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Decreased expression of tumor suppressive miR-874 and its clinical significance in human osteosarcoma

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ABSTRACT. Dysregulation of microRNAs (miRs) is associated with cancer development and progression and aberrant expression of miR-874 have been found in some types of cancer. However, the expression and function of miR-874 in osteosarcoma remain unclear. The aim of this study was to explore the effects of miR-874 in osteosarcoma tumorigenesis and development. The expression level of miR-874 was quantified by real-time reverse transcription-polymerase chain reaction (RT-PCR) in human osteosarcoma cell lines and tissues. Using a miR-874 mimic, cell proliferation and migration assays were performed in an osteosarcoma cell line and tumorigenicity was observed *in vivo* in order to determine the effects of miR-874 in osteosarcoma cell lines and tissues. MiR-874 was significantly downregulated in osteosarcoma cell lines and clinical

specimens. Decreased miR-874 expression was significantly associated with large tumor size, distant metastasis, and advanced clinical stage, and was an independent predictor of poor survival. Overexpression of miR-874 inhibited cell proliferation, invasion and migration *in vitro*, promoted cell apoptosis *in vitro*, and suppressed tumorigenicity *in vivo*. These findings indicate that miR-874 may act as a tumor suppressor in osteosarcoma and could serve as a novel therapeutic agent for miR-based therapy.

Key words: MiR-874; Osteosarcoma; Prognosis; Proliferation; Invasion

INTRODUCTION

Osteosarcoma is the most common type of primary malignant bone tumor and is a leading cause of cancer-related death among children and adolescents (Broadhead et al., 2011). Despite recent advances in multimodal treatments, particularly the combination of chemotherapy and aggressive surgical resection, the long-term outcomes of patients with local relapse or distant metastasis remain poor, with a 5-year overall survival rate of 50-60% (Gorlick, 2009). Therefore, a good understanding of the molecular mechanisms underlying osteosarcoma pathogenesis is important to improve diagnosis, treatment, and prevention of this disease.

MicroRNAs (miRs) are a class of short (~22 nucleotides in length), endogenous, single-stranded, and non-protein-coding RNA molecules. They can regulate gene expression at the post-transcriptional level by base pairing to the 3'-UTR region of the target gene messenger RNA (mRNA), resulting in mRNA degradation or translational suppression (Bartel, 2009). Growing evidence indicates that miRs play a crucial role in normal developmental processes, differentiation and tumorigenesis (Esquela-Kerscher and Slack, 2006). Some highly expressed miRs could function as oncogenes by repressing tumor suppressor genes, whereas miRs with lower expression could function as tumor suppressors by negatively regulating oncogenes. MiRs are thought to influence cell proliferation, apoptosis, chemo- and radiation-sensitivity, and metastasis in cancers and could even potentially define the phenotype of cancer stem cells (Esquela-Kerscher and Slack, 2006; Fang et al., 2015; Garofalo and Croce, 2015; Yang et al., 2015). Recently, reports of the correlation between dysregulated miRs and osteosarcoma initiation and progression are increasing, providing new insights for osteosarcoma treatment (Bilbao-Aldaiturriaga et al., 2015; Sun et al., 2015; Zhang et al., 2015; Zhao et al., 2015).

MiR-874 is located on chromosome 5q31.2, a well-known fragile site in the human genome that is often deleted in cancers and genetic disorders and specifically correlates with chromosomal rearrangements in cancer (Fundia et al., 1992; Thorland et al., 2003). It has been observed to be downregulated in maxillary sinus squamous cell carcinoma (Nohata et al., 2011), gastric cancer (Jiang et al., 2014; Zhang et al., 2015), non-small cell lung cancer (Kesanakurti et al., 2013), and breast cancer (Wang et al., 2014), where it acts as a candidate tumor suppressor. However, the potential role of miR-874 in the regulation of osteosarcoma tumorigenesis is still unknown.

In the present study, we examined miR-874 expression in osteosarcoma tissue samples and cell lines using real-time polymerase chain reaction (PCR). The association of miR-874 levels with clinicopathological features and prognosis was also analyzed. Furthermore, we investigated the effects of miR-874 on the biological behavior of osteosarcoma cells.

MATERIAL AND METHODS

Tissue samples and cell lines

Paired osteosarcoma and corresponding noncancerous bone tissue samples were obtained from 106 patients with osteosarcoma at The People's Hospital of Weifang (Weifang, China) from 2007 to 2010. The samples were biopsy materials and all patients did not previously receive radiotherapy, chemotherapy or immunotherapy. The fresh specimens were flash frozen immediately after collection and stored in liquid nitrogen until RNA was extracted. The backgrounds and clinicopathological characteristics of all patients are summarized in Table 1. Written informed consent was obtained from all study participants. The use of tissue samples was approved by the ethical committees of The People's Hospital of Weifang.

Human osteosarcoma cell lines (HOS, Saos-2, U2OS and MG-63) and human osteoblasts (HOB) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were maintained in Dulbecco's modified Eagle media (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin G sodium, and 100 µg/mL streptomycin sulfate. Cultures were incubated in a humidified atmosphere of 5% CO_2 at 37°C.

Table 1. Correlation of miR-874 expression with clinicopathological features of osteosarcoma.

Clinicopathological features	Number of cases	MiR-874 expression		P value
		High N (%)	Low N (%)	
Age				
<20 years	44	19 (43.2%)	25 (56.8%)	0.306
≥20 years	62	34 (54.8%)	28 (45.2%)	
Sex				
Male	59	26 (44.1%)	33 (55.9%)	0.241
Female	47	27 (57.4%)	20 (42.6%)	
Tumor Size				
>8 cm	41	12 (29.3%)	29 (60.7%)	0.001
≤8 cm	65	41 (63.1%)	24 (26.9%)	
Anatomical Location				
Tibia/Femur	69	31 (44.9%)	38 (55.1%)	0.221
Else where	37	22 (59.5%)	15 (40.5%)	
Serum Level of Lactate Dehydrogenase				
Elevated	71	39 (54.9%)	32 (45.1%)	0.215
Normal	35	14 (40.0%)	21 (60.0%)	
Serum Level of Alkaline Phosphatase				
Elevated	77	40 (51.9%)	37 (48.1%)	0.663
Normal	29	13 (44.8%)	16 (55.2%)	
Clinical stage				
I/II	56	38 (67.9%)	18 (32.1%)	<0.001
III/IV	50	15 (30.0%)	35 (70.0%)	
Distant metastasis				
Absent	80	46 (57.5%)	34 (42.5%)	0.012
Present	26	7 (26.9%)	19 (73.1%)	

RNA extraction and quantitative real-time PCR

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer protocol. Reverse transcription (RT) was performed using gene-specific reverse primers and reverse transcriptase (Takara Bio, Japan). The resulting cDNA was PCR-amplified on an

ABI 7500 thermocycler (Applied Biosystems, USA). All experiments were performed independently in triplicate and U6 was used as an endogenous reference. The RT primers were 5'-GTCGTATCCAGTG CAGGGTCCGAGGTATTCGCACTGGATACGACTCTTAGG-3' for miR-874 and 5'-TGGTGTCGTGG AGTCG-3' for U6. The PCR primers for miR-874 and U6 were: miR-874 forward, 5'-TGCGGCGGCC CCACGCACCAG-3' and reverse, 5'-CCAGTGCAGGGTCCGAGGT-3', and U6 forward, 5'-TGCGGG TGCTCGCTTCGGCAGC-3' and reverse, 5'-CCAGTGCAGGGTCCGAGGT-3'. The relative amount of miR-874 to U6 was calculated using the $2^{-\Delta Ct}$ method where $\Delta Ct = (Ct^{miR-874} - Ct^{U6})$.

Cell transfection

MiR-874 mimic and a negative control (miR-NC) were obtained from GenePharma (GenePharma, China). MG-63 cells were seeded in a 24-well plate at a concentration of 1 x 10^5 cells/well and incubated for 24 h. Transfection with miR-874 mimic or miR-NC was performed using Lipofectamine 2000 (Invitrogen) in accordance with the manufacturer protocol. Cells were collected for further assays after 48 h.

Cell proliferation assay

After transfection, approximately 3 x 10^3 cells were seeded in 96-well plates. After incubation for 1 to 5 days, 20 µL MTT solution (5 mg/mL) was added to each well, and the plates were further incubated for another 4 hours at 37°C. Then, the media was replaced with 150 µL dimethyl sulfoxide (DMSO; Sigma), and the absorbance was measured at 570 nm using a microplate reader (Sunrise).

Flow cytometry assay

At 48 hours post-transfection, each sample containing 1 x 10^6 cells/mL was stained with annexin V-FITC (50 µg/mL; BD Biosciences) and propidium iodide (10 µg/mL; Sigma) and incubated at room temperature in the dark for 15 min. Data were acquired on a FACScan flow cytometer (Becton–Dickinson, Franklin Lakes, NJ, USA).

Transwell invasion and migration assays

Cell migration and invasion were evaluated using 6-well transwell chambers (8 µm pore size; Corning, NY, USA). For the migration assay, tumor cells transfected with miR-874 mimic or miR-NC were resuspended in DMEM with 0.1% FBS (5 x 10^4 cells/mL) and seeded into the upper chambers. DMEM containing 20% FBS was placed into the lower chambers as the chemotaxin. After 24 h of incubation at 37°C, cells on the upper surface of the membrane were scrubbed off with suitable cotton swabs, and the migrated cells were washed with PBS, fixed with 95% ethanol, stained with 0.1% crystal violet, and then counted using light microscopy (Olympus Corp., Tokyo, Japan). For the invasion assay, the upper chambers were first covered with 5 mg/mL Matrigel, and all subsequent steps were identical to the migration assay.

In vivo tumor formation study

Tumor formation was studied by establishing a xenograft model. Commercial lentiviral

vectors containing miR-874 (LV-miR-874) (GeneChem Co. Ltd., Shanghai, China) were used to infect MG-63 cells according to the manufacturer instructions. An empty lentiviral construct served as a negative control (LV-NC). BALB/c nude mice (4 weeks old) were purchased from the Animal Center of Chinese Academy of Science (Shanghai, China) and divided into 2 groups with 6 mice in each group. Infected MG-63 cells stably overexpressing miR-874 or miR-NC were injected subcutaneously into each nude mouse (1 x 10^6 cells/mouse). Tumor size was measured every 4 days using vernier calipers along two major axes, and the volume of the implanted tumor was calculated according to the formula: volume = (length x width^2)/2. The mice were sacrificed and the tumors were weighed 3 weeks after inoculation.

Statistics

All data are presented as the mean ± SD. Statistical analysis was carried out using SPSS 15.0 software (SPSS Inc., Chicago, IL, USA). Differences between groups were analyzed by Student *t*-test or chi-square (X^2) test. Survival curves were constructed using the Kaplan-Meier method and compared by log-rank tests. A Cox proportional hazards regression analysis was used for univariate and multivariate analyses of prognostic values. $P < 0.05$ was considered significant.

RESULTS

Decreased miR-874 expression in osteosarcoma tissues and cell lines

We performed quantitative RT-PCR analysis to detect the expression level of miR-874 in osteosarcoma tissues and cell lines. Our results showed that miR-874 was significantly decreased in osteosarcoma tissues (mean ± SD: 8.21 ± 2.11) when compared to paired noncancerous bone tissues (mean ± SD: 23.46 ± 4.98) ($P < 0.001$, Figure 1A). MiR-874 expression in four osteosarcoma cell lines was also clearly downregulated (Figure 1B). The MG-63 cell line, which possessed the lowest level of miR-874 expression among all tested cell lines, was selected for further studies.

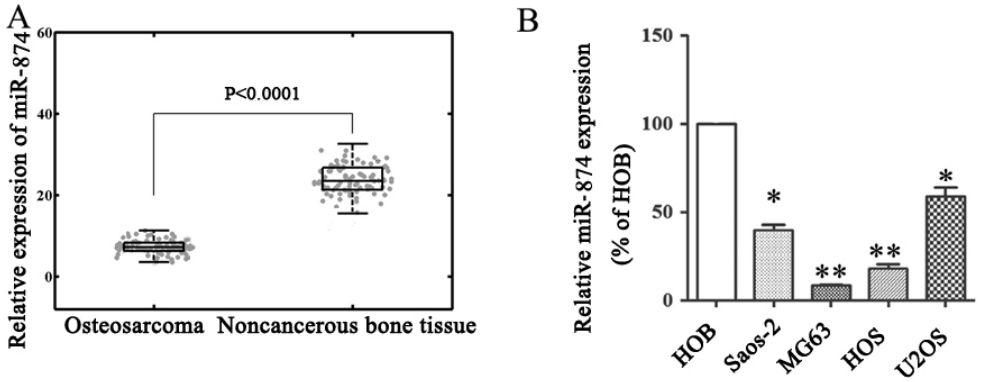

Figure 1. Relative expression levels of miR-874 in osteosarcoma tissues and cell lines. **A.** MiR-874 expression was significantly lower in osteosarcoma tissues than in the corresponding noncancerous bone tissues. MiR-874 expression levels were calculated by the $2^{-\Delta Ct}$ method and normalized to U6 small nuclear RNA. **B.** MiR-874 expression was downregulated in osteosarcoma cell lines (HOS, Saos-2, U2OS and MG-63) compared to normal human osteoblasts (HOB). *P < 0.05; **P < 0.01.

Correlation between miR-874 expression and clinical features and prognosis of osteosarcoma patients

To evaluate the correlation between miR-874 expression and clinicopathological characteristics, the 106 osteosarcoma patients were classified into two groups based on the median expression of miR-874. We compared the clinicopathological features of the high and low miR-874 expression groups (Table 1) and found that low expression of miR-874 significantly correlated with large tumor size (P = 0.001), positive distant metastasis (P = 0.012), and advanced clinical stage (P < 0.001). No significant difference was observed between miR-874 expression levels and patients' age, sex, anatomical location of tumor, and serum levels of lactate dehydrogenase or alkaline phosphatase.

Kaplan-Meier survival analysis showed that low miR-874 expression correlated with shorter overall survival (Figure 2). Univariate proportional hazard model analysis also revealed a statistically significant correlation between overall survival and tumor size, metastasis status, and tumor-node-metastasis (TNM) stage (Table 2). Multivariate Cox regression analysis using the above-mentioned significant parameters revealed that miR-874 expression, distant metastasis, and TNM stage were independent prognostic markers for overall survival of osteosarcoma patients (Table 2).

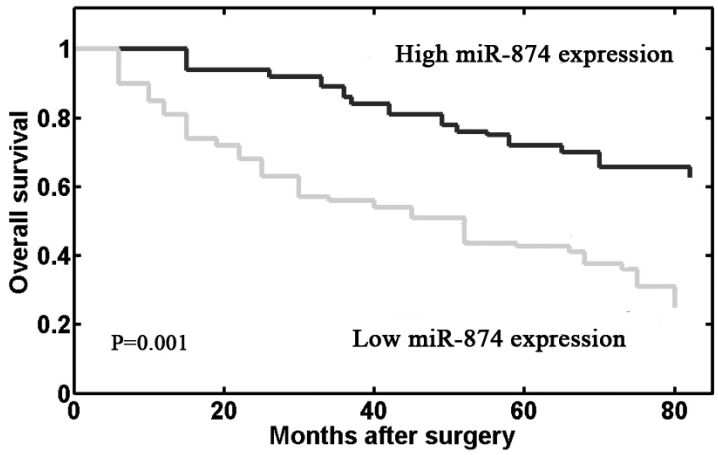

Figure 2. Kaplan-Meier survival curves of patients with osteosarcoma based on miR-874 expression status. Patients in the low expression group had significantly poorer prognosis than those in the high expression group (P = 0.001, log-rank test).

Effects of miR-874 on the biological behaviors of MG-63 cells

Next, we assessed the biological role of miR-874 in MG-63 cells. As shown in Figure 3A, the expression level of miR-874 in cells transfected with miR-874 mimic was significantly higher compared to miR-NC transfected cells (P < 0.001). The MTT assay showed that cell proliferation was significantly impaired after transfection with miR-874 mimic (Figure 3B). We also observed increased cell apoptosis in cells transfected with miR-874 mimic (Figure 3C). Transwell invasion and migration assays showed that upregulation of miR-874 impeded cell invasion and migration compared to control (Figure 3D and E).

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)	1.628	0.212	-	-
Gender	1.129	0.761	-	-
Anatomical location	1.698	0.169	-	-
Tumor size	2.331	0.024	2.125	0.081
Serum LDH	1.669	0.176	-	-
Serum AKP	1.405	0.272	-	-
Clinical stage	2.981	0.018	5.245	0.001
Distant metastasis	5.398	0.003	4.962	0.008
MiR-874	5.744	0.001	2.833	0.024

LDH = lactate dehydrogenase; AKP = alkaline phosphatase.

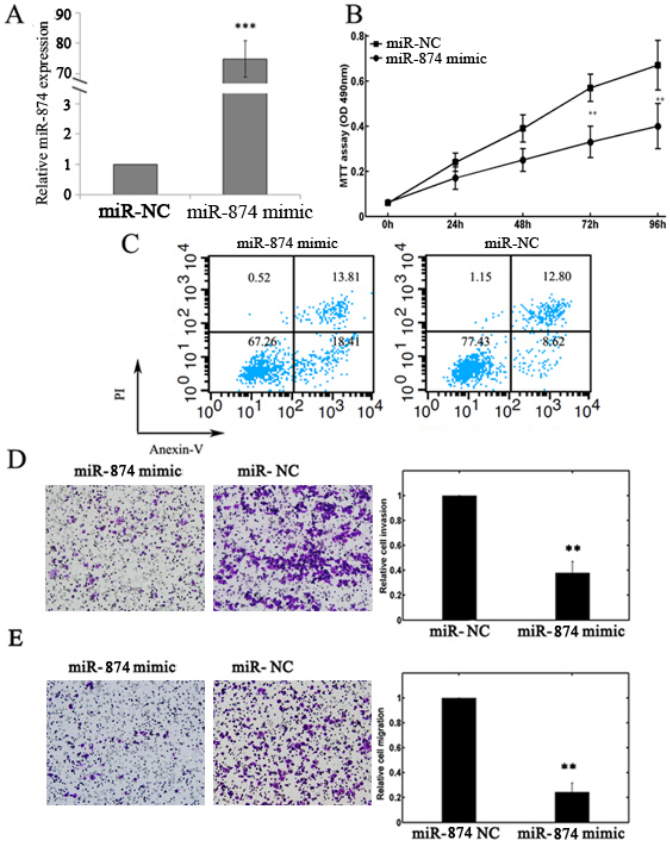

Figure 3. Effects of miR-874 on the biological behaviours of MG-63 cells. **A.** The expression level of miR-874 in cells transfected with miR-874 mimic was significantly higher compared to cells transfected with miR-NC. ***P < 0.001. **B.** Cell proliferation was measured by the MTT assay in MG-63 cells transfected with miR-874 mimic or miR-NC. **P < 0.01. **C.** Apoptosis of MG-63 cells was detected by flow cytometry analysis after transfection with miR-874 mimic or miR-NC. **D. E.** MiR-874 suppressed MG-63 cell invasion and migration *in vitro*. The transwell assays showed that the number of invading or migrating cells was significantly lower in the miR-874 mimic-transfected group than in the miR-NC-transfected group. **P < 0.01.

Increased miR-874 expression suppresses xenograft tumor formation

To further evaluate the effects of miR-874 on tumor growth *in vivo*, MG-63 cells stably overexpressing miR-874 or miR-NC were injected subcutaneously into nude mice to form ectopic tumors. As shown in Figure 4A-C, the tumors formed by miR-874-overexpressing MG-63 cells were smaller and had lower tumor weights than control (miR-NC) tumors. Quantitative RT-PCR analysis of the tumor tissues confirmed elevated miR-874 in miR-874-overexpressing tumors (Figure 4D).

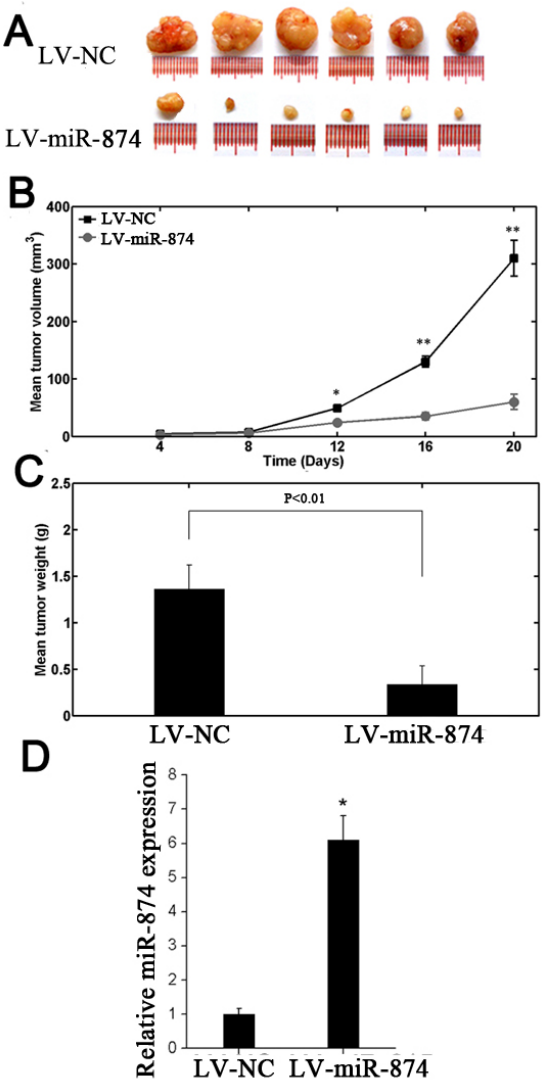

Figure 4. Upregulation of miR-874 resulted in inhibition of xenograft tumor growth *in vivo*. **A. B.** The tumors formed by miR-874-overexpressing MG-63 cells were significantly smaller than the control group. **C.** Tumors were weighed 3 weeks after inoculation. The average tumor weight is indicated as mean ± SD. **D.** Quantitative RT-PCR analysis of the tumor tissues confirmed elevated miR-874 in miR-874-overexpressing tumors. *P < 0.05.

DISCUSSION

Dysregulation of miR expression plays important roles in the initiation and development of human cancers (Calin and Croce, 2006). In the present study, we showed that miR-874 expression was significantly downregulated in osteosarcoma tissues. Decreased miR-874 expression was correlated with aggressive clinicopathological features and poor prognosis. Furthermore, we demonstrated that miR-874 could regulate cell proliferation, apoptosis, migration and invasion *in vitro*, and suppress tumorigenicity *in vivo*. To our knowledge, this is the first report investigating aberrant miR-874 expression and its biological functions in osteosarcoma.

Previous studies have reported miR-874 downregulation in many human malignancies and its function as a tumor suppressor by targeting a number of oncogenic genes. Nohata et al. (2011) found that miR-874 was downregulated in maxillary sinus squamous cell carcinoma (MSSCC) cells and ectopic expression of miR-874 significantly inhibited cell proliferation and invasion by targeting oncogene protein phosphatase 1, catalytic subunit, A isozyme (PPP1CA). Their follow-up study showed that restoration of miR-874 in SAS (derived from a primary lesion of tongue squamous cell carcinoma) and FaDu (derived from a primary lesion of hypopharyngeal squamous cell carcinoma) cell lines significantly inhibited cell proliferation and induced G2/M arrest and cell apoptosis (Nohata et al., 2013). They further identified histone deacetylase 1 (HDAC1) as a direct target of miR-874 (Nohata et al., 2013). In gastric cancer, decreased miR-874 expression was associated with poor histological type, lymph node metastasis, and advanced tumor stage (Jiang et al., 2014). Functional analyses indicated that overexpression of miR-874 suppressed the growth, migration, invasion and tumorigenicity of gastric cancer cells by regulating aquaporin-3 (AQP3), a water transporting protein which plays an oncogenic role in several malignant tumors. MiR-874 also inhibited the tumor angiogenesis of gastric cancer cells by targeting signal transducer and activator of transcription 3 (STAT3) (Zhang et al., 2015), a key transcription factor that plays a vital role in human gastric cancer angiogenesis (Judd et al., 2006; Giraud et al., 2012). In non-small cell lung cancer, restoration of miR-874 expression drastically reduced the ability of tumor cells to invade by suppressing the protein levels of MMP-2, and *in vivo* experiments revealed that miR-874 treatment decreased orthotopic tumor growth in nude mice (Kesanakurti et al., 2013). Furthermore, overexpression of miR-874 in breast cancer cells can lead to suppressed cell growth and increased cell apoptosis (Wang et al., 2014). Taken together, miR-874 may act as a potential tumor suppressor and further understanding of miR-874 could lead to the discovery of new molecular mechanisms in human malignancies.

In general, miRs exert their functions by binding to the 3'-UTR of the target gene to repress its expression (van Kouwenhove et al., 2011). Some oncogenes have been identified as direct targets of miR-874. However, miRs may function in accordance with the combinatorial circuit model, in which a single miR targets multiple mRNAs and several coexpressed miRs may target a single mRNA. Therefore, the potential regulatory circuit associated with miR−874 is vast and how miR-874 affects cancer progression specifically is still not well understood. The elucidation of the molecular characteristics of miR-874 remains an important facet in future investigations.

In conclusion, our results reveal that miR-874 was downregulated in osteosarcoma cell lines and clinical samples. Decreased miR-874 expression was associated with aggressive clinicopathological features and poor prognosis. The overexpression of miR-874 exhibited anti-tumor effects *in vitro* and *in vivo.* Our findings demonstrate that miR-874 may be a potential novel target for gene therapy of osteosarcoma.

Conflicts of interest

The authors declare no conflict of interest.

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