS[11-13]. So far, drugs targeting the immune system, intracellular and extracellular signaling for OS therapy have been well explored[14,15]. In recent years, microRNAs, a small non-coding ribonucleic acid containing about 22 nucleotides, have been found to be crucial in the process of tumor pathophysiology[16].

MicroRNAs are a class of newly discovered non-coding small RNAs with approximately 19-22 nucleotides in length, which are widely found in eukaryotes[16,17]. Most microRNAs originate from gene spacer sequence, and are distributed in single-copy, multi-copy and other forms. By completely or incompletely binding to the 3'UTR of target genes, microRNAs degrade or inhibit the translation of the corresponding target genes. They may eventually regulate cell proliferation, apoptosis and differentiation. Unlike siRNA, microRNAs have multiple target genes. Meanwhile, their regulation on cell proliferation, apoptosis and cell cycle are [illegible] conditions[19]. Studies have shown the involvement of microRNAs in the incidence and development of human diseases, especially in tumor diseases[20]. It is suggested that the functions of different types of microRNAs vary greatly and they may serve as oncogenes or tumor-suppressor genes[21]. MicroRNA-1269a is highly expressed in a variety of malignant tumors as an oncogene[22]. In this study, we explored the expression of microRNA-1269a in OS tissues and cell lines. Moreover, we also explored its [illegible] role in the development of OS. Our study aimed to provide new directions for the treatment of OS.

Transforming growth factor-β (TGF-β1) is a member of the TGF superfamily, and its potential role in tumors has been well concerned[23]. It is reported that TGF-β1 is highly expressed in multiple malignant tumors, such as colon cancer, bladder cancer, ovarian cancer, kidney cancer, lymphoma, etc. TGF-β1 also exerts an essential role in tumor invasion and metastasis[24-26]. The effects of TGF-β1 on tumors are not only complicated, but also contradictory. In the early stage of tumorigenesis, TGF-β1 can inhibit cell proliferation, initiate cell differentiation and induce apoptosis. However, TGF-β1 promotes the invasion and metastasis of tumor cells during the malignant transformation[27]. Therefore, we speculated whether microRNA-1269a regulated TGF-β1 expression, thereafter influencing the biological performances of OS.

Patients and Methods

Patients and Samples

61 OS patients who underwent surgical resection in our hospital were enrolled, including 39 male and 22 female cases aged 6-48 (mean age of [illegible]±9.58 years). All patients were diagnosed and confirmed as OS. No significant differences in age and gender were found in these patients. OS tissues and para-cancerous tissues were harvested and preserved in -80°C. Informed consent was obtained from enrolled patients and their families. This study was approved by the Ethics Committee.

Cell Lines

OS cell lines (HOS, U2OS, SOSP-9607, MG63, 143B and SaOS-2) and human osteoblast cell line (hFOB) were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). Cells were cultured in Roswell Park Memorial Institute-1640 (RPMI -1640) (HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) and maintained in a 5% CO_2 incubator at 37°C. Culture medium was replaced every 2-3 days. Cell passage was performed until 90% of confluence.

Cell Transfection

Negative control (si-NC) and si-microRNA-1269a were provided by GenePharma Biotechnology Co., Ltd. (Shanghai, China). Cells were first seeded into 6-well plates. When the confluency was up to 70%, cell transfection was performed according to the manufacturer's instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Cells were harvested 48 h later for the following experiments.

Cell Counting Kit-8 (CCK-8)

48 h after transfection, OS cells were seeded into 96-well plates with 2000 cells per well. After

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culturing for 6 h, 24 h, 48 h and 72 h, respectively, CCK-8 (Dojindo Laboratories, Kumamoto, Japan) reagent was added in each well, followed by incubation for 2 h in the dark. Finally, the optical density (OD) value of each well was measured using a microplate reader.

Colony Formation Assay

48 h after transfection, OS cells were seeded into 6-well plates with 200 cells per well. OS cells were cultured in complete medium for 2 weeks. Culture medium should not be replaced in the first week to prevent discarding un-adherent cells. Meanwhile, the medium was replaced twice in the second week. Until colony formation, the cells were washed with phosphate-buffered saline (PBS) twice and fixed with 2 ml of methanol for 20 min. Subsequently, the cells were washed with PBS and stained with 0.1% crystal violet staining solution for 20 min. Finally, formed colonies were observed and captured using a microscope.

Transwell Assay

48 h after transfection, OS cells were digested and re-suspended in serum-free medium [illegible] density was adjusted to 5.0×10^5/mL. Tr[illegible] chamber containing Matrigel (BD Biosc[illegible]es, Franklin Lakes, NJ, USA) or not was plac[illegible] 24-well plates. 200 µL of cell suspension co[illegible]ning 1.0×10^5 cells was added to [illegible]per cha[illegible]ber. Meanwhile, 700 µl of [illegible]ntaining 20% FBS was added to t[illegible] lower c[illegible]ber. 48 hours later, the chambe[illegible] remo[illegible] and the cells were fixed with 4[illegible] pa[illegible] min. After washin[illegible]e cells, [illegible]ere stained with 0.2% cryst[illegible]let for 20 [illegible] The inner layer cells we[illegible] removed. [illegible]ds were randomly sele[illegible]ed for e[illegible]ample. The cells were captured [illegible] the number [illegible]netrating cells was calcula[illegible].

Qu[illegible]tativ[illegible]eal Time-polymerase Chain Reac[illegible]T-PCR)

Total [illegible] was e[illegible]acted from OS cell lines [illegible]ssues u[illegible]zol reagent (Invitrogen, Carlsb[illegible] CA, US[illegible] Extracted RNA was reversely tra[illegible]ribed into cDNA using Primescript RT Rea[illegible], Otsu, Shiga, Japan). QRT-PCR was [illegible]rformed using SYBR® Premix Ex TaqTM (Ta[illegible], Otsu, Shiga, Japan), and StepOne Plus Re[illegible] PCR System (Applied Biosystems, Foster City, CA, USA). Primers used in this study were as follows: microRNA-1269a: 5'-CCCTCACAGCAATTTTATAGCATCT-3'; U6: 5'-TGCGGGTGCTCGCTTCGGCAGC-3'; TGF-β1: forward: 5'-CTCTCCGACCTGCCACAGA-3', reverse: 5'-AACCTAGATGGGCGCGATCT-3'; β-actin: forward: 5'-CCTGGCACCCAGCACAAT[illegible] verse: 5'-TGCCGTAGGTGTCCCTTT[illegible] relative expression levels of mRNA [illegible]ere calculated by the $2^{-\Delta\Delta Ct}$ method and a[illegible]zed by ABI Step One software.

Western Blot

OS cells were lysed, [illegible]ken on ice for [illegible] and centrifuged at 4[illegible]000 g [illegible]n for 15 m[illegible]n. The concentration of to[illegible] was dete[illegible]mined using the bicinc[illegible]nic ac[illegible]A) pro[illegible] determination kit [illegible]rce, Rockf[illegible]SA). The extracted p[illegible]s separated [illegible]0% sodium dodecyl s[illegible]hate-[illegible]crylamide gel electrophoresis (SDS-PAGE) [illegible] transferred onto polyvin[illegible] difluoride ([illegible]) membranes (Milli[illegible]e, Billerica, MA, US[illegible]). Western blot was [illegible]ducted according to the standard procedures. [illegible]mary antibo[illegible] (TGF-β1, p-Smad2, p-Smad3, N[illegible] Vimenti[illegible]MMP9 and GAPDH) and secon[illegible]antibo[illegible]s (anti-mouse and anti-rabbit) were p[illegible] by Cell Signaling Technology (Danvers, MA, USA).

[illegible]al Analysis

GraphPad Prism 6 V6.01 software (La Jolla, CA, USA) was used for all statistical analyses. Data were expressed as mean ± standard deviation. *t*-test was used to compare the difference between the two groups followed by Least Significant Difference as the Post-hoc test. Kaplan-Meier method was used to calculate the survival time of OS patients, and the difference between the curves was compared by Log-rank test. $p<0.05$ was considered statistically significant.

Results

MicroRNA-1269a was Highly Expressed in OS Tissues and Cell Lines

We first examined the expression of microRNA-1269a in 61 pairs of OS tissues and para-cancerous tissues by qRT-PCR. The results showed that microRNA-1269a was highly expressed in OS tissues when compared with the para-cancerous ones, and the difference was statistically significant (Figure 1A, 1B). Similarly, microRNA-1269a expression in OS cell lines was determined as well. Compared with human osteoblast cell line (hFOB), microRNA-1269a was highly

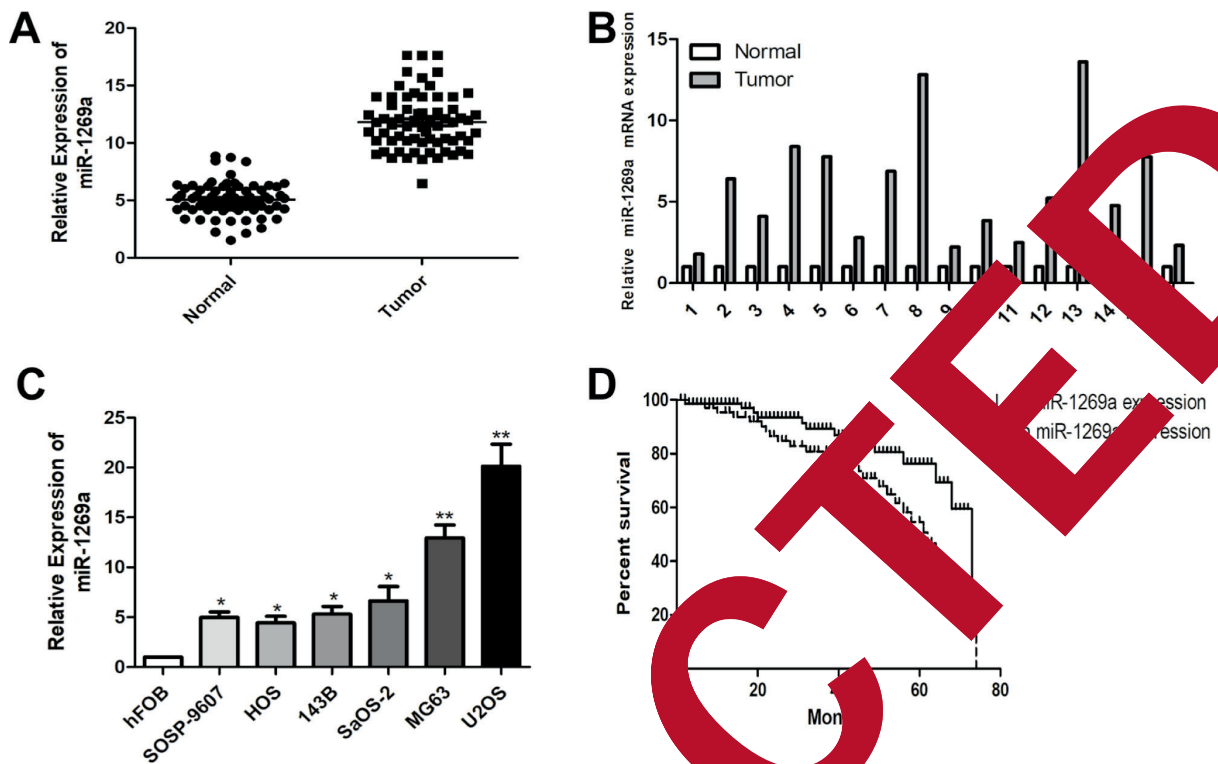

Figure 1. **MicroRNA-1269a was highly express[illegible] tissues and ce[illegible]. *A-B,*** MicroRNA-1269a expression in OS tissues and para-cancerous tissues detected by qR[illegible]R. [illegible]NA-1269a expression in OS cell lines. ***D,*** Survival curves of microRNA-1269a expression in OS patients. Da[illegible]e expr[illegible] ± SD, *p<0.05, **p<0.01, ***p<0.001.

expressed in OS cells, and th[illegible] was statistically significant (Figu[illegible]C). A[illegible]g them, MG63 and U2OS cell lin[illegible]ressed [illegible] highest level of microRNA-1[illegible]9a, [illegible] for subsequent exp[illegible]ents.

MicroRNA-1[illegible]ression w[illegible] Correlated [illegible]ith Cl[illegible] Stage, Lymph Node M[illegible]stasis, Dis[illegible] Metastasis, and O[illegible]rall Survival in [illegible]atients

B[illegible] on the expression of microRNA-1269a in [illegible]sues [illegible]s patients were divided into the high [illegible]on grou[illegible]nd the low expression [illegible]up. T[illegible]mber [illegible]atients in each group was [illegible]ded. C[illegible] test was used to analyze the rel[illegible]nship be[illegible]en microRNA-1269a expressio[illegible]nd the clinical characteristics of OS patien-[illegible]ge, sex, clinical stage and distant [illegible]etastasis. As shown in Table I, high expression [illegible]icroRNA-1269a was positively correlated w[illegible]oung OS patients and high rate of distant metastasis, whereas was not correlated with age, sex and Enneking stage. Subsequently, follow-up data were collected to explore the relationship between microRNA-1269a expression and the prognosis of OS patients. Kaplan-Meier survival curves showed that high expression of microRNA-1269a was significantly associated with poor prognosis of OS patients. The higher expression level of microRNA-1269a indicated remarkably worse prognosis of OS (p<0.05) (Figure 1D). These results indicated that microRNA-1269a might serve as a new biological indicator for predicting OS prognosis.

Knockdown of MicroRNA-1269a Inhibited Cell Proliferation

To explore the effect of microRNA-1269a on the proliferation of OS cells, we first constructed the microRNA-1269a interference model. Meanwhile, the transfection efficiency of si-microRNA-1269a was determined (Figure 2A, 2B). The CCK-8 results found that OS cells transfected with si-microRNA-1269a present a significantly decreased proliferative rate than those transfected with si-NC (Figure 2C, 2D). Similarly, microRNA-1269a knockdown remarkably decreased the colony formation ability of the OS cells

(Figure 2E).

Knockdown of MicroRNA-1269a Inhibited Cell Migration and Invasion

Transwell assay was conducted to explore the regulatory effect of microRNA-1269a on the migratory and invasive abilities of OS cells. Compared with MG63 cells transfected with si-NC, the number of penetrating cells in those transfected with si-microRNA-1269a was markedly decreased, suggesting the inhibited migratory and invasive abilities (Figure 3A, 3B). Similar results were obtained in U2OS cells (Figure 3C, 3D).

TGF-β1 Modulated MicroRNA-1269a Expression in Human Osteosa[illegible]ma Cells

To further explore the mechan[illegible] of microRNA-1269a in promoting the malign[illegible]gression of OS, we found a possible [illegible]lations[illegible]tween TGF-β1 and microRNA-1[illegible]a through [illegible]for-

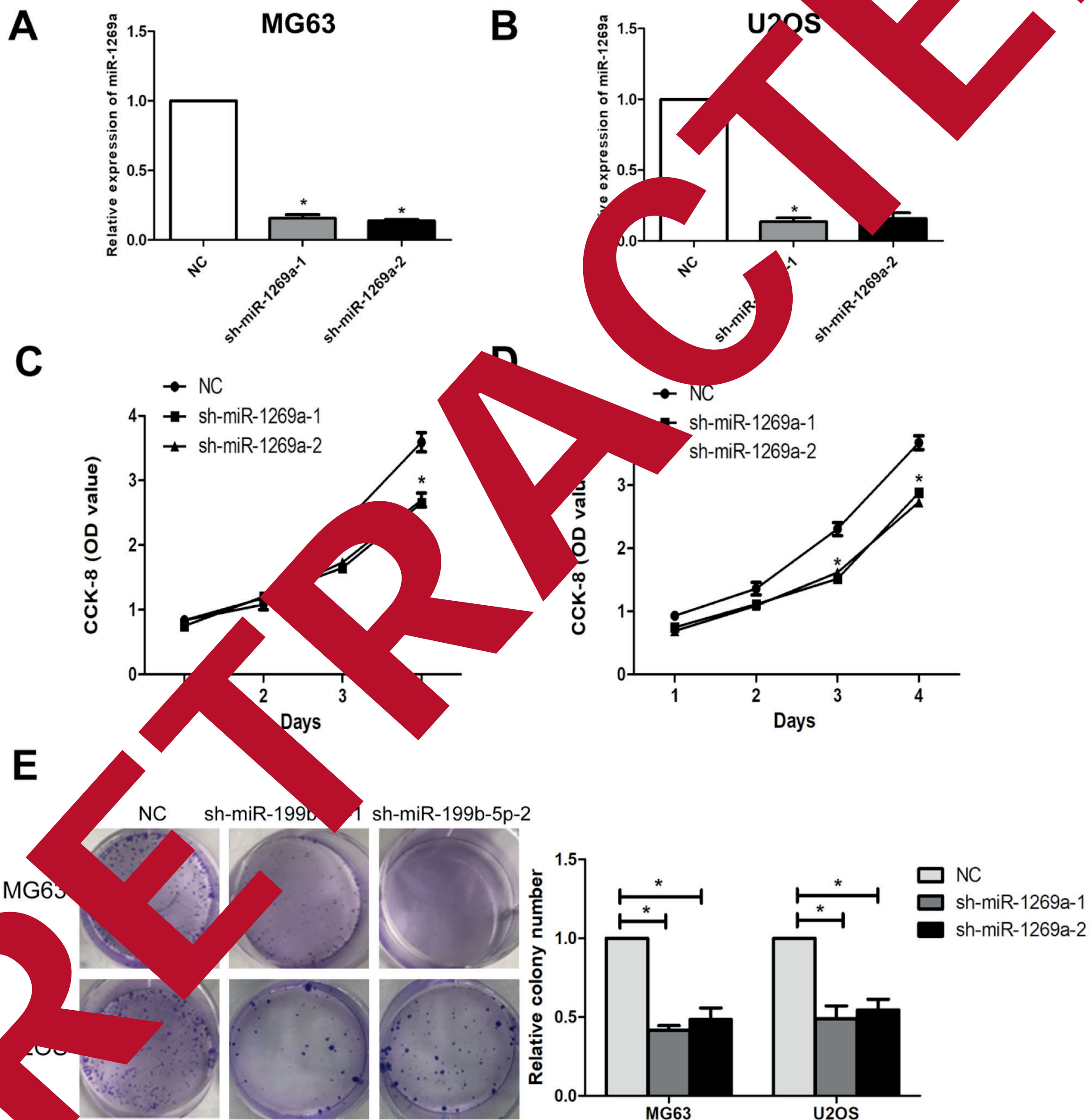

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Figure 2. Knockdown of microRNA-1269a inhibited cell proliferation. ***A-B,*** Transfection efficacy of si-microRNA-1269a. ***C-D,*** Proliferative rate of MG63 and U2OS cells detected by CCK-8 assay after transfection of si-microRNA-1269a. ***E-F,*** Colony formation of MG63 and U2OS cells after transfection of si-microRNA-1269a. Data were expressed as mean ± SD, $*p<0.05$.

Table I. Association of miR-1269a expression with clinicopathologic characteristics.of osteosarcoma.

Parameters	Number of cases	miR-1269a expression		*p*-value
		Low (%)	High (%)	
Age (years)				[illegible]
<21	27	10	17	
≥21	34	22	12	
Gender				[illegible]
Male	39	19	20	
Female	22	10	12	
Enneking stage				0.796
IA	8	4	4	
IIA	15	5	10	
IIB	29	9	20	
III	9	3	[illegible]	
Distance metastasis				[illegible]
No	34	20	14	
Yes	27	9	[illegible]	

matics analysis. QRT-PCR and Western blot results detected the mRNA and protein expressions of TGF-β1 in 61 pairs of OS tissues and para-cancerous tissues. Compared with para-cancerous tissues, TGF-β1 was lowly expressed in O[illegible] sues at both mRNA and protein levels [illegible] [illegible]. Additionally, TGF-β1 was also lowly expres-s[illegible] in OS cell [illegible]s than that of hFOB cell line ([illegible]re 4B). Su[illegible]quently, we selected 16 pairs fro[illegible] pairs [illegible]OS tissues and para-cancerous tissues [illegible]ore the correlation between mi-[illegible]roRNA-1269a and TGF-β1. QRT-PCR data indi-

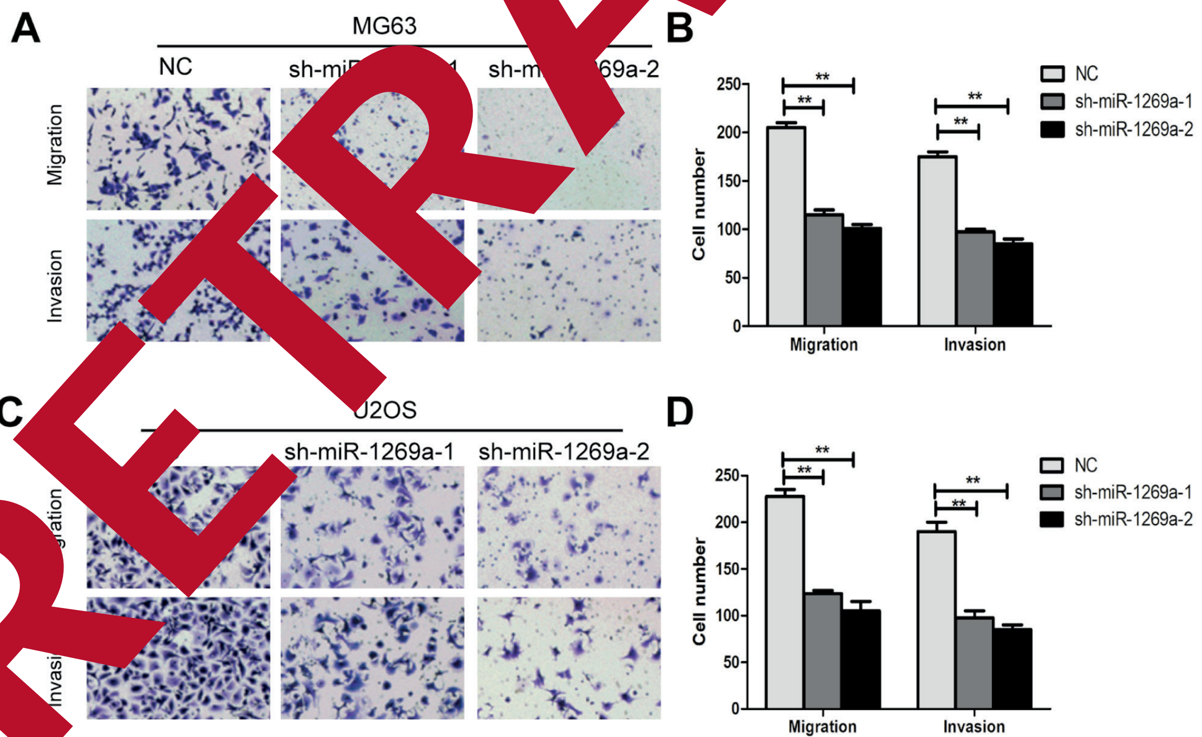

Figure 3. Knockdown of microRNA-1269a inhibited cell migration and invasion. ***A-B,*** Invasive and migratory abilities in MG63 cells after transfection of si-microRNA-1269a. ***C-D,*** Invasive and migratory abilities in U2OS cells after transfection of si-microRNA-1269a. Data were expressed as mean ± SD, $**p<0.01$.

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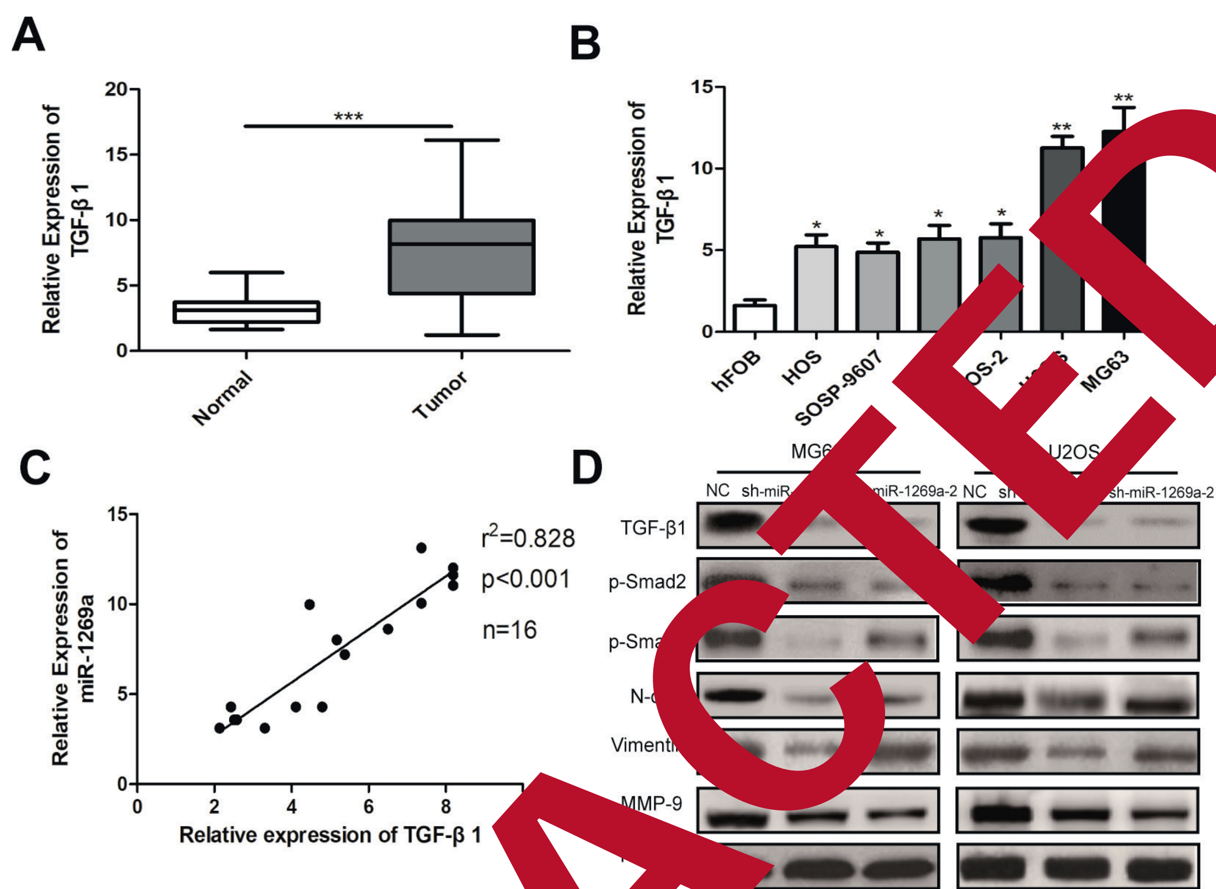

Figure 4. **TGF-β1 modulated microR 269a expr n human osteosarcoma cells.** ***A,*** TGF-β1 expression in OS tissues and para-cancerous tissues. ***B,*** T ession in lls. ***C,*** MicroRNA-1269a expression was negatively correlated with TGF-β1 expression in OS cell , Knoc n of mic -1269a downregulated the expressions of TGF-β1, p-Smad2, p-Smad3, N-cad, Vimentin and P9 in OS s. Data we xpressed as mean ± SD, *p<0.05, **p<0.01, ***p<0.001.

cated that the exp on of micror 269a was negatively cor th TGF-β1 e ession in OS tissues (Figure 4C).

Further re, small rence plasmid of TGF-β1 as constructed. transfection efficac f si-TGF-β1 was verified by qRT-PCR and tern (Figure 5A, 5B). Further results sho knockd n of TGF-β1 markedly creased migra and invasive abilities of MG63 s cells (Figure 5C, 5D).

Kn kdown of MicroRNA-1269a ated Expressions of Key enes in EMT/Smad Pathway

analyze the potential mechanism of micr A-1269a in regulating the biological performances of OS cells, we detected the expressions of relative genes in the EMT/Smad pathway. Western blot results revealed that microRNA-1269a knockdown in OS cells markedly downregulated the protein expression levels of TGF-β1, p-Smad2, p-Smad3, N-cad, Vimentin and MMP9 (Figure 4D).

Discussion

OS is a primary malignancy with high heterogeneous, which mainly affects adolescents and children[1,4]. Long bones, such as femur, tibia, fibula and ilium, are frequently involved in OS. The prognosis and survival rate of OS patients remain poor even after surgery[4,6]. Currently, surgery combined with multiple chemotherapy drugs is the preferred treatment for OS. However, drug resistance and adverse events resulted from chemotherapy drugs, including methotrexate, doxorubicin, cisplatin and ifosfamide, have greatly

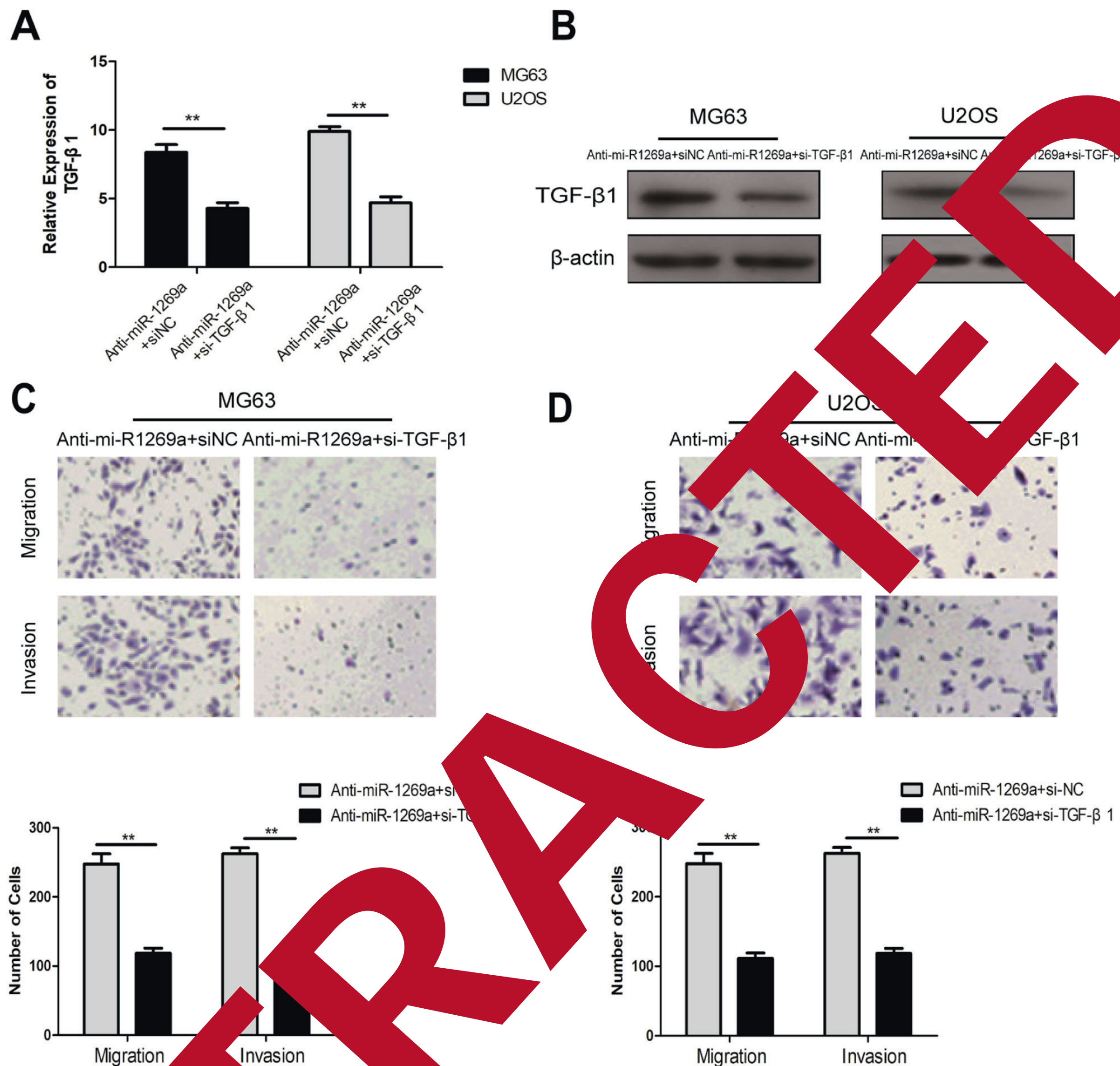

Figure 5. TGF[illegible] effec[illegible] migration and invasion of OS. ***A-B,*** Transfection efficacy of TGF-β1. ***C-D,*** Migration and invasion of [illegible]MG63 and U2[illegible]lls after knockdown of TGF-β1. Data were expressed as mean ± SD, *p<0.05, **p<0.01, ***p<0.00[illegible]

restric[illegible] therape[illegible]e efficiency[8,9]. Therefo-[illegible] it is u[illegible] to ful[illegible]lucidate the pathogenesis [illegible] to se[illegible] novel therapeutic targets[11,12]. Ex[illegible]ations o[illegible]e molecular mechanism, intera[illegible]n between genome and proteome, as well [illegible]r signaling pathways lay the solid [illegible]undation for developing a new treatment for [illegible]. Furthermore, ideal treatment should reduce th[illegible]e of lung metastasis and tumor recurrence, thereby improving the survival rate of OS[12,15].

Site-specific gaps or cleavage regions that occur on the metaphase chromosomes of microRNA-1269a are regions of genomic instability[22]. Fragile sites are usually stable in somatic cells, but are deleted or rearranged in many tumor cells. MicroRNAs near the fragile sites are closely related to tumorigenesis, often accompanied by chromosome breakage[19,20]. Previous studies have found that under pathological conditions, microRNA-1269a is closely related to the malignant progression of tumor cells. MicroRNA-1269a can regulate cell cycle, tumor invasion, hematopoietic differentiation, immune response and other biological functions[16-18]. Multiple target

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genes of microRNA-1269a form a complex network regulation system, including c-Myc, BNIP3, IRS-1 and other cell cycle-related genes. This may eventually affect the incidence and development of tumors[19,21,22]. However, the effects of microRNA-1269a on OS cells and its specific mechanism have not been reported yet. Previous studies have mostly focused on the role of microRNA-1269a in regulating the proliferation and apoptosis of tumor cells. Few studies have reported the regulatory effects of microRNA-1269a on cell invasion and metastasis. The strong invasive and metastatic abilities of OS are the main reasons for its poor prognosis. In this work, we explored the role of microRNA-1269a in the proliferation, invasion and metastasis of OS cells. The results showed that microRNA-1269a was highly expressed in OS tissues, which was correlated with tumor stage, lymph node metastasis, distant metastasis and poor prognosis. We observed that microRNA-1269a might be an oncogene in the OS development. Furthermore, the knockdown of microRNA-1269a significantly inhibited the proliferation, migration and invasion of OS cells. However, the specific mec[illegible]sm remains unclear.

The TGF-β superfamily is a type of cyt[illegible]es with multiple biological effects. Mature T[illegible]β includes 5 subtypes, namely TGF-β1-5. Am[illegible] them, TGF-β1 is currently kn[illegible] be ass[illegible]ciated with malignant tum[illegible]anwhil[illegible] TGF-β1 exerts a dual eff[illegible] on tum[illegible]. In the early stage of tumorige[illegible] it ca[illegible]ibit the proliferation of tumo[illegible]ells[illegible]tes the proliferatio[illegible] tumor [illegible] the advanced stage[25,26]. In[illegible]ion, besides [illegible]β1 itself, TGF-β1 recep[illegible] and Smad [illegible]lso important in tu[illegible]origene[illegible]. In this experiment, rescue ex[illegible]iments foun[illegible]t there was a mutual re[illegible]ation between m[illegible]NA-1269a and TGF[illegible]. In-depth researches on the regulatory rol[illegible]TGF-[illegible] in the incidence and progression o[illegible] may co[illegible]ibute to improving the [illegible]gnosi[illegible]tmen[illegible]nd prognosis of tumors. [illegible]undou[illegible]rings good news to tumor pa[illegible]ts and th[illegible]amilies.

[illegible] verify whether microRNA-1269a promoted t[illegible]al progression of OS by regulating [illegible]F-β1, we detected the TGF-β1 expression in [illegible]ells after microRNA-1269a knockdown. Our st[illegible]ndicated that the TGF-β1 expression was regulated by microRNA-1269a, showing that microRNA-1269a promoted the malignancy of OS probably by regulating TGF-β1.

Conclusions

We showed that microRNA-1269a is highly expressed in OS, which is remarkably co[illegible] with tumor stage, distant metastasis an[illegible] gnosis of OS. In addition, microRN[illegible]269a promotes the malignant progression [illegible]S by regulating TGF-β1 expression.

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Conflict of [illegible]

The Author[illegible]eclare [illegible]y have no co[illegible]lict of interest.

Refere[illegible]ces

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