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Decreased miR-134 expression and its tumor-suppressive function in human osteosarcoma

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ABSTRACT. Dysregulation of microRNA (miR) is often associated with cancer development and progression. Aberrant expression of miR-134 has been found in some types of cancer. However, its expression and function in osteosarcoma remain unclear. The aim of this study was to explore the effects of miR-134 in osteosarcoma tumorigenesis and development. The expression level of miR-134 was quantified by real-time reverse transcription-polymerase chain reaction in human osteosarcoma cell lines and tissues. The effects of miR-134 on MG-63 cell phenotypes and tumorigenicity *in vivo* were observed using flow cytometry, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, transwell invasion, migration, and scratch migration assays. MiR-134 was significantly downregulated in osteosarcoma cell lines and clinical specimens. Decreased miR-134 expression was significantly associated with large tumor size, positive distant metastasis, and advanced clinical stage. Low miR-134 expression in osteosarcoma was an independent

predictor of poor survival. Overexpression of miR-134 inhibited MG-63 cell proliferation, invasion, and migration, promoted cell apoptosis *in vitro*, and suppressed tumorigenicity *in vivo*. These findings indicate that miR-134 may act as a tumor suppressor in osteosarcoma and could serve as a novel therapeutic agent for miRNA-based therapy.

Key words: MiR-134; Osteosarcoma; Prognosis; Proliferation; Metastasis

INTRODUCTION

Osteosarcoma is the most common primary bone tumor that occurs predominantly in adolescents and young adults (Mirabello et al., 2009). It frequently localizes to the distal femur and proximal tibia regions, and has a highly malignant tendency to rapidly destroy the surrounding tissues and to metastasize (Ottaviani and Jaffe, 2009). Despite advances in multiple therapeutic strategies, including chemotherapy, surgery, and sometimes radiotherapy, overall clinical outcomes for osteosarcoma patients remain unsatisfactory, especially for patients with metastasis or recurrent osteosarcoma. Previous studies have demonstrated diverse genetic alterations in osteosarcoma, but the highly complex molecular mechanisms underlying its initiation and progression have not been fully elucidated. Therefore, it is necessary to find novel markers for osteosarcoma which can accurately identify biological characteristics of tumors, improve therapeutic strategies, and predict clinical outcomes.

MicroRNAs (miRs) are a class of short (about 22 nucleotides), endogenous, single-stranded, non-protein-coding RNAs that directly bind to the 3' untranslated regions of target mRNAs, leading to mRNA degradation or translational suppression (Bartel, 2009). It is now well known that miRs are involved in many cellular biological processes such as cell growth, apoptosis, development, differentiation, and tissue homeostasis (Bartel, 2004). Accumulating evidence also suggests that miRs play an essential role in the biology of human cancers, and may provide a new and promising way to deal with them (Heneghan et al., 2010). Gene expression profiling studies have revealed that miR expression may be an excellent biomarker for cancer diagnosis and prognosis estimation (Eisenmann et al., 2012; Takahashi et al., 2012). In terms of osteosarcoma, abnormal expression of several miRs such as miR-206 (Bao et al., 2013), miR-34a (Wu et al., 2013), and miR-145 (Tang et al., 2013) have been reported. Novello et al. (2013) found that miR-1 and miR-133b may regulate cell proliferation of osteosarcoma cells (Novello, et al., 2013). Cai et al. (2013) indicated that overexpression of miR-210 is strongly associated with large tumor size, chemotherapy resistance, positive metastasis, and decreased overall survival in pediatric osteosarcoma (Cai, et al., 2013). In Wang et al. (2013) study, upregulated expression of miR-214 was linked to tumor progression and shorter overall and progression-free survival (Wang, et al., 2013). These findings suggest that miRs act not only as diagnostic and prognostic markers but also as potential therapeutic targets of osteosarcoma.

The miR-134 gene is located at 14q32, and is involved in several physiological and pathological processes. For example, miR-134 can promote vertebrate central nervous system development (including neuron, cylindrical axon, and dendrite) (Gaughwin et al., 2011); miR-134 is regarded as a potential plasma biomarker for the diagnosis of acute pulmonary embolism (Xiao et al., 2011). Recently, miR-134 has been implicated in the pathogenesis of several types of tumors and plays dual roles in different tumors. It is significantly increased in head and neck squamous-cell carcinoma (HNSCC) and acts as a potential oncogene (Liu et al., 2014), while it is downregulated and

acts as a candidate tumor suppressor in gastrointestinal stromal tumors (GISTs) (Haller et al., 2010), gliomas (Lages et al., 2011), non-small cell lung cancer (Li et al., 2012), hepatocellular carcinoma (Yin et al., 2013), and prostate cancer (Wang et al., 2011). However, very little is currently known about the links of miR-134 dysregulation to clinicopathological characteristics of osteosarcoma, and the functional attributes of miR-134 associated with osteosarcoma progression remain elusive.

In the present study, we examined miR-134 expression in osteosarcoma tissue samples and cell lines using real-time reverse transcription-polymerase chain reaction (RT-PCR). The association of miR-134 levels with clinicopathological features and prognosis was also analyzed. Furthermore, we investigated the effects of miR-134 on the biological behavior of osteosarcoma cells.

MATERIAL AND METHODS

Tissue samples and cell lines

A total of 112 pairs of osteosarcoma specimens and corresponding noncancerous bone tissue samples were collected from the Department of Orthopedics, The Affiliated Hospital of Yangtze University, between January 2006 and December 2009. All the samples were biopsy materials and had received no therapy. They were frozen and stored at -80°C until RNA extraction. The clinicopathological information for these patients is shown in Table 1.

Clinical stage was classified according to the sixth edition of the tumor-node-metastases (TNM) classification of the International Union Against Cancer. Follow-up information was available for all patients. Overall survival was defined as the time from the date of diagnosis to death or, for living patients, the date of last follow-up. Informed consent was obtained from each patient, and the experiments were approved by the ethics committee of the Medical School of Yangtze University, Jingzhou, China.

Human osteosarcoma cell lines (HOS, Saos-2, U2OS, and MG-63) and human osteoblasts were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were maintained in Dulbecco's modified Eagle medium (DMEM), supplemented with 10% heat-inactivated fetal bovine serum, 100 units/mL penicillin G sodium, and 100 µg/mL streptomycin sulfate. Cultures were incubated in a humidified atmosphere of 5% CO₂ at 37°C.

RNA extraction and quantitative real-time RT-PCR

Total RNA was extracted from the cells and tissues with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer instructions. cDNA was reverse-transcribed from total RNA samples using specific miR primers from the TaqMan MicroRNA Assays and reagents from the TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). Products were amplified by PCR using a TaqMan Universal PCR Master Mix kit (Applied Biosystems). Small nucleolar RNA U6 was used as an internal standard for normalization. All reactions were carried out in triplicate, and the 2^{-ΔΔCT} method ($\Delta CT = CT_{miR-134} - CT_{U6}$) was used to quantify the relative amount of miR-134.

Cell transfection

MiR-134 mimics and negative control (NC) RNA-oligonucleotides, which are non-homologous to the human genome, were obtained from GenePharma, China. After seeding into each well of a

24-well plate and incubating overnight, MG-63 cells were transfected with miR-134 mimics or NC at a concentration of 50 nM using Lipofectamine 2000 (Invitrogen) transfection reagent according to the manufacturer instructions. Cells were collected for further experiments 48 h post-transfection.

Analysis of cell proliferation *in vitro*

The *in vitro* cell proliferation was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. Briefly, cells were seeded on a 96-well plate (2×10^4 cells per well) and incubated at 37°C after transfection. At different time points (24, 48, 72, or 96 h), the culture medium was removed and replaced with fresh medium containing 0.5 mg/mL MTT. Cells were then incubated for another 4 h and resolved by dimethyl sulfoxide (Sigma, St Louis, MO, USA). The absorbance was measured at 490 nm.

Apoptosis assay

For analysis of apoptosis, MG-63 cells were collected, diluted to a concentration of 1×10^6 cells/mL, and washed three times with ice-cold phosphate-buffered saline. 48 h after transfection. The cell suspension (200 μ L) was then taken and the cells were labeled with annexin V-fluorescein isothiocyanate and propidium iodide using an apoptosis detecting kit (Invitrogen). Finally, the samples were incubated for 15 min at room temperature in the dark. Stained cells were detected by FACSCalibur and data were analyzed with the CellQuest software (both from Becton, Dickinson and Company (Franklin Lakes, NJ, USA)).

Transwell invasion and migration assays

Cell migration and invasion assays were performed using a 24-well transwell plate (8-mm pore size, Corning, New York, NY, USA). For the migration assay, 4×10^5 MG-63 cells suspended in serum-free DMEM were added to the upper chamber lined with non-coated membrane. DMEM containing 20% fetal bovine serum was added to the lower chambers as a chemoattractant. For the invasion assay, chamber inserts were coated with 2 mg/mL of Matrigel (Becton, Dickinson and Company). After 24 h transfection, the non-filtered cells were gently removed with a cotton swab. Filtered cells located on the lower side of the chamber were stained with 0.1% crystal violet (Sigma) and counted under a microscope (Olympus Corp., Tokyo, Japan).

Scratch migration assay

A scratch migration assay was performed to observe the influence of miR-134 on MG-63 cell migration. When the cells transfected with miR-134 mimics or NC were grown to confluence, a scratch in the cell monolayer was made with a cell scratch spatula. After the cells had been incubated under standard conditions for 24 h, pictures of the scratches were taken using a digital camera system coupled with a microscope (Olympus Corp., Tokyo, Japan).

Tumorigenicity *in vivo*

Tumor formation was studied by establishing a xenograft model. Animal experiments were

approved by the Committee of Animal Ethics of our hospital, and were conducted in accordance with the Guide for the Care and Use of Animals for research purposes. Commercial lentiviral vectors containing miR-134 (LV-miR-134) (GeneChem Co. Ltd, Shanghai, China) were used to infect MG-63 cells according to the manufacturer instructions. The empty lentiviral construct served as a negative control (LV-NC). The stably transfected cells were selected using puromycin (1.5 $\mu\text{g}/\text{mL}$). Eight female BALB/c athymic nude mice (3-4 weeks old) were purchased from the Model Animal Research Center of Nanjing University. MG-63 cells (1×10^6 cells; 100 μL cell suspension) stably overexpressing miR-134 or NC were inoculated subcutaneously into the left or right flanks of the mice, respectively. Bidimensional tumor measurements were taken with vernier calipers every 4 days, and tumor volume (mm^3) was calculated using the formula $\text{volume} = (\text{length} \times \text{width}^2)/2$. The mice were killed and the tumors were weighed 3 weeks after inoculation.

Statistical analysis

All data are reported as means \pm standard deviation. Statistical analysis was carried out using the SPSS 15.0 software (SPSS Inc., Chicago, IL, USA). Differences between groups were analyzed by the Student *t*-test or chi-square test. Survival curves were constructed using the Kaplan-Meier method and compared by log-rank tests. Cox proportional hazards regression analysis was used for univariate and multivariate analyses. Probabilistic values (*P*-values < 0.05) were considered to be significant.

RESULTS

Decreased miR-134 expression in osteosarcoma tissues and cell lines

We performed a quantitative RT-PCR analysis to determine the expression level of miR-134 in osteosarcoma tissues and cell lines. Our results showed that the level of miR-134 was significantly reduced in osteosarcoma tissues (8.69 ± 2.01) compared with that in the paired noncancerous bone tissues (19.58 ± 3.99) ($P < 0.001$, Figure 1A).

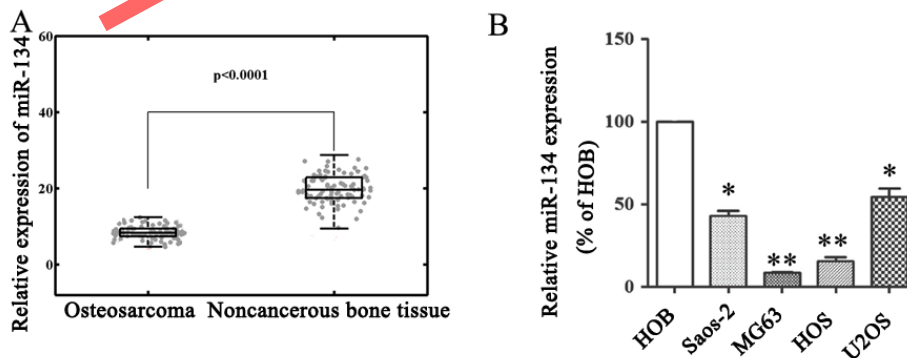


Figure 1. Relative expression levels of miR-134 in osteosarcoma tissues and cell lines. **A.** MiR-134 expression was significantly lower in osteosarcoma tissues than in the corresponding noncancerous bone tissues. MiR-134 expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method and normalized to U6 small nuclear RNA. **B.** MiR-134 expression was downregulated in osteosarcoma cell lines HOS, Saos-2, U2OS, and MG-63, compared with human osteoblasts (HOB). * $P < 0.05$; ** $P < 0.01$.

The miR-134 expression in four osteosarcoma cell lines (HOS, Saos-2, U2OS, and MG-63) was also clearly downregulated (Figure 1B). The MG-63 cell line, which possessed the lowest level of miR-134 expression among all tested cell lines, was selected for further studies.

Correlation between miR-134 expression and clinical features and prognosis of osteosarcoma patients

To evaluate the correlation between miR-134 expression and clinicopathological characteristics, the 112 osteosarcoma patients were classified into two groups according to the median expression of miR-134. We compared the clinicopathological factors of the high and low miR-134 expression groups (Table 1) and found that low expression of miR-134 was significantly correlated with large tumor size ($P = 0.001$), positive distant metastasis ($P = 0.03$), and advanced clinical stage ($P < 0.001$).

Table 1. Correlation of miR-134 expression with clinicopathological features of osteosarcoma.

Clinicopathological features	Number of cases	miR-134 expression		P
		High [N (%)]	Low [N (%)]	
Age				
<20 years	47	30 (42.6)	27 (57.4)	0.251
≥20 years	65	42 (64.6)	29 (44.6)	
Gender				
Male	62	27 (43.5)	35 (56.5)	0.183
Female	50	29 (58.0)	21 (42.0)	
Tumor size				
>8 cm	34	13 (29.5)	31 (70.5)	0.001
≤8 cm	68	43 (63.2)	25 (36.8)	
Anatomic location				
Tibia/femur	72	32 (44.4)	40 (55.6)	0.167
Elsewhere	40	24 (60.0)	16 (40.0)	
Serum level of lactate dehydrogenase				
Elevated	74	41 (55.4)	33 (44.6)	0.162
Normal	38	15 (39.5)	23 (62.5)	
Serum level of alkaline phosphatase				
Elevated	80	42 (52.5)	38 (47.5)	0.531
Normal	32	14 (43.8)	18 (56.2)	
Clinical stage				
I/II	59	40 (67.8)	19 (32.2)	< 0.001
III/IV	53	16 (30.2)	37 (69.8)	
Distant metastasis				
Absent	83	47 (56.6)	36 (43.4)	0.03
Present	29	9 (31.0)	20 (69.0)	

No significant difference was observed between miR-134 expression levels and patients' age, gender, anatomic location, and serum levels of lactate dehydrogenase and alkaline phosphatase.

Kaplan-Meier survival analysis showed that low miR-134 expression correlated with shorter overall survival (Figure 2). A univariate proportional hazard model also revealed a statistically significant correlation between overall survival and tumor size, metastasis status, and TNM stage (Table 2).

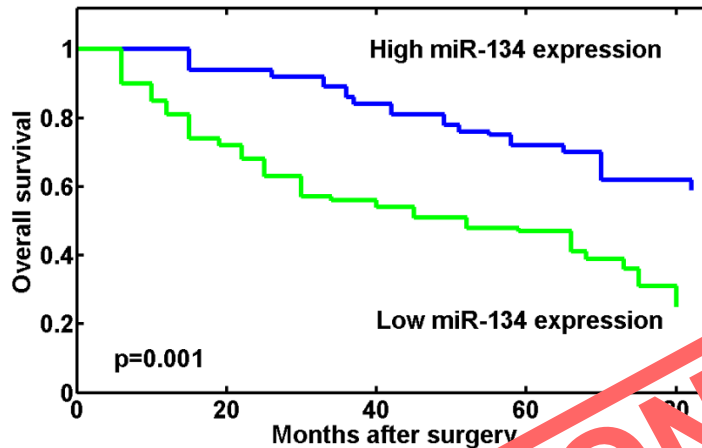


Figure 2. Kaplan–Meier survival curves of patients with osteosarcoma based on miR-134 expression status. Patients in the low expression group had significantly poorer prognosis than those in high expression group ($P = 0.001$, log-rank test).

Table 2. Univariate and multivariate analyses of prognostic factors in patients with osteosarcoma.

Variable	Univariate analysis		Multivariate analysis	
	HR	P value	HR	P value
Age (years)	1.032	0.215	–	–
Gender	1.004	0.759	–	–
Anatomic location	1.704	0.173	–	–
Tumor size	2.116	0.021	2.116	0.078
Serum LDH	1.676	0.188	–	–
Serum AKP	1.395	0.269	–	–
Clinical stage	2.973	0.015	5.232	0.001
Distant metastasis	5.395	0.004	4.958	0.005
MiR-134	5.728	0.001	2.825	0.026

HR = hazard ratio; LDH = lactate dehydrogenase; AKP = alkaline phosphatase.

Multivariate Cox regression analysis employing the above-mentioned significant parameters revealed that miR-134 expression [relative risk (RR) 2.825; $P = 0.026$], distant metastasis (RR 4.958; $P = 0.005$), and TNM stage (RR 5.232; $P = 0.001$) were independent prognostic markers for overall survival of osteosarcoma patients (Table 2).

Effects of miR-134 on the biological behavior of MG-63 cells

Further, we assessed the biological role of miR-134 in MG-63 cells. As shown in Figure 3A, the expression level of miR-134 in MG-63 cells transfected with miR-134 mimics was significantly higher compared with NC-transfected cells ($P < 0.001$). The MTT assay showed that cell proliferation was significantly impaired after miR-134 mimics transfection (Figure 3B). We also observed promoted cell apoptosis in miR-134 mimics-transfected cells (Figure 3C). Transwell invasion and migration assays were performed to investigate whether miR-134 had a direct influence on MG-63 cell migration and invasion. As shown in Figure 3D and E, upregulation of miR-134 impeded

cell invasion/migration compared with the control. The scratch migration assay also confirmed the inhibitory effect of miR-134 on MG-63 cell migration (Figure 3F).

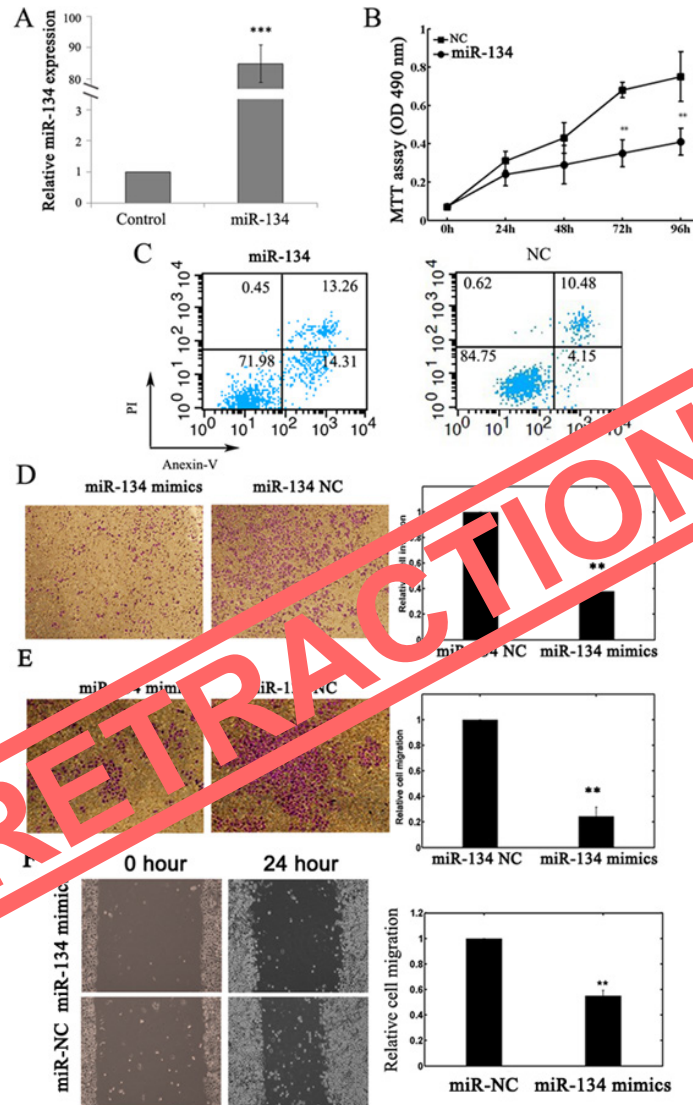


Figure 3. Effects of miR-134 on the biological behaviors of MG-63 cells. **A.** Expression level of miR-134 in MG-63 cells transfected with miR-134 mimics was significantly higher compared with NC-transfected cells. *** $P < 0.001$. **B.** Cell proliferation was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays in MG-63 cells transfected with miR-134 mimics or negative control. ** $P < 0.01$. **C.** Apoptosis of MG-63 cells was detected by flow cytometry analysis after transfection with miR-134 mimics or negative control. **D. E.** MiR-134 suppressed MG-63 cell invasion and migration *in vitro*. The transwell invasion and migration assays showed that the number of invaded or migrated cells was significantly lower in the miR-134-transfected group than in the NC-transfected group. ** $P < 0.01$. **F.** Scratch migration assay confirmed the inhibitory effect of miR-134 on MG-63 cell migration. ** $P < 0.01$.

Increased miR-134 expression suppresses xenograft tumor formation

To further evaluate the effects of miR-134 on tumor growth *in vivo*, we engineered MG-63 cells to stably overexpress miR-134 by lentivirus infection. These cells were injected subcutaneously into nude mice to form ectopic tumors. The cells transfected with negative LV-NC were also inoculated. As shown in Figure 4A-C, the tumors formed by miR-134-overexpressing MG-63 cells were smaller and had lower tumor weights than the control tumors. Real time RT-PCR analysis of the tumor tissues confirmed elevated miR-134 in miR-134-overexpressing tumors (Figure 4D).

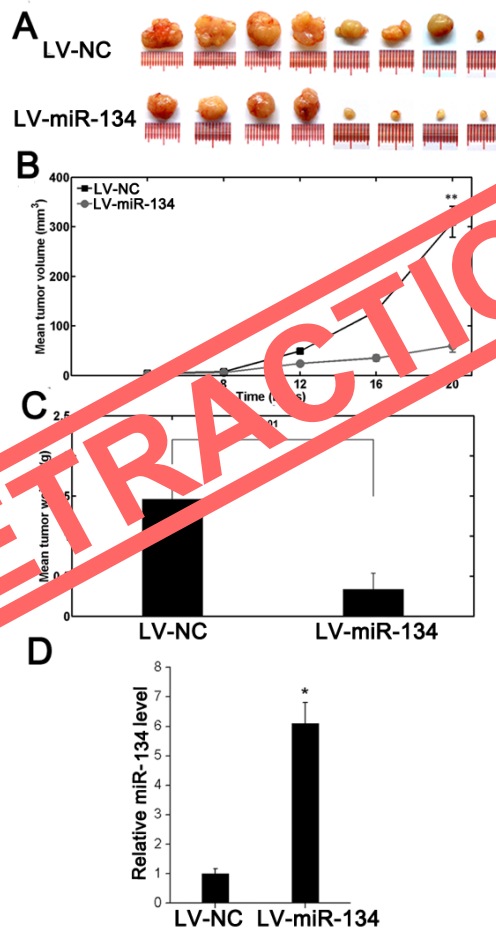


Figure 4. Upregulation of miR-134 resulted in inhibition of xenograft tumor growth *in vivo*. **A, B.** Tumors formed by miR-134-overexpressing MG-63 cells were significantly smaller than in the control group. **C.** Tumors were weighed 3 weeks after inoculation. The average tumor weight is indicated as means \pm standard deviation. **D.** Real-time reverse transcription-polymerase chain reaction analysis of the tumor tissues confirmed elevated miR-134 in miR-134-overexpressing tumors. * $P < 0.05$. ** $P < 0.01$.

DISCUSSION

It has been demonstrated that dysregulation of miRs is involved in tumorigenesis and progression in various types of tumors. However, elucidation of the potential roles of miRs in osteosarcoma remains in the early stage of development. In the present study, we confirmed that miR-134 expression is significantly downregulated in osteosarcoma tissue samples and cell lines, and that loss of miR-134 expression is associated with large tumor size, positive distant metastasis, advanced clinical stage, and shorter overall survival. This indicates that miR-134 might be involved in osteosarcoma progression and could be used as a potential prognostic biomarker. Furthermore, overexpression of miR-134 could significantly inhibit cell proliferation, invasion, and migration, and promote cell apoptosis *in vitro*, and suppress tumorigenicity *in vivo*. To the best of our knowledge, this study showed for the first time that miR-134 may function as a tumor suppressor in osteosarcoma.

Previous research has reported dysregulated miR-134 expression in many human malignancies, and miR-134 functions as a tumor suppressor targeting a number of oncogenic genes. Yin et al. (2013) confirmed decreased miRNA-134 expression in HCC tissues and its association with more aggressive pathological features, including liver metastasis, high levels of α -fetoprotein, large tumor size, advanced tumor stage, presence of tumor microsatellites, and absence of tumor encapsulation (Yin et al., 2013). Overexpression of miR-134 in HCC cells arrested cell growth and decreased cell migration and invasion by downregulating the oncoprotein KRAS. Zha et al. (2014) also revealed that miR-134 suppressed the invasion and metastasis of HCC cells *in vitro* and *in vivo*, and integrin beta 1 was a direct and functional target gene of miR-134 (Zha, et al., 2014). In non-small cell lung cancer, miR-134 inhibits the invasive potential and epithelial-to-mesenchymal transition phenotypes of tumor cells, and directly targets *FOXM1* (Li et al., 2012), a potential metastasis promoter. Low miR-134 expression in malignant pleural effusion is associated with poor survival in non-small cell lung cancer patients (Wang et al., 2012). In prostate cancer, increased expression of miR-134 contributes to the activity of testosterone and $1,25(\text{OH})_2\text{D}_3$ to suppress tumor growth (Wang et al., 2011). Moreover, downregulation of miR-134 has been reported in GISTs and gliomas (Haller et al., 2010; Lages, et al., 2011), and decreased miR-134 expression indicates shorter disease-free survival in patients with GIST.

In contrast to the antitumor properties mentioned above, miR-134 is significantly increased in HNSCC and acts as a potential oncogene. Liu et al. (2014) found that miR-134 is upregulated in HNSCC specimens relative to matched noncancerous tissues (Liu, et al., 2014). High expression of miR-134 is associated with nodal metastasis and mortality of patients. Decreased plasma miR-134 levels after tumor ablation indicate a better prognosis for patients. Multivariate analysis has shown that high miR-134 expression in HNSCC is an independent predictor of poor survival. Furthermore, ectopic miR-134 expression enhances the oncogenic phenotypes, including growth, mobility, and invasion of HNSCC cells via the downregulation of the WW domain-containing oxidoreductase gene - a tumor suppressor. Thus, miR-134 has dual functions in cancer pathogenesis and progression, and the role of miR-134 should be tumor-specific and possibly dependent on its targets in different cancer types.

In conclusion, our results reveal that miR-134 is downregulated in osteosarcoma cell lines and clinical samples. Decreased miR-134 expression is associated with aggressive clinicopathological features and poor prognosis. Overexpression of miR-134 exhibits anti-tumor effects *in vitro* and *in vivo*. Our findings demonstrate that miR-134 may be a potential novel target for the gene therapy of osteosarcoma.

Conflicts of interest

The authors declare no conflict of interest.

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