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ORIGINAL ARTICLE

The tumor suppressor miR-124 inhibits cell proliferation and invasion by targeting B7-H3 in osteosarcoma

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Abstract Our previous studies have shown that the expression level of B7 homolog 3 (B7-H3) was correlated with clinical staging and prognosis of osteosarcoma (OS) patients, and its silencing inhibited the proliferation and invasion of OS cells in vitro. However, its overexpression mechanism behind was far from elucidated. On the basis of bioinformatics and the preliminary screening data, we hypothesized that miR-124 might play an important role in OS development and as a lead candidate for modulating B7-H3 expression. In this study, we found that miR-124 was downregulated significantly in OS tumor tissue, compared to normal adjacent tissues (NATs). Lower miR-124 expression levels were associated with advanced Ennecking stage, lower tumor differentiation, and common pulmonary metastasis. The 5-year overall survival rate in the miR-124 upregulated group was 61.5 %, while with low miR-124 expression, only 11.8 % survived. Further studies in vitro showed that B7-H3 was a direct target of miR-124. Overexpression of miR-124 decreased B7-H3 mRNA and protein level and inhibited B7-H3 3′-UTR reporter activity. Treatment of OS cells with miR-124 mimics induced the inhibition of cell growth and invasion in vitro, which could be abrogated by transfected by B7-H3 expression vector. Our findings highlight the potential application of miR-124 as a novel onco-miRNA in OS, and its oncogenic effects are mediated chiefly through downregulation of B7-H3, thus suggesting a model for identifying miR-124 that can be exploited to improve the therapeutic potential efficacy of mAb targeting to B7-H3.

Keywords Osteosarcoma · miR-124 · B7-H3 · Proliferation · Invasion

Introduction

Osteosarcoma (OS) is the most common primary musculoskeletal malignant tumor mainly affecting children and young adults with complicated pathogenesis and frequent distal metastasis [1, 2]. Despite dramatic advances in wide-margin surgery and intensification of chemotherapeutic treatment, a 5-year survival rate for OS remains approximately 65–70 % for localized disease, but only 20 % for metastatic disease [3, 4]. Herein, there is an urgent need for developing more targeted treatment approaches that effectively inhibit metastasis. Over the last decade, immunotherapies were introduced and had achieved great success in various malignancies, such as leukemia, melanoma, prostate, and breast cancer [5–8]. However, there are not adequate immunotherapy modules for OS patients, since the immune escape mechanism of OS is complex and has not been fully elucidated.

As we know, T cells are activated not just by the T-cell antigen-specific receptors (TCRs) but also by costimulatory molecules [9, 10]. B7 costimulatory family members are important components involving in the immune response process [11, 12]. Our previous study showed that B7-H3 expression is significantly

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upregulated in primary tumor lesions of OS in comparison to osteochondroma and bone fibrous dysplasia tissues and inversely associated with the intensity of infiltrating $CD8^+$ T lymphocytes in tumor site [13]. Our findings indicated that B7-H3 might play an important role in OS progression, might act as a negative regulator of T cells, and play an important role in shielding tumors from immune surveillance. However, the reason for overexpression of B7-H3 on membrane and cytoplasm in OS cells remains far from elucidated.

MicroRNAs (miRNA) are a class of naturally occurring 21–23 nt small noncoding RNAs and regulate gene expression based on their "seed region" complementarity to the 3′-untranslated region (UTR) of target mRNAs resulting in mRNA cleavage and/or translational suppression [14, 15]. miRNAs play critical roles not only in tumorigenesis, but also in the regulation of host immune response [16, 17]. For example, miR-124 and miR-200 have been implicated in the regulation of IL-6-mediated cholangiocyte proliferation [18]. Besides, the induction of miR-155 during the macrophage inflammatory response suggests its potential involvement in the regulation of inflammation [19, 20]. As for B7-H3, there are several studies reporting that miRNA can regulate B7-H3 expression in nervous system tumor, renal cell carcinoma, and breast cancer [21–23]. Nevertheless, whether miRNAs involved in the posttranscriptional regulation of B7-H3 expression in OS remain unclear.

In the work described here, we showed that miR-124 was capable of targeting a predicted site in the B7-H3 3′ UTR, thus further leading to translational repression. Transfection of a miR-124 mimics or antisense could induce the downregulation or upregulation of B7-H3 protein expression. miR-124 precursor transfection can also consequently influence B7-H3-associated cell growth and invasion in OS cells. Thus, our findings provide valuable information that miR-124 acts as a tumor suppressor by directly targeting B7-H3 in OS, indicating that miR-124 has potential diagnostic and therapeutic value for OS.

Materials and methods

Cell lines

Human OS MG-63 cells were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and cultured according to the instructions from American Type Culture Collection (ATCC). Cell lines were all maintained in suitable medium supplemented with 10 % fetal bovine serum and 1 % penicillin/streptomycin.

Patients

A total of 69 paraffin-embedded tumor with normal adjacent tissues (NATs) specimens were retrieved from OS patients after surgery, from the department of the division of surgical pathology, the third hospital of Hebei Medical University respectively from 2004 to 2009, with complete histopathology and follow-up information. None of the patients received pre-operative chemotherapy or radiotherapy before surgery. All patients provided written informed consent for tissue sample analysis. The study protocol was approved by the Institutional Ethics Committee at Third Hospital of Hebei Medical University.

RNA isolation and quantitative real-time PCR

Total RNA from paraffin-fixation samples was isolated using miRNeasy FFPE Kit (Qiagen tec, China). Before performing Quantitative RT-PCR assays (qPCR), RNA was purified by RNeasy Plus Mini Kits (Qiagen tec, China). For qPCR analysis of miR-124, an amount of 0.25 μg of total RNA was reverse transcribed by using the miScript II RT Kit (Qiagen tec, China) and performed by using the miScript SYBR Green PCR Kit on the Applied Biosystems 7500 FAST real-time PCR System. Specific primers and probes for mature miR-124 and small nuclear RNA RNU6B (endogenous reference) were purchased from Sangon Biotech. All reactions were tested in triplicate. The amount of miR-124 was obtained by normalizing to small nuclear RNA RNU6B as control.

For qPCR analysis of B7-H3 mRNA, an amount of 200 ng of total RNA was reverse transcribed by using the RT^2 Easy First Strand Kit (Qiagen tec, China) and conducted in triplicate using the RT^2 qPCR Primer Assays (Qiagen tec, China) on Bio-Rad PCR instrument. The primers were as follows: 5′- CAAAGGAT GCGATACACAGACCAC -3′ (forward) and 5′-CAGCAGGCAGGATGACTTAGAGAA -3′ (reverse) and annealing temperature was 60 °C for human B7-H3, and 5′-TGCACCACCAACTGCTTAGC-3′ (forward) and 5′-GGCATGGACTGTGGTCATGAG-3′ (reverse) and annealing temperature was 60 °C for human GAPDH. The threshold cycle (Ct) values were analyzed using the comparative Ct ($^{\Delta\Delta}$Ct) method.

Dual-luciferase reporter assay

As illustrated in result figure model, oligonucleotides corresponding to the miR-124 binding site in the B7-H3 3′UTR or a single-base mutant were synthesized and inserted into the pGL3-control vector (Promega). MG-63 cells were co-transfected in 48-well plates using

Table 1 The relationship between miR-124 and B7-H3 expression and clinicopathological parameters in osteosarcoma

Clinical features	Number of cases	B7-H3			miR-124		
		Low	High	*p* value	Low	High	*p* value
Age							
≤20	34	13	21	0.367	21	13	0.08
>20	35	11	24		28	7	
Gender							
Male	39	16	23	0.162	27	12	0.46
Female	30	8	22		22	8	
Site							
Femur	33	13	20	0.138	23	10	0.265
Tibia	21	9	12		13	8	
Others	15	2	13		13	2	
Ennecking stage							
I	6	5	1	0.003	1	5	*0.002*
II	48	18	30		34	14	
III	15	1	14		14	1	
Histologic type							
Osteoblastic	39	17	22	0.155	26	13	0.36
Chondroblastic	16	4	12		12	4	
Fibroblastic	8	3	5		5	3	
Others	6	0	6		6	0	
Differentiation status							
High	48	22	26	0.003	28	19	*0.001*
Low	21	2	19		21	1	
Pulmonary metastasis							
Yes	33	2	31	0.001	30	3	*0.002*
No	36	22	14		19	17	
Response to chemotherapy							
Good	39	18	21	0.021	26	13	0.262
Poor	30	6	24		23	7	

Significant difference ($p < 0.05$) is indicated in italic

lipofectamine^{3000} reagent (Invitrogen), together with 40 ng of the firefly luciferase reporter plasmid, 1 ng of the renilla luciferase reporter plasmid as transfection control, and 100 nM miR-124 mimics. Firefly and renilla luciferase activities were measured consecutively using dual-luciferase assays (Promega) 24 h after the transfection. The experiments were replicated in triplicate.

Transient transfection of miRNA

The miR-124 mimics, anti-miR124, and non-specific miRNA negative control were synthesized and purified by Sangon Biotech (Shanghai, China). In brief, about 5×10^5 cells per well in a 6-well plate were seeded and transfected miRNA mimics or anti-miR124 complex at working concentrations of 40 nM using lipofectamine^{3000} reagent (Invitrogen, CA, USA), according to the manufacturer's protocol.

Western blotting analysis

Protein extracted from OS cells was detected for western blotting as previously described [24, 25]. The primary antibodies used included antibodies to B7-H3, PCNA, Cyclin D1, PARP, MMP-9, E-cadherin, vimentin, and GAPDH were purchased from Abcam company.

Cell proliferation assay

Cell proliferation was tested using a Cell Counting Kit-8 (CCK-8, Sigma) according to the manufacturer's instructions. MG-63 cells were planted into 96-well plates at the concentration of 1×10^4 cells per well and

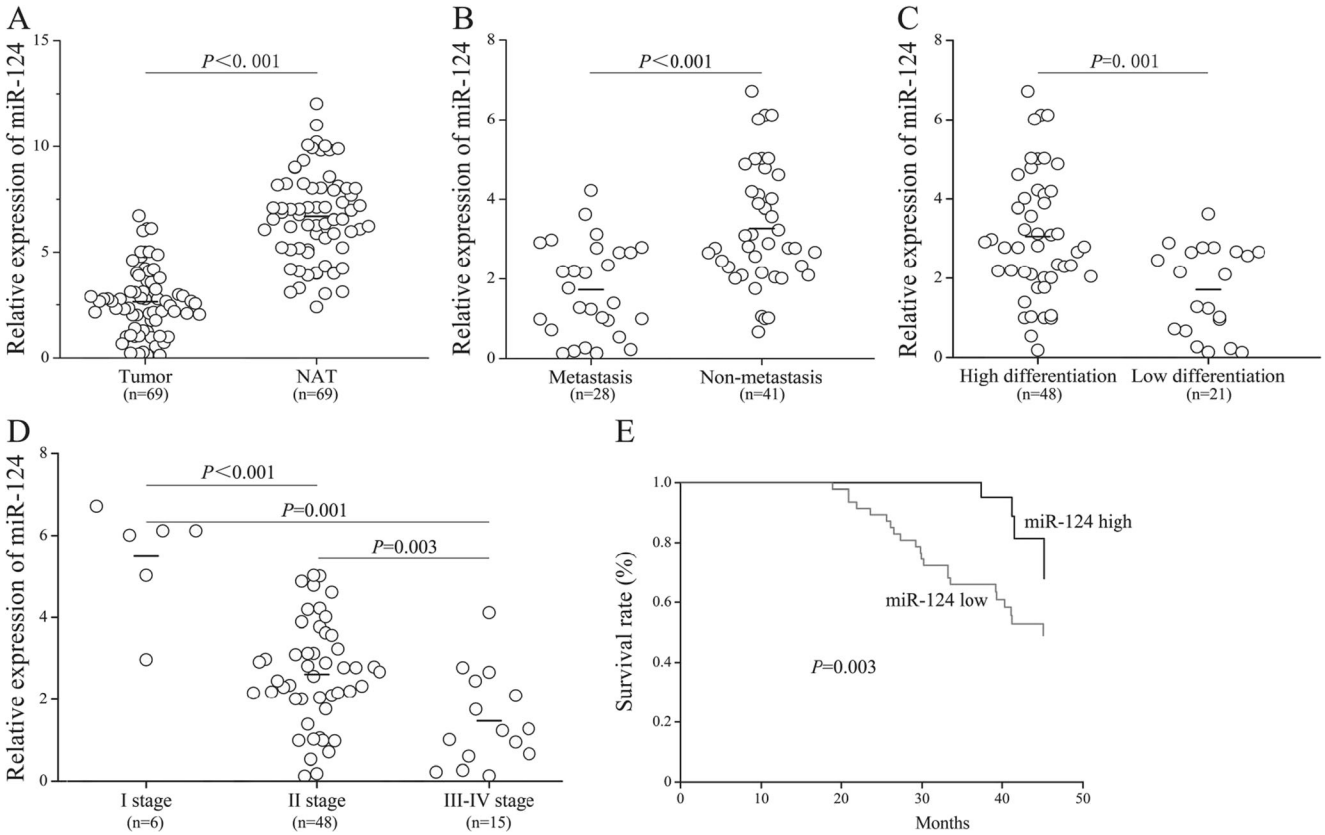

Fig. 1 The relative expression levels of miR-124 were detected by real-time qPCR in OS. **a** miR-124 was downregulated in OS compared to paired NATs in 69 patients ($p < 0.05$). **b** Lower miR-124 expression level was associated with the tendency of tumor metastasis ($p < 0.05$). **c** Higher miR-124 expression level was associated with higher tumor differentiation ($p < 0.05$). **d** Lower miR-124 expression level was associated with higher tumor stage ($p < 0.05$). **e** Expression levels of miR-124 are associated with 5-year survival of OS patients. The survival rate of the group with low expression of miR-124 was higher than that of the group with high expression of miR-124 ($p < 0.05$)

treatment with 100 nM miR-124 mimics together with 1.5 μg B7-H3 cDNA expression plasmid for 24, 48, and 72 h. After transfection, 10 μl/well CCK-8 solution was added and incubated in 37 °C, 5 % CO_2 humidified incubator for 2–4 h. The absorbance value at 450 nm was read using a microplatereader (Bio-Rad, CA, USA). Cell growth rate was calculated. The experiment was repeated three times.

Invasion assay

Invasion assays were done using transwell matrigel invasion chambers with a pore size of 8 μm coated with 1 to 2 μg/cm^2 martigel (BD, USA). OS cells were detached and resuspended in serum-free DMEM. A total of 2×10^4 cells in 100 μl of serum-free media were seeded onto the upper portion of a 24-well matrigel chamber. The lower compartment contained DMEM with 10 % FBS. After incubation at 37 °C for 24 h, the filter was removed and the cells that migrated to the lower surface were kept for further examine. Then, 500 μl complete DMEM with 0.5 mg/ml CCK-8 solution were added in 24-well lower compartment and the filter was dipped into it. Appropriate amount of DMSO was added to each well to dissolve the crystals. Optical density (OD) value was determined by scanning with a microplate reader at a wavelength of 450 nm. All experiments were performed at least in triplicate.

Statistical analysis

Results are reported as mean ± SEM. All the experimental data were analyzed by the SPSS 20.0 statistical software package. The Mann-Whitney U test, χ^2 test, Pearson chi-square test, or Spearman rho test were performed for comparative statistical evaluations among groups and for correlation analysis with histological and clinical parameters (age, gender, tumor stage, tumor grade, and postoperative survival). Survival periods were counted in months from the date of first visit to date of death or last follow-up before study closure. We used Kaplan-Meier method to estimate the overall survival for low and high levels of miR-124 expression. A p value <0.05 was considered as statistically significant.

Results

miR-124 is downregulated in clinical OS specimens and associated with advanced clinical stage and pulmonary metastasis

In our study, a total of clinical human OS tissues and their paired normal adjacent tissues (NATs) were enrolled in this cohort for assessing the expression levels of miR-124. The relationship between the miR-124 expression levels and clinicopathologic characteristics in OS patients are summarized in Table 1. As shown in Fig. 1a, the average expression level of miR-124 was significantly downregulated in OS tissues compared with paired NATs. According to analyzing the expression differences and tumor features, we found that the expression of miR-124 in the non-metastasis group and high-differentiation group was even higher than that of metastasis positive group and low differentiation group, respectively (Fig. 1b, c). In addition, we divided 69 OS cases into three groups according to the stages of Ennecking I stage, II stage, and III–IV stage. We found that the expression of miR-124 was significantly lower in advanced Ennecking stage OS patients (III–IV stage) than that of early stage patients (stages I and II, Fig. 1d). In addition, we found that the 5-year overall survival rate in the miR-124 upregulated group was 61.5 %, respectively, but the rate was only 11.8 % in the miR-124 downregulated group (Fig. 1e).

Relationship between B7-H3 protein and miR-124 expression in clinical OS specimens

In our previous study, B7-H3 was showed to be overexpressed in the majority OS lesions, and the intensity of B7-H3 expression in OS was significantly increased compared with NATs [13]. Here, pertinent clinicopathological findings of the enrolled patients and miR-124 and B7-H3 expression levels are further analyzed in Table 1. In order to investigate the relationship between miR-124 and B7-H3 in human OS, Spearman's rank-order correlation analysis was used and showed that B7-H3 expression in OS tissues was inversely correlated with the expression of miR-124 (R^2 = 0.5082, p = 0.004, Fig. 2a). In order to further clarify their relationship under different conditions, the analysis was performed according to the different Ennecking stage, differentiation, and

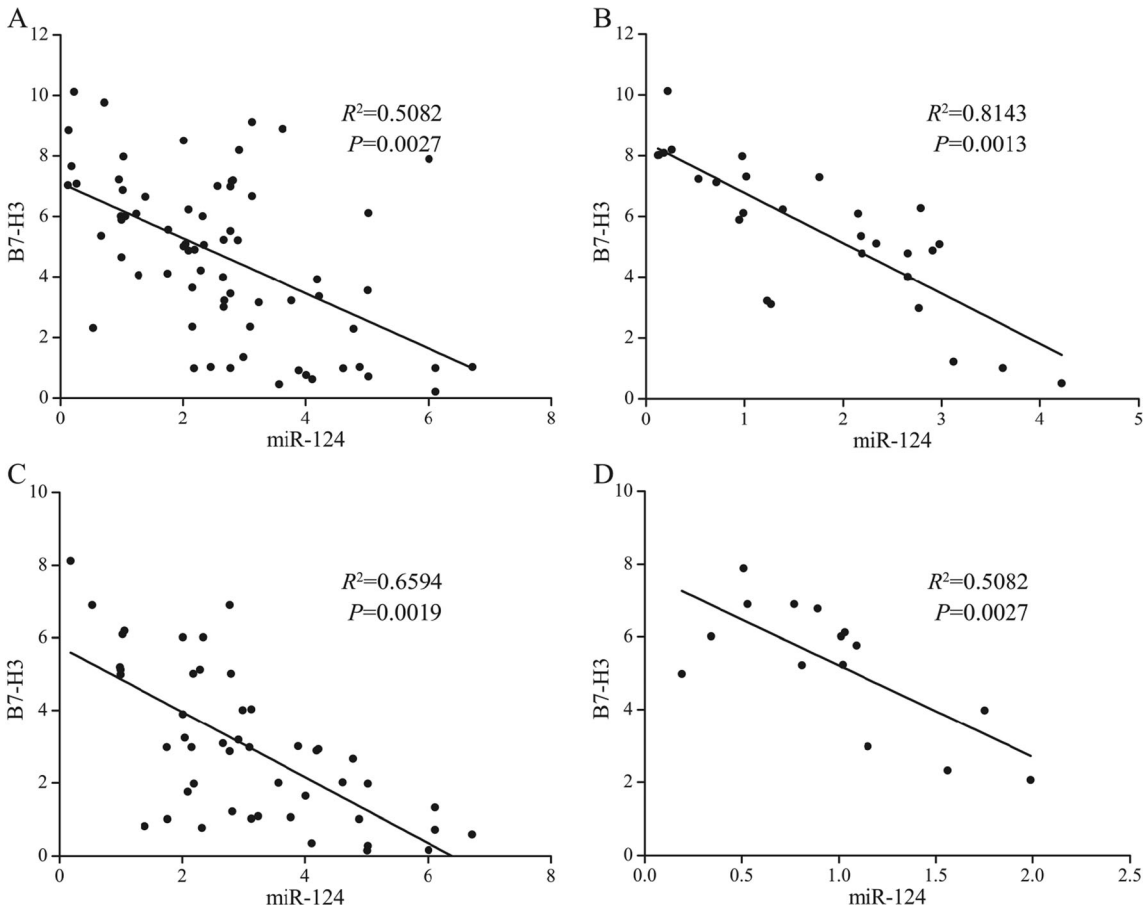

Fig. 2 The relationship between miR-124 and B7-H3 in the whole cohort (**a**), metastatic (**b**), high-differentiation (**c**), and III–IV stage group OS patients (**d**)

metastasis status. Results showed that the inverse correlation seemed much more significant in metastatic, high-differentiation, and advanced stage group (Fig. 2b–d). Taken together, these results indicated that reduced expression of miR-124 may play an important role in regulating B7-H3 in the progression and metastasis of OS.

miR-124 directly targets B7-H3 by binding to its 3′-UTR

In order to analyze the potential interaction between miR-124 and B7-H3, we used TargetScan target prediction software (http://www.targetscan.org/) to predict the alignment of miR-124 binding with the B7-H3 3′UTR target site (Fig. 3a, b). To verify whether B7-H3 is a direct target of miR-124, B7-H3 3′-UTR and mutant constructs were cloned into pGL3-control vector and then co-transfected with miR-214 mimics into MG-63 cells and performed reporter assays. The result showed that the relative luciferase activity of the wild-type B7-H3 3′UTR reporter construct was a significant decrease compared with the empty vector control ($p < 0.05$). However, in the mutant B7-H3 3′UTR group, there was no difference in luciferase activity was found in cells transfected with the mutant constructs compared with the empty vector control (Fig. 3c).

To further test if miR-124 acts directly on B7-H3 expression, we transfected the OS cell line, MG-63, with miR-124 mimics, anti-miR-124, or the respective controls. Results showed that miR-124 mimics could significantly inhibit B7-H3 mRNA and protein expression in MG-63 cells, while anti-miR-124 obviously could elevate B7-H3 expression (Fig. 3d, e). Our data strongly suggest that miR-124 negatively regulates B7-H3 expression via direct binding to putative binding sites in the 3′-UTR region.

B7-H3 is involved in miR-124-induced repression of OS cell growth and invasion

To investigate the possible effects of miR-124 on the OS cell malignancy, cell growth and invasion of MG-63 cells were determined by CCK-8 and transwell assays, respectively. As our expected, exogenous transfected miR-124 mimics could significantly inhibit the growth and invasive ability of MG-63 cells, compared with negative control-transfected cells. Accompanied with the cell viability changing, a set of protein markers related with proliferation and apoptosis were also changed. PCNA and Cyclin D1 are considered to be important factors involving in OS tumorigenesis. Here, the expression of PCNA and Cyclin D1 was inhibited markedly in OS cells after

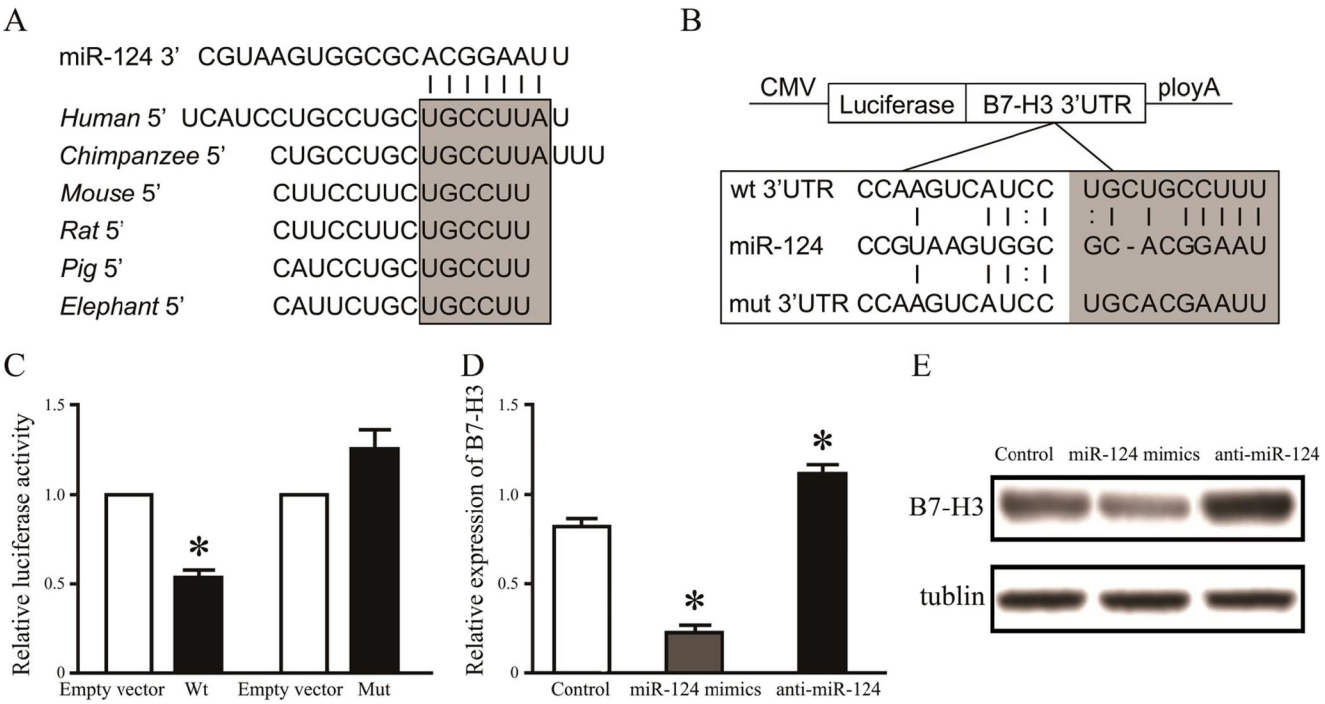

Fig. 3 miR-124 directly targets B7-H3 by binding to the B7-H3 3′-UTR. Potential miR-124 binding site in the 3′-UTR region of B7-H3 and conservation of seed matching sequences among different interspecies (**a**). Diagram of oligonucleotides containing the predicted miR-124 binding sites in the 3′-UTR of B7-H3 mRNA together with mutations on the "seed" sequences are designed (**b**). MG-63 cells were co-transfected with 100 nM of either miR-124 mimics or 100 ng empty expression vector with Wt or Mut 3′-UTR of B7-H3. The relative luciferase activity was measured 24 h after transfection (**c**). mRNA (**d**) and protein (**e**) expression levels of B7-H3 are tested after miR-124 mimics and anti-miR-124 transfected in MG-63 cells by qPRC and western blot assay, respectively. *$p < 0.05$ compared with control

miR-124 mimic treatment. In addition, the classic apoptotic marker PARP was upregulated in transfected miR-124 mimic group. As for invasion, representative candidate MMP-9 and mesenchymal cell marker vimentin were downregulated significantly after miR-124 mimics transfected, while the epithelial marker E-cadherin was enhanced on the contrary.

Next, to elucidate whether miR-124 exerts its function via B7-H3 on growth and invasion of human OS cells, ectopically expressed B7-H3 plasmid together with miR-124 was transfected into MG-63 cells. Results showed that the cell growth and invasive ability of B7-H3 cDNA + miR-124 group were remarkably more increased than empty vector + miR-124 group, suggesting that overexpression of B7-H3 abrogated the reduction of cell growth and invasion caused by ectopic expression of miR-124 in MG-63 cells (Fig. 4). Besides, the notably changes of protein markers related with proliferation and invasion were altered correspondingly.

Discussion

OS is the most common primary malignant bone tumor originating in the metaphysis of the long bones and accounts for 2.4 % of all malignancies in pediatric patients. OS has a highly malignant propensity to rapidly destroy the surrounding tissues and to metastasize; therefore, patients with metastasis have a less than 20 % chance of long-term survival despite the use of chemotherapeutic drugs [26, 27]. The initiation and development of OS metastasis consist of multiple sequential steps that are not completely elucidated to date, and further investigation of this mechanism is urgently needed. Our previous study showed that the B7-H3 expression level correlated with clinical staging and prognosis, and its silencing inhibited the invasion and migration of OS cells in vitro [13]. B7-H3, also known as B7 homolog 3 or CD276, was initially described during a research for homolog genes of the B7 family using nucleic acid sequence analysis in a human dendritic cell (DC)-derived cDNA library [28, 29]. B7-H3 transcripts are ubiquitously expressed in a wide spectrum of tissues, while its normal protein expression is relatively limited and maintained at low levels [30]. However, B7-H3 protein overexpression has been described in numerous human malignancies of the esophagus, stomach, breast, prostate, and other tissues [25, 31–33]. Related to this, aberrant tumor cell B7-H3 expression has recently emerged as a possible mechanism whereby human tumors might escape host immune surveillance.

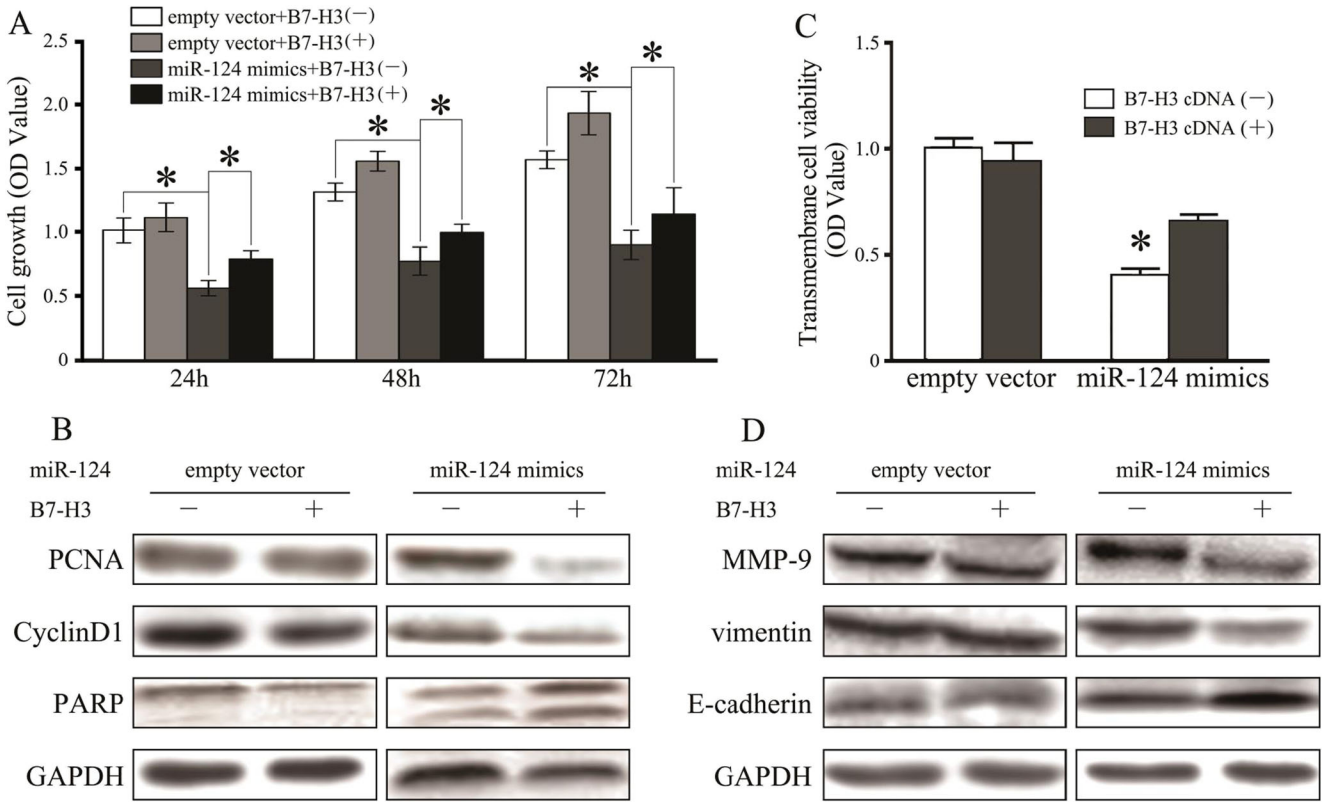

Fig. 4 miR-124 inhibits cell growth and invasion in OS cells. **a**, **b** CCK-8 assay showed that cell proliferation was changed in MG-63 cells accordingly after transfection of miR-124 mimics and B7-H3 cDNA at different time point. The proliferative index molecules PCNA, Cyclin D1, and PARP were detected after transfection of miR-124 mimics and B7-H3 cDNA via western blotting. **c**, **d** Transwell assay showed that cell invasion was changed in MG-63 cells accordingly after transfection of miR-124 mimics and B7-H3 cDNA at 48 h. The invasive index molecules MMP-9, E-cadherin, and Vimentin were detected after transfection of miR-124 mimics and B7-H3 cDNA via western blotting

Nevertheless, the reason that B7-H3 expression was differentially expressed between normal and malignant cells is still elusive.

An emerging paradigm for protein regulation is through miRNA [34, 35]. It has been demonstrated that dysregulation of miRNAs is involved in tumorigenesis and progression in various types of tumors including OS. Qu et al. showed that miRNA-150 targets IGF2BP1 to regulate proliferation, migration, and invasion in OS [36]. miR-664 was also approved to be an oncogene miRNA and involved in promoting human OS cell invasion and migration by suppressing SOX7 expression [37]. miR-124, first demonstrated to be a "brain-specific" miRNA although not been studied as extensively as some other miRNAs, is known to be downregulated in breast cancer, lung cancer, and prostate cancer [38–40]. Our data showed that the expression of miR-124 was significantly downregulated in OS tissues and cell lines, and low miR-124 expression had much lower tumor differentiation, advanced Ennecking stage, and common pulmonary metastasis. In addition, OS patients with high miR-124 expression had a significantly longer survival time compared with the patients with low miR-124 expression. Moreover, this downregulation was inversely correlated with high protein expression of B7-H3 in OS. Subsequently, we demonstrated whether B7-H3 was as direct target of miR-124 in human OS. Our findings showed that miR-124 mimics could significantly inhibit B7-H3 mRNA and protein expression in MG-63 cells, while anti-miR-124 obviously could elevate B7-H3 expression. Besides, a significant decrease of luciferase activity was detected in OS cells transfected with a pGL3 luciferase construct that contains the putative miR-124 binding site in the 3′-UTR of B7-H3 compared with cells transfected with the control empty vector. This is probably due to the fact that only a single conserved miR-124 binding site is in B7-H3 3′UTR. However, we cannot rule out the possibility of involvement of other miRNA families in the regulation of B7-H3 expression, for TargetScan software also predicted dozens of poorly conserved sites for the conserved miRNA families in the human B7-H3 3′UTR. Therefore, it does seem that miR-124 probably plays a critical role in determining the differential protein expression of B7-H3 in tumor tissues versus normal tissues.

The capability of cells to proliferate, migrate, and invade is considered an important determinant in the process of tumorigenesis and progression [41, 42]. Given that B7-H3 plays crucial roles in regulation of cell proliferation and invasion [43], we further investigated the effects of miR-124 on these biological behaviors of OS cells. Notably, overexpression of miR-124 significantly suppressed in vitro cell proliferation and invasion. Our study suggested that miR-124 might downregulated the expression of Cyclin D1 and PCNA and upregulated the apoptotic protein PARP, contributing to the normal cell cycle. Yin et al. [44] and Liu et al. [45] demonstrated that the importance of miRNA-triggered downregulation of transcriptional factors related to EMT during cancer cell invasion and metastasis. Here, our immunoblotting results showed that miR-124 significantly increased the expression of E-cadherin and suppressed the expression levels of MMP-9 and vimentin. Furthermore, restoration of B7-H3 expression abrogated miR-124-inhibited OS cell proliferation and invasion. Our results establish a functional association between miR-124 and B7-H3 and confirm that miR-124 acts as an onco-miRNA in OS cells by targeting B7-H3.

In conclusion, we demonstrated that miR-124 might play a tumor suppressor role in OS and target the 3′-UTR of B7-H3 resulting in translational repression. The dysregulation of miR-124 gains the expression of B7-H3, which promotes cell proliferation and invasion uncontrollably. This study suggests that miR-124 downregulated may play an important role in tumor proliferation and migration and may be a novel diagnostic marker and potential therapeutic target in OS. In the future, modulating B7-H3 protein expression by administering miR-124 mimics might improve the therapeutic potential efficacy of mAb targeting to B7-H3.

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Compliance with ethical standards

Conflicts of interest None

Authors' contributions The conception and design were initiated by FBK and LW; NS, JW, and WC performed clinical data acquisition and drafted the manuscript; DL contributed in the statistical analysis; BES directed the study and helped revised the paper. All authors read and approved the final manuscript.

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