Pathology & Oncology Research (2020) 26:533–539
https://doi.org/10.1007/s12253-018-0556-8

ORIGINAL ARTICLE

MiR-374a Activates Wnt/β-Catenin Signaling to Promote Osteosarcoma Cell Migration by Targeting WIF-1

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Received: 15 August 2018 / Accepted: 19 November 2018 / Published online: 6 December 2018

Abstract

MiR-374a was proved to take part in the initiation and development of several cancers. However, the molecular mechanism of miR-374a in osteosarcoma (OS) cells remains unclear. The aim of our research was to investigate the role of miR-374a in OS cells migration and clarify the potential mechanisms. Quantitative real-time PCR (qRT-PCR) and western blot analysis were applied to evaluate the expression of miR-374a and Wnt inhibitory factor-1 (WIF-1). Bioinformatical methods and luciferase reporter assay were carried out to predict and confirm the combination of miR-374a and WIF-1. Transwell and wound healing assays were performed to detect the migration capacity of OS cells. Lithium chloride (LiCl) was used to investigate the role of LiCl-activated Wnt/β-catenin signaling pathway in regulating cell migration. Our studies revealed that miR-374a was up-regulated whereas WIF-1 was down-regulated in OS cells. Besides, WIF-1 was the target of miR-374a by performing luciferase reporter assay. By transfection of miR-374a inhibitor and/or WIF-1 siRNA to OS cells, we found that miR-374a promoted the migration of OS cells. In addition, the inhibition of WIF-1 abolished the miR-374a inhibitor-induced migration suppression of OS cells. LiCl experiment revealed that miR-374a promoted OS cells migration by regulating Wnt/β-catenin signaling. In conclusion, miR-374a promotes OS cells migration by activating Wnt/β-catenin signaling pathway via targeting WIF-1.

Keywords Osteosarcoma (OS) · miR-374a · Migration · Wnt inhibitory factor-1 (WIF-1) · Wnt/β-catenin · Signaling pathway

Introduction

Osteosarcoma (OS) is known as the most frequent malignant bone tumor worldwide, with the incidence of 3.4 per million people each year [1–3]. For most years of 1990s, the five-year survival of OS was as low as about 50%. In the twenty-first century, the survival of OS increases to about 65% [1]. New therapeutic methods and biotherapy have been appeared recently, whereas the therapeutic effect is not ideal [4]. Thus, novel therapeutic strategies and biomarkers for OS are still strongly required to enhance the treatment effect of osteosarcoma.

MiRNAs are small non-coding RNAs that are transcribed from introns or non-protein-coding genes, and are reported frequently in recent research [5]. MiRNAs mediate translational cleavage or the suppression of their target mRNAs in the way of binding to their 3'UTR complementary sites [6, 7]. Accumulating evidences have demonstrated that miR-374a expression in cancer cells is higher than that in normal cells. For instance, miR-374a is up-regulated in colonic cancer cells and promotes colonic cancer cells proliferation [8]. In breast cancer, miR-374a is also overexpressed and acts as an oncogene [9]. In OS cells, miR-374a is also observed to be up-regulated and promotes the proliferation of OS by targeting Axin2 [10].

Wnt/β-catenin signaling pathway has been reported to be involved in embryonic development [11–13]. WIF-1 has been reported in previous studies as an important negative regulator of Wnt/β-catenin signaling pathway [14–16]. Wnt/β-catenin signaling pathway can be inhibited by WIF-1 combining with Wnt signaling proteins directly [11, 17]. Accumulating evidences suggested that Wnt/β-catenin signaling pathway was

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regulated aberrantly in OS cells [4, 18, 19]. Furthermore, the aberrant regulation of Wnt/β-catenin signaling pathway has been also found to result in the tumorigenesis of OS cells.

To investigate the role of miR-374a in OS progression and clarify the mechanism of migration regulated by miR-374a in OS cells, our research was then designed and performed.

Materials and Methods

Cell Culture

Human OS cell lines (U2OS, MG63, HOS, Saos-2) and human osteoblastic cell line (HFOB1.19) and were obtained from the cell bank of Chinese Academy of Science (Shanghai, China). All cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen, CA, USA), 100 mg/mL streptomycin (Invitrogen, CA, USA), and 100 U/mL penicillin (Invitrogen, CA, USA). Cells were cultured at 37 °C with 95% air and 5% CO_2 in a humidified incubator.

Reverse Transcription and qRT-PCR

TRIzol reagent (Sigma, St. Louis, MO, USA) was used to extract total RNAs of cell lines following the manufacturer's protocols. SuperScript III reverse transcriptase kit (Sigma, St. Louis, MO, USA) was used to synthesize cDNA according to the protocol of manufacturer. qRT-PCR was then performed using Taq-Man Universal PCR master mix (Sigma, St. Louis, MO, USA). Relative transcript levels of miR-374a and WIF-1 mRNA expression were then calculated. U6 and GAPDH were respectively used as the normalization of miRNA and WIF-1 expression.

Western Blotting

Cells were first washed with precooled buffer PBS, and then lysed with the mammalian protein extraction regent RIPA buffer (Invitrogen, Carlsbad, CA, USA) supplemented with phenylmethylsulfonylfluoride and protease inhibitor cocktail (Sigma, St. Louis, MO, USA). 50 micrograms of protein were first separated by 10% SDS-PAGE, and then were transferred to PVDF membranes. The membranes were incubated with primary antibodies against WIF-1 (Abcam, Cambridge, UK) overnight at the atmosphere of 48 °C, and then incubated with secondary antibodies. The membranes were then visualized by using ECL western blotting detection kit (Sigma, St. Louis, MO, USA). Gel Image system software (Invitrogen, CA, USA) was used for the analysis of western blotting. Nuclear extracts were prepared by applying the Nuclear Extraction Kit (Abcam, Cambridge, UK) according to the manufacturer's instructions.

Luciferase Reporter Assay

The WIF-1 3'UTR containing predicted miR-374a binding sites were first amplified by PCR from human cDNA using primers. After that, WIF-1 3'UTR was inserted into pMIR-REPORT luciferase reporter vectors (Invitrogen, CA, USA) to acquire constructs containing wild-type WIF-1 3'UTR (WIF-1-wt). Recombination constructs, miR-374a and pRL-TK (Invitrogen, CA, USA) were co-transfected into U2OS and MG63 cells by applying lipofectamine 2000 (Invitrogen, CA, USA). For internal control, plasmids of pRL-TK containing Renilla luciferase were applied. 24 h after transfection, firefly and enilla luciferase activity were measured by dual luciferase assay (Invitrogen, CA, USA) following the manufacturer's instructions.

Knock-Down of WIF-1

For WIF-1 knock-down, siRNA of WIF-1 was acquired from RiboBio (Guangzhou, Guangdong, China). Next, the WIF-1 siRNA and negative control was transfected separately into MG63 and U2OS cells via Lipofectamine 2000 (Invitrogen, CA, USA) according to the manufacturer's instructions.

Transfection of miRNA Inhibitor

For the investigation of miRNA function, miR-374a inhibitor and a non-targeting control miRNA inhibitor (NC) were acquired from RiboBio (Guangzhou, Guangdong, China). U2OS and MG63 cells were then transfected by miR-374a inhibitor or NC in the presence of 8 mg/mL Polybrene (Invitrogen, CA, USA) overnight, separately.

Transwell Assay

Migration of cells were analyzed using the Transwell chambers (Costar, Corning Inc., Corning, NY, USA), with or without coated Matrigel (BD Biosciences, CA, USA). The lower chamber of the experiment device was filled with 10% FBS and 500 μl DMEM. After incubation for 24 h, cells that have invaded into the bottom side of the inserts were fixed. Next, the cells were stained and photographed. Finally, the cells were quantified by counting them in 5 random high-power fields.

Wound Healing Assay

To evaluate the migration ability of cells, scratch wound healing assay was performed. Indicated cells were cultured in 6-well plates. A pipette tip was used to create streaks in the center of monolayer. After wounding for 24 h, the progression of migration was observed and then photographed. The percentage of the uncovered area was calculated using the following equation: uncovered area (%) = [wound width (0 h)/wound width (24 h)] × 100%.

Data Analysis

All data of this study was presented as mean ± SEM, and Graphpad (Ver. Prism 6, GraphPad Prism Software, CA, USA) was used to statistical analysis. Student's t test was used to evaluate the difference between means of different groups. Differences between comparing groups were considered significant if $p < 0.05$.

Results

MiR-374a Expression is Up-Regulated whereas WIF-1 is Down-Regulated in OS Cell Lines

Relative miR-374a mRNA expression level was evaluated by performing qRT-PCR in osteoblastic cell line HFOB1.19 and OS cell lines (U2OS, HOS, SaOS2, KHOS, and MG-63). Results showed that mRNA expression of miR-374a was significantly up-regulated to various degrees in OS cell lines compared with HFOB1.19 cells ($p < 0.01$, Fig. 1a).

WIF-1 mRNA and protein expression were tested by qRT-PCR and western blotting, respectively. Relative expression of WIF-1 mRNA in OS cell lines (U2OS, HOS, KHOS, SaOS2 and MG-63) was significantly lower than that in HFOB1.19 cells ($p < 0.05$, $p < 0.01$, Fig. 1b). WIF-1 protein expression in OS cell lines was also significantly decreased compared to HFOB1.19 cells ($p < 0.01$, Fig. 1c and d).

WIF-1 was the Target Gene of miR-374a

To validate the regulatory role of miR-374a in OS cell, a specific inhibitor against miR-374a was used to inhibit the expression of miR-374a in U2OS and MG63 cells. qRT-PCR showed that expression of miR-374a was decreased successfully ($p < 0.01$, Fig. 2a). Relative level of WIF-1 mRNA expression in cells increased significantly after the transfection of miR-374a inhibitor ($p < 0.01$, Fig. 2b). These results revealed that WIF-1 mRNA expression may be regulated by miR-374a. To further investigate the molecular mechanism of miR-374a regulating WIF-1 expression, we explored the targets of miR-374a by using bioinformatics algorithm. Corresponding results demonstrated that WIF-1 was a potential target of miR-374a (Fig. 2c). To confirm WIF-1 as the target gene of miR-374a, we constructed luciferase reporter constructs containing wild-type or mutant 3'UTR of the WIF-1 gene. Luciferase reporter assay demonstrated that miR-374a decreased the luciferase activity of wild-type WIF-1, whereas the luciferase activity of mutant WIF-1 has no significant difference between mimic and NC groups in both MG63 and U2OS cell lines ($p < 0.01$, Fig. 2d). Thus, WIF-1 was a direct target gene of miR-374a.

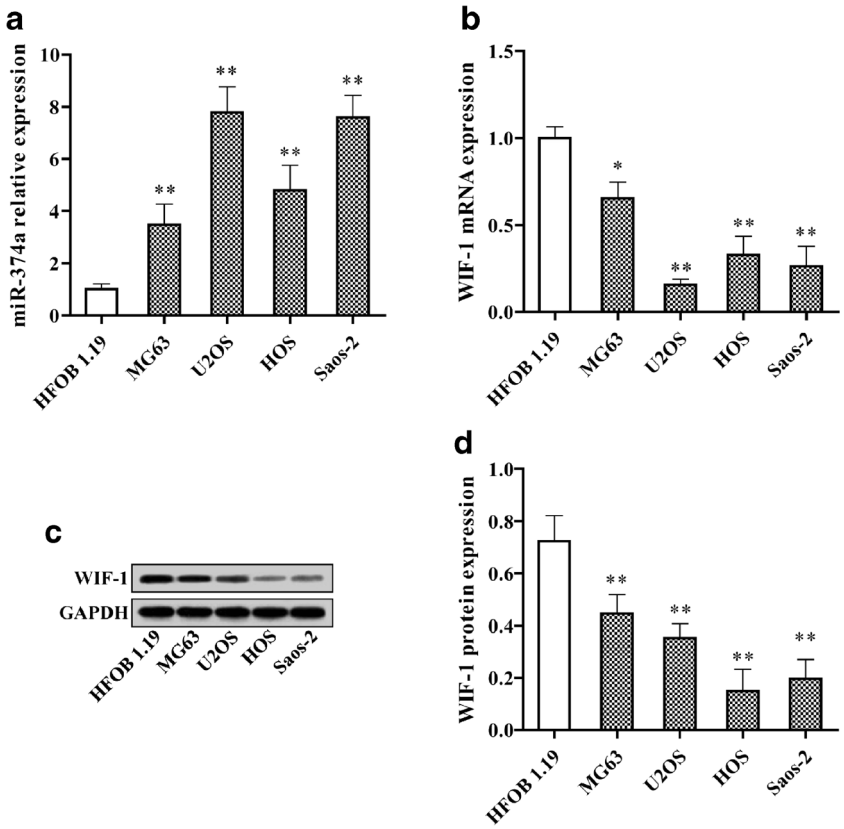

Fig. 1 Relative expression of miR-374a and WIF-1 in osteosarcoma cell lines. a qRT-PCR analysis of miR-374a relative expression in OS cell lines (U2OS, MG63, HOS and Saos-2) compared with HFOB 1.19 cells. **b** qRT-PCR analysis of WIF-1 mRNA level in OS cell lines (U2OS, MG63, Saos-2 and HOS) compared with HFOB 1.19 cells. **c** Western blot analysis of WIF-1 protein expression in OS cell lines (U2OS, MG63, Saos-2 and HOS) and HFOB 1.19 cells. **d** Statistical result of WIF-1 protein expression in OS cell lines (U2OS, MG63, Saos-2 and HOS) and HFOB 1.19 cells. * $P < 0.05$, ** $P < 0.01$, compared with HFOB 1.19

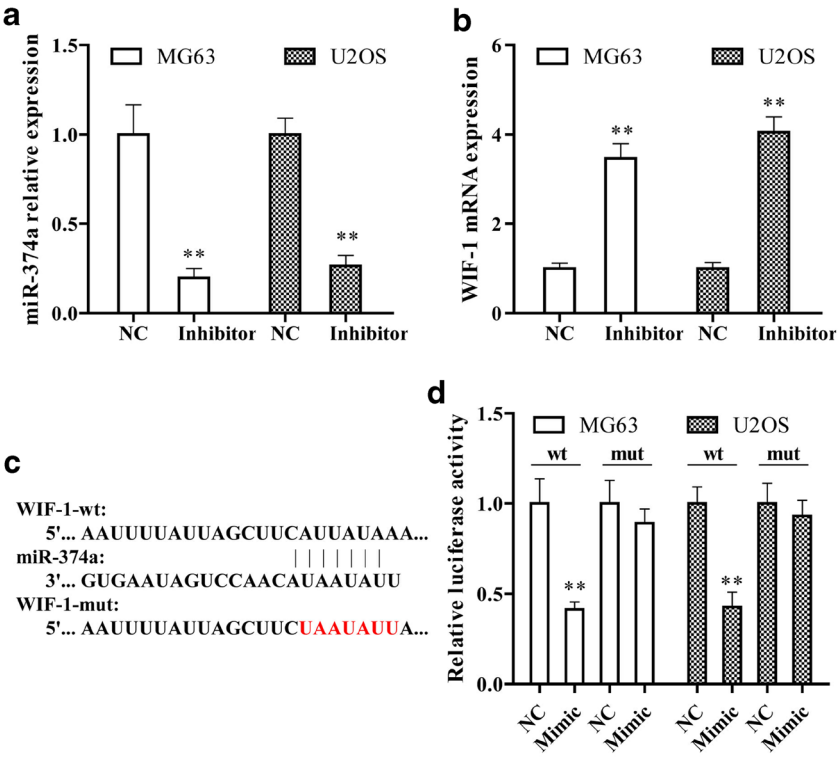

Fig. 2 WIF-1 is a target of miR-374a. a Relative expression of miR-374a after the transfection of miR-374a inhibitor and NC in cell lines MG63 and U2OS. **b** Relative expression of WIF-1 mRNA after the transfection of miR-374a inhibitor and NC in cell lines MG63 and U2OS. **c** The potential binding sites between miR-374a and WIF-1, and the sequence of WIF-1-mut. **d** The validation of target by performing luciferase reporter assay in MG63 and U2OS cells. The relative luciferase activities of luciferase reporters containing WT or Mut WIF-1 were assayed 48 h after co-transfection with miR-374a mimics or NC RNA. Relative activity of renilla luciferase was used to normalize that of firefly luciferase. * $P < 0.05$, ** $P < 0.01$, compared with NC

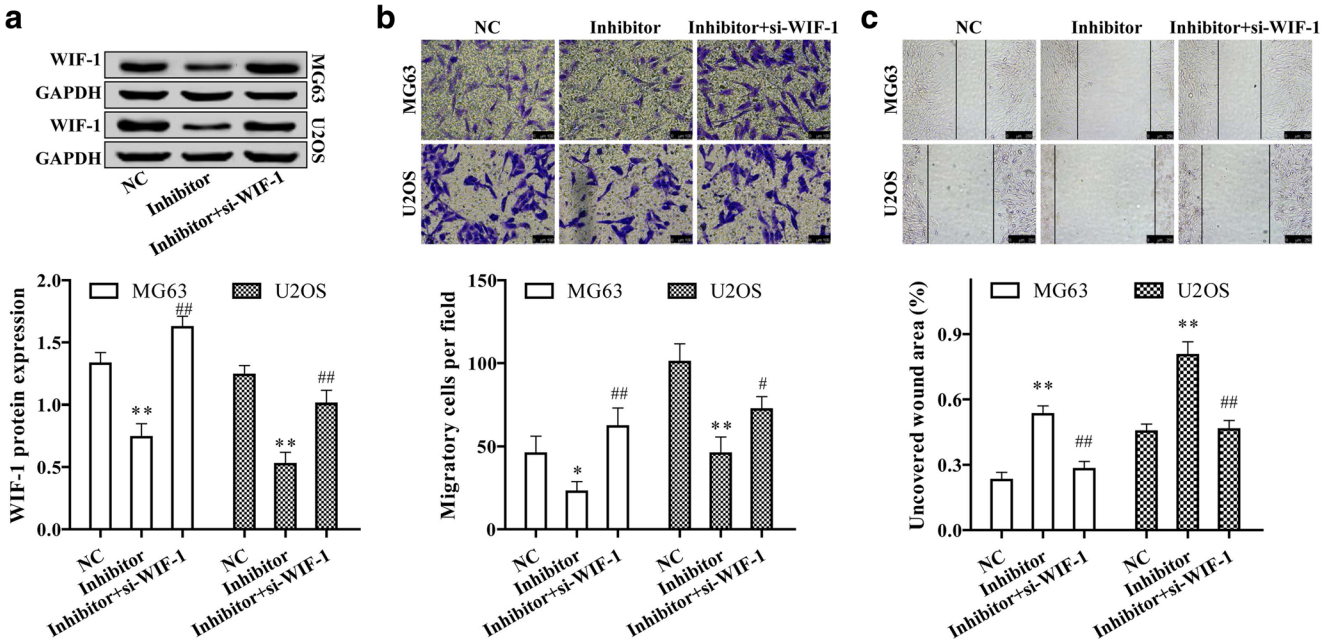

Fig. 3 Knock-down of WIF-1 abolished the inhibition of OS cell by miR-374a knockdown. a Western blot analysis of WIF-1 expression after the transfection of miR-374a inhibitor and/or WIF-1 siRNA in MG63 and U2OS cells. WIF-1 protein expression was enhanced after transfected with miR-374a inhibitor. **b** Transwell assay shows that WIF-1 knock-down increased the migration of MG63 and U2OS cells. Migrated cells per field were more than NC after transfection of miR-374a inhibitor and WIF-1 siRNA. **c** Micrographs of wound healing. Wound closures were photographed 24 h after scratching. Uncover wound area percentage of the cells after transfection of miR-374a inhibitor was larger than NC significantly, whereas knock-down of WIF-1 abolished the migration ability inhibited by miR-374a know-down. * $P < 0.05$, ** $P < 0.01$, compared with NC. # $P < 0.05$, ## $P < 0.01$, compared with inhibitor

Knockdown of WIF-1 Abolished the Migration Inhibition of OS Cell by miR-374a Knockdown

Next, miR-374a inhibitor and/or siRNA of WIF-1 were/was transfected into MG63 and U2OS cells. WIF-1 expression of cells transfected with miR-374a inhibitor was lower than cells transfected by NC in MG63 and U2OS cell lines ($p < 0.01$, Fig. 3a). According to the results of transwell assay, migratory cells per field of U2OS and MG63 cells transfected by miR-374a inhibitor was lower than NC ($p < 0.01$, $p < 0.01$). However, migrated cells per field of cells transfected by miR-374a inhibitor and WIF-1 siRNA were more than NC ($p < 0.05$, $p < 0.01$, Fig. 3b). According to the results of wound healing assay, miR-374a inhibitor decreased the migratory speed of MG63 and U2OS cells ($p < 0.01$), whereas knock-down of WIF-1 abolished the inhibition of miR-374a inhibitor in speed of migration ($p < 0.01$, Fig. 3c).

MiR-374a Promoted OS Cell Migration by Regulating Wnt/β-Catenin Signaling Pathway

It has been known that Wnt/β-catenin signaling pathway plays an important role in regulating the migration of cells. Therefore, we tried to investigate whether miR-374a takes part in activating Wnt/β-catenin signaling pathway. Western blot analysis was performed to evaluate the nuclear fractions of β-catenin in U2OS and MG63 cells. Results showed that miR-374a inhibitor decreased β-catenin nuclear translocation significantly in U2OS and MG63 cells ($p < 0.01$, Fig. 4a and b). On the contrary, knock-down of WIF-1 abolished the inhibition of miR-374a inhibitor in β-catenin nuclear translocation ($p < 0.01$, Fig. 4a and b). These results demonstrated that miR-374a plays an important role in promoting nuclear translocation of β-catenin, and consequently, promoting cell migration.

Lithium chloride (LiCl) was then used to investigate the role of LiCl-activated Wnt/β-catenin signaling pathway in regulating cell migration. As we expected, results of transwell assay showed that LiCl rescue the inhibition of miR-374a inhibitor in cell migration ($p < 0.01$, Fig. 4c). In addition, results of wound healing assay showed that LiCl abolished the inhibition of miR-374a inhibitor in cell migration ($p < 0.01$, Fig. 4d). These results revealed that miR-374a promoted the migration of OS cells by regulating Wnt/β-catenin signaling pathway.

Discussion

OS is one of the most common primary bone malignant tumors with extremely low survival rate [1, 20, 21]. Despite new therapeutic strategies have been developed in recent years, the

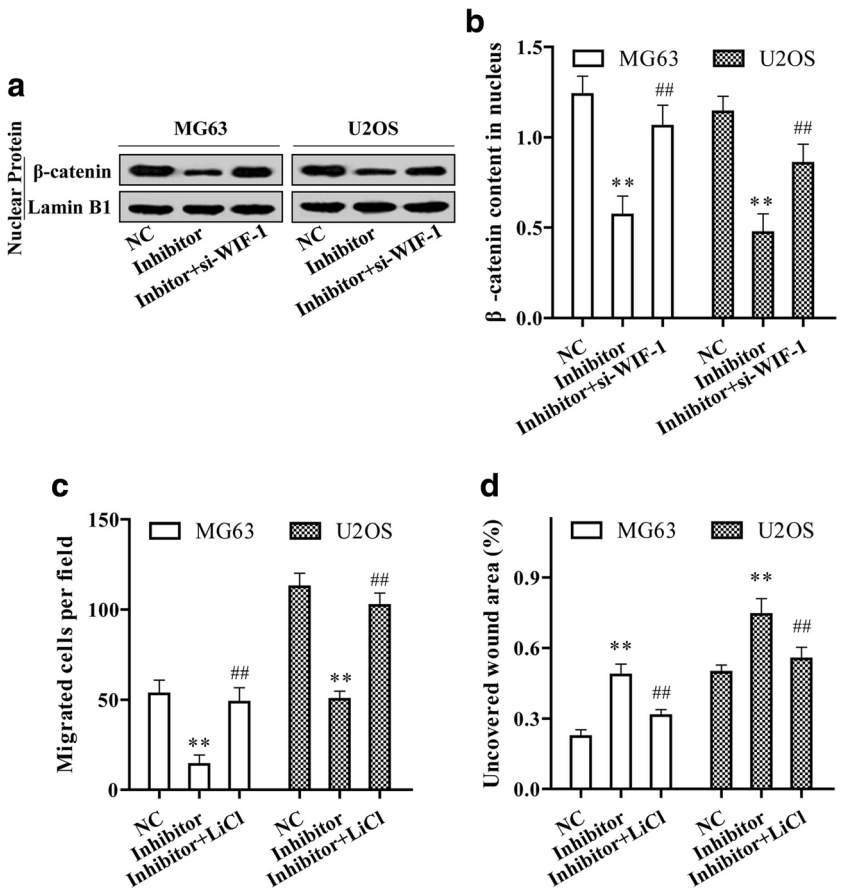

Fig. 4 MiR-374a promoting the migration of OS cells by regulating Wnt/β-catenin signaling pathway. a Altered nuclear translocation of β-catenin after transfection of miR-374a inhibitor and/or WIF-1 siRNA in MG63 and U2OS cells. Nuclear fraction of cells was analyzed by western blot analysis. Lamin B1 was used as a loading control. **b** Statistical result of β-catenin content in nucleus. **c** Transwell assay shows that the migration of MG63 and U2OS cells was rescued after LiCl was applied to activate the Wnt/β-catenin signaling pathway. Migrated cells per field were more than miR-374a group after the transfection of miR-374a inhibitor and LiCl. **d** Uncovered wound area after transfection of miR-374a inhibitor was larger than NC significantly, whereas LiCl rescued the migration ability inhibited by miR-374a know-down by activating the Wnt/β-catenin signaling pathway. * $P < 0.05$, ** $P < 0.01$, compared with NC. # $P < 0.05$, ## $P < 0.01$, compared with inhibitor

curative effect is still not ideal. Novel therapeutic strategies or biomarkers for OS remain urgently needed [22]. Thus, our study aims to illuminate the molecular mechanism of tumorigenesis of OS cells and provide potential therapeutic strategies for OS.

MiRNAs are non-coding RNAs that regulate gene expression at post transcriptional level [5]. In recent years, a lot of researches have indicated that miRNAs and their target genes may represent new therapeutic targets or biomarkers for OS [2]. Accumulating evidences have shown that miR-374a plays a vital role in several types of tumor progression [17]. MiR-374a was observed to promote the proliferation of OS cells by targeting FOXO1 and Axin2 in previous studies [10, 23]. In addition, miR-374a plays varying roles in different kinds of cancers [8–10]. For instance, miR-374a acts as an oncogene in the proliferation of colonic cancer and breast cancer cells [10, 24]. On the contrary, miR-374a was also found to be down-regulated in the tissues of lung cancer [25]. In this research, miR-374a was up-regulated in OS cells and promoted the migration of OS cells.

Wnt signaling pathways play vital roles in regulating the development of embryonic [26]. WIF-1 protein, encoded by gene Wnt inhibitory factor 1, is one of the important regulatory factor for Wnt signaling pathways [16], which inhibits the activation of Wnt signaling pathways by combining with Wnt signaling proteins directly [15]. As an inhibitor of cancer, WIF-1 has been reported in several studies to be down-regulated in various kinds of cancers [16]. In our study, WIF-1 expression was observed to be down-regulated in OS cells by performing qRT-PCR and western blotting. Besides, we noticed that WIF-1 was a potential target of miR-374a via the bioinformatical tool TargetScan (http://www.targetscan.org/). Later, we confirmed that WIF-1 was a target gene of miR-374a and directly regulated by miR-374a using luciferase reporter assay. These results demonstrated that WIF-1 takes part in the development of OS cancer.

To further investigate the role of miR-374a in regulating WIF-1 expression, miR-374a inhibitor was transfected into OS cells. As expected, WIF-1 protein expression was down-regulated significantly and the migratory ability decreased as well. To further confirm the role of WIF-1 in regulating migration, miR-374a inhibitor and siRNA of WIF-1 were co-transfected into MG63 and U2OS cells. According to the results, knock-down of WIF-1 abolished the inhibition of miR-374a inhibitor in speed of migratory. Thus, we can draw a conclusion that miR-374a promotes migration of OS cells by inhibiting WIF-1 expression. These results were of importance to confirm the regulatory function of WIF-1 in regulating cell migration.

Wnt/β-catenin signaling pathway has been identified for its role in the initiation and development of cancer [13]. It participates in the process of tumor development by controlling cell proliferation, differentiation, and migration [12]. Increasing evidences have suggested that Wnt/β-catenin signaling pathway was activated in OS cells and the aberrant regulation of Wnt/β-catenin signaling pathway results in the tumorigenesis of OS cells [11]. To confirm the involvement of Wnt/β-catenin signaling pathway in regulating migration of OS cells, western blotting was performed to analyze the nuclear fractions of β-catenin. We found that miR-374a inhibitor decreased β-catenin nuclear translocation whereas WIF-1 knock-down abolished the inhibitory effect of miR-374a inhibitor on cell migration. Lithium chloride (LiCl) was then used to investigate the role of LiCl-activated Wnt/β-catenin signaling pathway in regulating the migration of OS cells [27]. Transwell and wound healing assays showed that LiCl abolished the inhibition of miR-374a knock-down on cell migration. These results demonstrated that miR-374a promotes the migration of OS cells by regulating Wnt/β-catenin signaling pathway and confirmed the important role of Wnt/β-catenin signaling pathway in OS development.

In this study, miR-374a was up-regulated and WIF-1 was down-regulated by miR-374a up-regulation in OS cells. Thus, Wnt/β-catenin signaling pathway was activated and the migration of OS cells was promoted. In conclusion, miR-374a promotes OS cells migration by activating Wnt/β-catenin signaling pathway via targeting WIF-1.

Acknowledgements This work was supported the Development Program of Kunming University of Science and Technology (Grant No.: KKSY201560051), Yunnan Provincial Science and Technology Department-Kunming Medical University Joint Special Project (Grant No.: 2015FB095) and the Basic Research Project of Yunnan Provincial Science and Technology (Grant No.: 2018FB119).

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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