

Original Article

Targeted inhibition of miR-340 on β -catenin to potentiate Adriamycin sensitivity of osteosarcoma Saos-2 cells

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Abstract: Wnt/ β -catenin pathway regulates cell proliferation and apoptosis via facilitating Survivin transcription. Over-expression of β -catenin is correlated with osteosarcoma onset and drug resistance. MiR-340 is down-regulated in osteosarcoma, and has targeted relationship with 3'-UTR of β -catenin. This study investigated the role of miR-340 in regulating β -catenin expression, and affecting osteosarcoma Saos-2 cells drug resistance to Adriamycin (ADM). MiR-340 and β -catenin expression was compared between Saos-3 and hFOB1.19 cells. Dual luciferase gene reporter assay demonstrated regulatory relationship between miR-340 and β -catenin. ADP-resistant cell line Saos-2/ADM1.0 and Saos-2/ADM4.0 was generated for quantifying miR-340, β -catenin and Survivin expression. Drug resistant index was calculated and compared. 4.0 mg/L ADM was used to treat Saos-2/ADM4.0 cells, which were then treated with miR-340 mimic and/or si- β -catenin. Cell expression of β -catenin and Survivin, and cell proliferation/apoptosis were compared. Saos-2 cells had lower miR-340 and higher β -catenin expression compared to hFOB1.19 cells. A targeted regulation relationship existed between miR-340 and β -catenin. MiR-340 expression in Saos-2/ADM4.0 (RI=44.528) and Saos-2/ADM1.0 (RI=21.937) cells was lower than Saos-2 cells, whilst β -catenin and Survivin expression was higher. Transfection of miR-340 mimic and/or si- β -catenin lowered β -catenin and Survivin expression, weakened cell proliferation potency, and increased ADM-induced apoptosis. MiR-340 can decrease Saos-2 cell proliferation, elevate apoptosis and ADM sensitivity via targeted inhibition on β -catenin and downstream anti-apoptotic protein Survivin expression.

Keywords: MiR-340, β -catenin, survivin, ADM, osteosarcoma

Introduction

Osteosarcoma is one primary malignant bone tumor commonly occurred in children and young population [1]. Although wide usage of chemotherapy significantly improves survival and prognosis of osteosarcoma patients, certain cases still present drug resistance to chemotherapy. Therefore, the investigation of mechanism underlying drug resistance of osteosarcoma is one major challenge of current research [2]. Wnt/ β -catenin signal pathway is involved in regulating cell proliferation and apoptosis. This pathway is correlated with occurrence, progression and metastasis of various tumors such as colorectal carcinoma [3], pulmonary cancer [4], pancreatic cancer [5] and endometrial carcinoma [6]. β -catenin is the

core effector of Wnt/ β -catenin signal transduction pathway, as its up-regulation induces abnormal activation of this pathway, and potentiates expression of downstream targeted genes such as c-myc, cyclin D1 and Survivin, thus playing an important role in tumor occurrence, invasion, metastasis and chemotherapy resistance.

Survivin is one anti-apoptotic protein and can inhibit Caspase activity for antagonizing cell apoptosis, thus is related with lower drug sensitivity of tumor cells [7]. Previous study showed significantly elevated β -catenin protein expression in osteosarcoma cells, and its correlation with lower chemotherapy sensitivity and acquirement of drug resistance [8, 9]. MicroRNA (miR) is one small molecule non-coding RNA

with 22~25 nucleotides in eukaryotes. It can bind with 3'-untranslated region (3'-UTR) of target gene mRNA via complete or incomplete base pairing to degrade or inhibit target mRNA translation, thus mediating about 30% of human genes [10]. Abnormal expression or function of miR in osteosarcoma occurrence is drawing lots of focus [11]. Previous study showed significantly lower miR-340 expression in tumor tissues of osteosarcoma patients [12], indicating its tumor inhibitory role. Moreover, miR-340 has been reported to mediate chemotherapy drug sensitivity of tumor cells [13]. Bioinformatics analysis showed complementary binding sites between miR-340 and 3'-UTR of β -catenin mRNA. This study thus investigated if miR-340 played a role in mediating expression of β -catenin and downstream anti-apoptotic factor Survivin, and sensitivity towards ADM of osteosarcoma cells.

Materials and methods

Reagent and materials

Human osteosarcoma cell line Saos-2 was purchased from Boster (China). Normal human osteoblast cell line hFOB1.19 was purchased from Hongshun Biotech (China). DMEM medium, RPMI 1640 medium and DMEM/F12 (1:1) medium were purchased from Bosheng Biotech (China). Fetal bovine serum (FBS) was purchased from Bioind (Israel). Streptomycin-penicillin mixture and L-glutamine were purchased from Cellgro (US). G418 was purchased from Amresco (US). DharmaFECT Duo Transfection Reagent was purchased from GE (US). QuantiTect SYBR Green RT-PCR Kit was purchased from Qiagen (Germany). MiR-340 nucleotide fragment was synthesized by Gimma (China). PCR primers were synthesized by Sangco (China). Mouse anti-human β -catenin polyclonal antibody was purchased from Abnova (US). Rabbit anti-human Survivin monoclonal antibody was purchased from Weiming Biotech (China). Mouse anti-human β -actin polyclonal antibody was purchased from Merck Millipore (US). Goat anti-rabbit and anti-mouse IgG-HRP (H + L) were purchased from Shengxing Biotech (China). ADM was purchased from Hengrui Pharma (China). CCK-8 reagent was purchased from Dojindo (Japan). Dual-luciferase activity assay kit and pGL3 plasmid were purchased from Promega (US).

Cell culture

Saos-2 cells were kept in RPMI 1640 medium containing 10% FBS and 1% streptomycin, and in an incubator with 5% CO₂ at 37°C. After paving all dishes, cells were passed at 1:4 ratio. hFOB1.19 cells were kept in DMEM/F12 (1:1) medium containing 10% FBS, 2.5 mM L-glutamine and 0.3 mg/mL G418, and in an incubator with 5% CO₂ at 34°C. After paving all dishes, cells were passed at 1:4 ratio.

Induction of ADM resistant osteosarcoma cells

Saos-2 cells at log-growth phase with good status were treated with 0.1 mg/L ADM as the starting concentration. 24 h later, PBS was used to wash out, for changing fresh, ADM-free medium. When cell status returned to normal, cells were repeatedly treated with same concentrations for six times. ADM drug concentration was then gradually increased to 0.4, 1.0 and 4.0 mg/L until Saos-2 cells can maintain normal growth status. Those Saos-2 cells that can normally grow at 1.0 and 4.0 mg/L ADM were named as Saos-2/ADM1.0 and Saos-2/ADM4.0.

Assay for drug sensitivity

In vitro cultured Saos-2, Saos-2/ADM1.0 and Saos-2/ADM4.0 cells were treated for 72 h at different concentrations of ADM. CCK-8 approach was used to calculate relative proliferation activity of all cells. Relative inhibition rate (%) = 1 - relative proliferation activity (%). IC₅₀ value was calculated as the drug concentration required for inhibiting 50% cell growth. Resistance index (RI) = IC₅₀ of drug resistant cells / IC₅₀ of home line cells.

Dual luciferase activity assay

Full length fragment of 3'-UTR of β -catenin was sub-cloned into pGL3 vector, which was named as β -catenin-UTR-wt. Luciferase reporter vector containing mutant form of 3'-UTR of β -catenin gene was also constructed as β -catenin-UTR-mut. DharmaFECT Duo Transfection Reagent was used to transfect β -catenin-UTR-wt (or β -catenin-UTR-mut) and miR-340 mimic into HEK293T cells. After 48 h, dual luciferase activity assay kit was used to test dual luciferase activity following cell lysis.

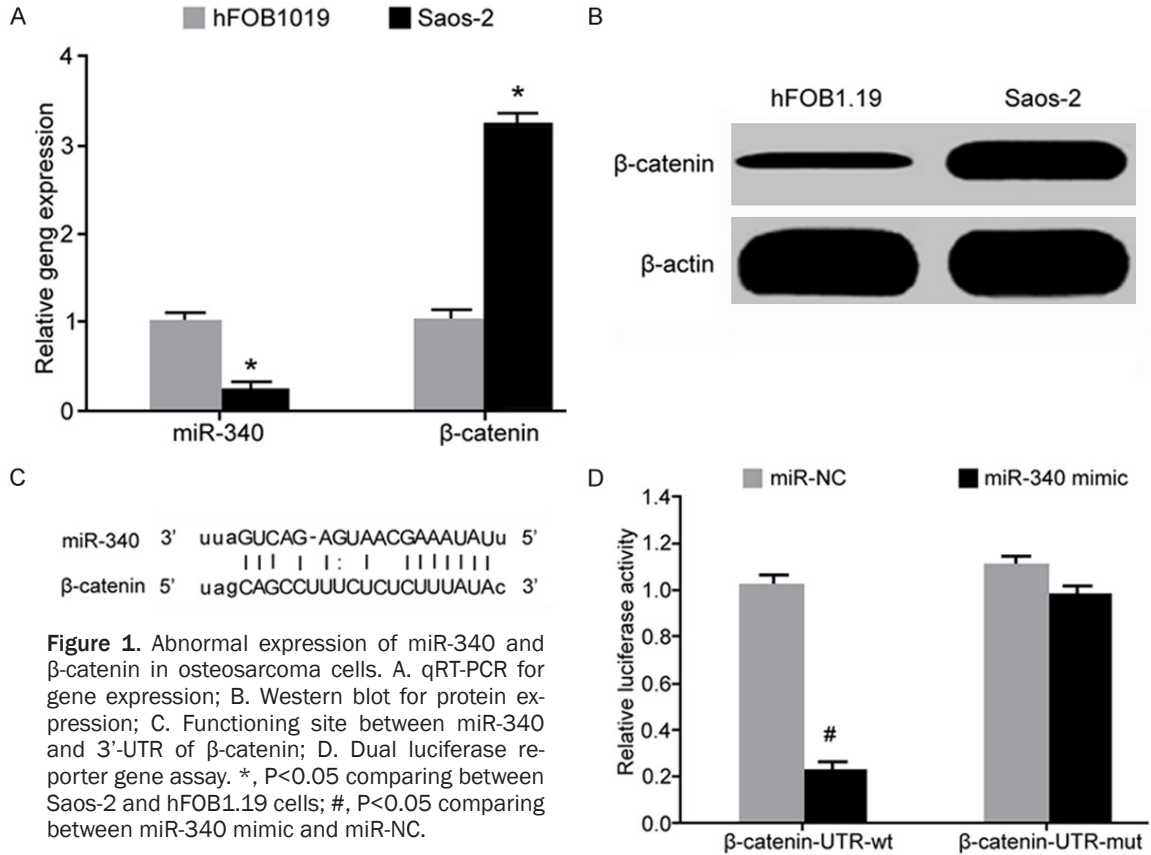


Figure 1. Abnormal expression of miR-340 and β-catenin in osteosarcoma cells. A. qRT-PCR for gene expression; B. Western blot for protein expression; C. Functioning site between miR-340 and 3'-UTR of β-catenin; D. Dual luciferase reporter gene assay. *, P<0.05 comparing between Saos-2 and hFOB1.19 cells; #, P<0.05 comparing between miR-340 mimic and miR-NC.

Cell transfection and grouping

In vitro cultured Saos-2/ADM4.0 cells were divided into five groups: miR-NC transfection group; miR-340 mimic transfection group; si-NC transfection group; si-β-catenin group; and miR-340 mimic + si-β-catenin group. 72 hours after transfection, cells were collected for assay. Primers sequences were: si-β-catenin sense, 5'-CATGU GUTGG UAAGC UCUAdT dT-3'; si-β-catenin anti-sense, 5'-GCAAC AGTTG CAGAG AGGUdT dT-3'; si-NC sense, 5'-AUGCU GATCA GUGUC GATUdT dT-3'; si-NC anti-sense, 5'-CAGAG AGCTC GUGAG AGTAdT dT-3'; miR-410 mimic, 5'-UUAUA AAGCA AUGAG ACUGA UU-3'; miR-NC, 5'-UUCUC CGAAC GUGUC ACGU-3'.

qRT-PCR for gene expression

QuantiTest SYBR Green RT-PCR Kit was used to test gene expression by qRT-PCR. In a 20 μL system, one added 10.0 μL 2× QuantiTest SYBR Green RT-PCR Master Mix, 1.0 μL of forward/reverse primers, 2 μg Template RNA, 0.5 μL QuantiTest RT Mix and ddH₂O. Primer

sequences used were: miR-340P_F: 5'-GCGGT TATAA AGCAA TGAGA-3'; miR-340P_R: 5'-GTGCG TGTCT GGGAG TCG-3'; U6P_F: 5'-ATTGG AACGA TACAG AGAAG ATT-3'; U6P_R: 5'-GGAAC GCTTC ACGAA TTTG-3'; β-cateninP_F: 5'-AGGAC CACCG CATCT CTACA T-3'; β-cateninP_R: 5'-GCAGT TTTGT CAGTT CAGGG A-3'; SurvivinP_F: 5'-AGGAC CACCG CATCT CTACA T-3'; SurvivinP_R: 5'-AAGTC TGGCT CGTTC TCAGT G-3'; β-actinP_F: 5'-GAACC CTAAG GCCAA C-3'; β-actinP_R: 5'-TGTCA CGCAC GATTT CC-3'. PCR conditions were: 95°C for 15 min pre-denature, followed by 40 cycles each containing 94°C 15 s denature, 60°C 30 s annealing and 72°C 30 s elongation. Applied Biosystems 7500 real-time quantitative PCR was used for testing gene expression.

Western blot

Cells were collected and digested using 0.25% trypsin buffer. By twice PBS rinsing, protein extraction lysis buffer was added for 20 min iced incubation. After BCA quantification, 40 μg samples were loaded with 5× buffer, boiled for 5 min at 99°C and separated in SDS-PAGE. Proteins were then transferred to PVDF mem-

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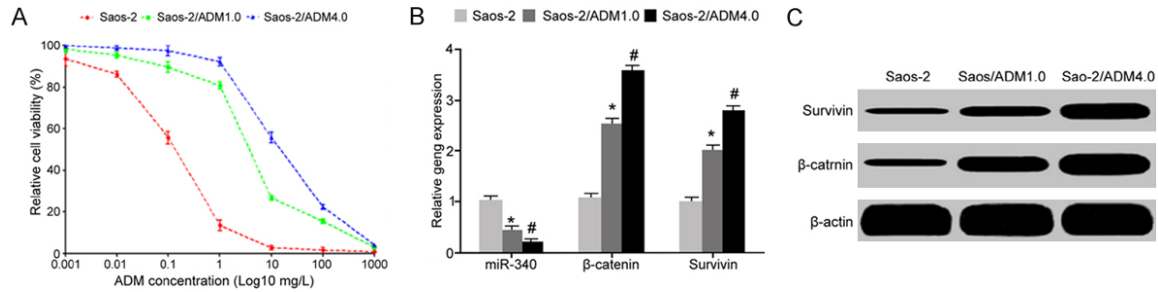


Figure 2. Lower miR-340 and up-regulation of β -catenin or Survivin is correlated with ADM resistance. A. CCK-8 assay for cell proliferation activity; B. qRT-PCR for gene expression; C. Western Blot for protein expression. *, $P < 0.05$ comparing between Saos-2/ADM1.0 and Saos-2 cells; #, $P < 0.05$ comparing between Saos-2/ADM4.0 and Saos-2/ADM1.0 cells.

Table 1. IC_{50} values of Saos-2/ADM4.0 and Saos-2/ADM1.0 cells

Saos-2	Saos-2/ADM1.0		Saos-2/ADM4.0	
IC_{50}	IC_{50}	RI	IC_{50}	RI
0.142	3.215	21.937	6.323	44.528

brane, which was blocked in 5% defatted milk powder for 60 min room temperature incubation. Primary antibody (β -catenin at 1:300, Survivin at 1:200, β -catenin at 1:500) was added for 4°C overnight incubation. After PBST rinsing, secondary antibody (1:10000 dilution) was added for 60 min incubation. The membrane was rinsed in PBST and quantified for protein expression using ECL method.

CCK-8 assay for cell proliferation activity

All cells were seeded into 96-well plate, which was incubated at 37°C chamber for 48 h. 10 μ L CCK-8 was added for 4 h continuous incubation. Absorbance value at 450 nm (A450) was measured by AMR-100 fully automatic microplate reader. Relative proliferation activity = (A450 of treated cells - A450 of blank group) / (A450 of control group - A450 of blank group) * 100%.

Cell apoptosis assay

Cells from all groups were collected and rinsed in PBS by centrifugation. Cells were re-suspended in Binding Buffer, with sequential addition of 5 μ L Annexin V-FITC and PI. After 15 min staining, Beckman Coulter Gallios flow cytometry was used to test cell apoptosis.

Statistical analysis

SPSS 18.0 was used for statistical analysis of data, of which measurement data were pre-

sented as mean \pm standard deviation (SD). Comparison of measurement data between groups was performed by student t-test. A statistical significance was defined when $P < 0.05$.

Results

Abnormal expression of miR-340 and β -catenin in osteosarcoma cells

qRT-PCR showed significantly lower miR-340 expression in Saos-2 cells compared to hFOB1.19 cells, whilst β -catenin mRNA level was remarkably increased (Figure 1A). Western blot results showed significantly elevated β -catenin protein expression in Saos-2 cells than hFOB1.19 cells (Figure 1B). These results indicated possible correlation between lower miR-340 and higher β -catenin expression with osteosarcoma onset. Moreover, online prediction by microRNA.org showed good targeted relationship between miR-340 and 3'-UTR of β -catenin gene (Figure 1C). Therefore, this study further studied the regulatory relationship. Results showed that transfection of miR-340 mimic significantly decreased relative luciferase activity in HEK293T cells (Figure 1D), indicating that miR-340 targeted 3'-UTR of β -catenin mRNA and inhibited its expression.

Lower miR-340 and up-regulation of β -catenin or survivin is correlated with ADM resistance

CCK-8 assay showed significantly lower ADM drug sensitivity of Saos-ADM4.0 cell than Saos-2/ADM1.0 cells, which further had lower ADM sensitivity than home strains Saos-2 cells (Figure 2A). RI of Saos-ADM4.0 cells and Saos-2/ADM1.0 cells were 21.937 and 44.528 relative to Saos-2 cells (Table 1). With higher drug resistance, miR-410 expression in Saos-2 cells

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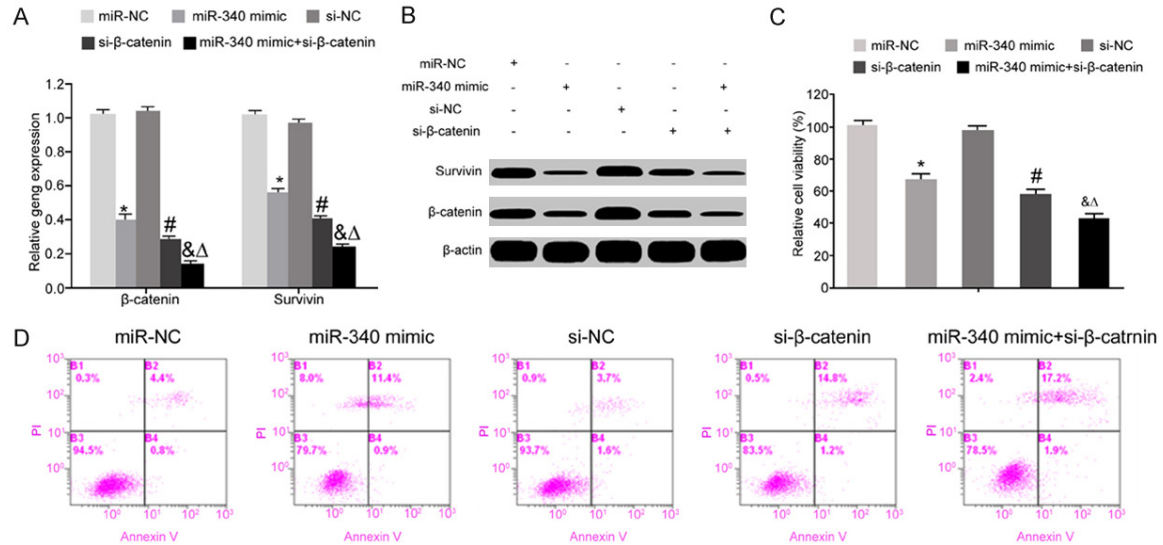


Figure 3. MiR-340 over-expression decreased β -catenin and Survivin expression and weakened ADM drug resistance. A. qRT-PCR for gene expression; B. Western Blot for protein expression; C. CCK-8 for cell proliferation activity; D. Flow cytometry for cell apoptosis. *, $P < 0.05$ comparing between miR-340 mimic and miR-NC groups; #, $P < 0.05$ comparing between si- β -catenin and si-NC groups; &, $P < 0.05$ comparing between miR-340 mimic + si- β -catenin and miR-340 mimic groups; Δ , $P < 0.05$ comparing between miR-340 mimic + si- β -catenin and si- β -catenin groups.

was gradually decreased, plus elevated expression of β -catenin and Survivin mRNA (Figure 2B). With more potent drug resistance, the expression of β -catenin and Survivin proteins was further enhanced (Figure 2C). These results indicated the possible role of miR-340 down-regulation in enhancing β -catenin and Survivin expression, and in decreasing drug sensitivity of Saos-2 cells.

MiR-340 over-expression decreased β -catenin and survivin expression and weakened ADM drug resistance

Using Saos-2/ADM4.0 cells as the research object, we set ADM treatment concentration at 4.0 mg/L and observed ADM drug resistance of all transfected Saos-2/ADM4.0 cells. Results showed that transfection of miR-340 mimic and/or si- β -catenin significantly depressed β -catenin and Survivin expression in Saos-2/ADM4.0 cells (Figure 3A and 3B), and further inhibiting cell proliferation potency (Figure 3C), eventually enhancing ADM (4.0 mg/L) induced cell apoptosis (Figure 3D).

Discussion

β -catenin is the core protein in canonical Wnt signal pathway, with its expression level as one determining factor in Wnt/ β -catenin pathway

activity [3]. In the absence of Wnt/ β -catenin signal pathway activating factor, β -catenin can be degraded by Axin-GSK β -APC complex via phosphorylation, thus keeping its cytoplasmic expression at relatively lower level [14]. In the presence of Wnt signal, extracellular Wnt protein can bind with Frz receptor and auxiliary protein LRP5/6 to further activate Dsh, which further inhibits kinase activity of GSK-3 β , thus stabilizing β -catenin and preventing its degradation. Elongated half-life time and cytoplasmic aggregation of β -catenin causes its nuclear entry for binding transcriptional factor TCF/LEF to activate transcription and expression of various target genes [15]. Among all these genes, anti-apoptosis factor Survivin is one important target gene for canonical Wnt/ β -catenin signal pathway [16]. Besides regulating proliferation, cell cycle and apoptosis, over-activation of Wnt/ β -catenin may also induce osteosarcoma via modulating osteoblast differentiation and bone formation [17, 18]. Previous studies showed significantly elevated β -catenin protein expression in osteosarcoma, plus its correlation with lower chemotherapy and drug resistance of osteosarcoma [8, 9]. One study also showed lower miR-340 expression in osteosarcoma tissues, indicating its tumor suppressor role. Moreover, the participation of miR-340 in regulating tumor cell chemotherapy sensitivity

has also been reported [13]. Bioinformatics analysis showed the existence of complementary binding sites between miR-340 and β -catenin mRNA 3'-UTR. Therefore, this study investigated if miR-340 played a role in mediating β -catenin expression and sensitivity of osteosarcoma cells against ADM.

Our results showed lower miR-340 expression in Saos-2 cells and significantly elevated β -catenin expression compared to hFOB1.19 cells. These results indicated that miR-340 down-regulation and elevated β -catenin expression might be related with osteosarcoma occurrence, and possibly targeted regulation between miR-340 and β -catenin. Cai et al showed significantly lower miR-340 expression in osteosarcoma tissues compared to adjacent tissues, plus lower miR-340 in metastatic osteosarcoma tissue than those in tumors without metastasis [12]. They also found lower overall survival rate and disease free survival rate in patients with lower miR-340 expression than those with relatively higher miR-340 [12], thus supporting the role of miR-340 expression level as independent predictive index for patient survival and prognosis. Zhou et al showed lower miR-340 expression in osteosarcoma tissues compared to normal osteoblast [19]. They also showed down-regulation of miR-340 in various osteosarcoma cell lines including HOS, SaOS2, MG63 and U2OS compared to hFOB1.19 cells [19]. All these studies revealed tumor suppressor role of miR-340 in osteosarcoma, as its down-regulation directly involved in tumor pathogenesis, as similar with this study. Haydon et al showed the participation of β -catenin up-regulation in osteosarcoma pathogenesis [20]. This study showed significantly elevated expression of β -catenin in osteosarcoma cell line Saos-2, as consistent with Haydon et al [20]. Dual luciferase gene reporter assay showed that miR-340 mimic transfection significantly depressed relative luciferase activity in HEK293T cells, indicating that miR-340 could target on 3'-UTR of β -catenin mRNA and inhibit its expression. When comparing ADM resistant and sensitive cell lines, this study revealed that with more potent drug resistance, Saos-2 cells had lower miR-340 expression and higher expressions of β -catenin and Survivin. Results showed the possible role of miR-340 down-regulation in elevating β -catenin/Survivin expression and acquirement of ADM resistance.

Further assays showed that transfection of miR-340 mimic and/or si- β -catenin remarkably decreased β -catenin and Survivin expression in resistant cell line Saos-2/ADM4.0, and inhibited cell proliferation potency, showing significant induction on cell apoptosis by ADM. Zhou et al showed that miR-340 over-expression could target and inhibit ROCK1 expression, thus suppressing proliferation of *in vitro* cultured osteosarcoma cell proliferation, migration and invasion, retarding tumor growth speed and suppressing distal metastasis potency. This study showed that elevation of miR-340 expression antagonized malignant biological properties of osteosarcoma cells, as consistent with Zhou et al [19]. Xia et al showed that inhibition of β -catenin expression remarkably inhibited osteosarcoma cell MG63 survival and induced apoptosis [21]. Liu et al showed β -catenin down-regulation significantly inhibited cycle progression of osteosarcoma cell U2OS and MG63, and weakening their proliferation potency or migration ability [22]. Xu et al found that β -catenin inhibition significantly decreased proliferation activity of osteosarcoma cell lines 143B and MG63 [23]. All these studies are similar with malignant biological features of osteosarcoma cells observed in our study with β -catenin down-regulation. In studies related with tumor drug resistance of miR-340 or β -catenin, Cai et al found those osteosarcoma patients with relatively lower miR-340 expression than those with higher miR-340 expression [12]. Shi et al found lower miR-340 expression in cisplatin resistant cell line HepG2/CDDP than parental HepG2 cells [13]. They also revealed that transfection of miR-340 mimic could significantly decreased resistance of HepG2/CDDP cells against cisplatin via targeted inhibition of Nrf2 expression [13]. Wozniak et al found the role of miR-340 down-regulation in potentiating drug resistant protein ABCB5, thus endowing melanoma cells with stem-cell like features and drug resistance [24]. Martins-Neves et al found positive correlation between β -catenin up-regulation in osteosarcoma stem cells and activation of Wnt/ β -catenin signal pathway or expression level of drug resistant transport ABCG2 [16], indicating the correlation between over-activation of Wnt/ β -catenin signal pathway and drug resistance. They further showed that the induction of osteosarcoma cells toward tumor stem cells by chemotherapy drugs is accompanied with

potentiated Wnt/ β -catenin pathway activity and higher ABCG2 expression, whilst inhibition of Wnt/ β -catenin activity can weaken the transition of osteosarcoma cells into tumor stem cells with drug resistance [9]. This study focused on the regulation of β -catenin and downstream Survivin expression by miR-340, and revealed the role of miR-340 down-regulation in acquisition of drug resistance by osteosarcoma cells, which has not been covered by previous studies.

Conclusion

MiR-340 can decrease proliferation activity of osteosarcoma Saos-2 cells via targeted inhibition on β -catenin and downstream anti-apoptotic protein Survivin expression, thus increasing apoptosis and elevating ADM sensitivity.

Disclosure of conflict of interest

None.

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