Suppression of adriamycin resistance in osteosarcoma by blocking Wnt/β-catenin signal pathway

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Abstract. – OBJECTIVE: Wnt/β-catenin signal pathway plays a role in regulating cell proliferation and apoptosis, and is correlated with tumor onset, progression and drug resistance. B-cell lymphoma 2 (Bcl-2) is an anti-apoptotic factor inducing tumor cell drug resistance. Wnt/β-catenin signal pathway can modulate Bcl-2 expression. This study established a cell model of drug resistance using adriamycin (ADM) treatment. Wnt/β-catenin signal pathway was intervened to discuss its role in drug resistance of osteosarcoma cells.

MATERIALS AND METHODS: Expression of β-catenin and Bcl-2 was compared between U2OS and hFOB1.19 cells. ADM resistant cell line U2OS/ADM was established for comparing β-catenin and Bcl-2 expression. Cell counting kit-8 (CCK-8) assay was used to test cell proliferation, followed by flow cytometry for apoptotic rate under ADM concentrations. U2OS/ADM cells were further treated with si-β-catenin and/or β-catenin inhibitor XAV939. β-catenin and Bcl-2 expression were measured, followed by CCK-8 and flow cytometry.

RESULTS: Comparing to hFOB1.19 cells, U2OS cells had significantly elevated β-catenin and Bcl-2 expression. U2OS/ADM cells had higher β-catenin and Bcl-2 expression than U2OS, plus lower ADM sensitivity and suppressed apoptotic rate. Transfection of si-β-catenin and XAV939 suppressed β-catenin and Bcl-2 expression, and significantly enhanced ADM sensitivity and ADM-induced apoptosis.

CONCLUSIONS: Up-regulation of β-catenin plays a role in potentiating expression and downstream anti-apoptotic factor Bcl-2, and in enhancing ADM resistance of osteosarcoma U2OS cells.

Key Words: β-catenin, Bcl-2, Adriamycin, U2OS, Osteosarcoma.

Introduction

Osteosarcoma is one of the primary malignant bone tumors derived from mesenchymal cells commonly in clinics, with features including high malignancy, rapid disease progression and high mortality. There are a certain number of patients suffering from failure of treatment due to tumor metastasis or recurrence. Drug resistance (DR) is an important reason for treatment failure. Adriamycin (ADM) is a common chemotherapy drug treating various tumors including osteosarcoma. With its wide application, drug resistance for ADM has become severe. Wnt/β-catenin is a signal pathway with high conservation in evolution, and is widely involved in regulation of cell proliferation, apoptosis, differentiation and invasion. Abnormal activation of Wnt/β-catenin signal pathway plays a role in occurrence and progression of multiple tumors such as pulmonary carcinoma, breast cancer, and liver cancer. Moreover, Wnt/β-catenin signal pathway is also correlated with mediation of osteoblast differentiation and bone formation. β-catenin is the core protein in Wnt/β-catenin signal pathway as induced by up-regulation of β-catenin. Wnt/β-catenin is involved in occurrence of osteosarcoma and tumor progression or metastasis. Previous studies showed that abnormal elevated activity of Wnt/β-catenin signal pathway is correlated with lower chemotherapy sensitivity of multiple tumors including colorectal carcinoma, pancreatic cancer, oral cavity cancer and glioma. Multiple mechanisms participate in tumor cell drug resistance, including drug intake and metabolism, and excluding cell proliferation and apoptosis. Among those apoptosis escape and suppressed apoptosis are important mechanisms for tumor cell drug resistance. B-cell lymphoma 2 (Bcl-2) is an important anti-apoptotic factor in regulation of mitochondrial dependent apoptotic pathway. Up-regulation of Bcl-2 can induce lower cell apoptosis and is closely correlated with acquire-
ment of drug resistance of tumor cells. Various studies showed that the activation of Wnt/β-catenin signal pathway plays a role in up-regulating expression of anti-apoptotic factor Bcl-2 and suppressing cell apoptosis. This study established ADM resistant cell model by drug treatment, and intervened Wnt/β-catenin signal pathway in those cells to investigate the role of Wnt/β-catenin signal pathway in drug resistance of osteosarcoma cells.

**Materials and Methods**

**Major Reagent and Materials**

Human osteosarcoma cell line U2OS and normal human osteoblast cell line hFOB1.19 were purchased from Yuxin Biotech (Guangzhou, China). RPMI 1640 medium, Dulbecco’s Modified Eagle Medium (DMEM)/F12 medium, fetal bovine serum (FBS), streptomycin-penicillin mixture and L-glutamine were purchased from Gibco (Grand Island, NY, USA). G418 was obtained from Amresco Inc. (Solon, OH, USA). Trizol and Lipofectamine 2000 were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). Reverse transcription reagent and SYBR Green dye were purchased from TaKaRa (Dalian, China). PCR primers were synthesized by Sango Co. Ltd. (Tokyo, Japan). Mouse anti-human Bcl-2 monoclonal antibody, β-catenin siRNA sequence, and negative control (NC) sequence, were purchased from Active Motif (Saranac Lake, NY, USA). Mouse anti-human β-catenin and β-actin polyclonal antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). HRP labeled secondary antibody was purchased from Shengxin Biotechnology (Shanghai, China). ADM was purchased from Hengrui Pharma (Shanghai, China). Annexin V-FITC/PI cell apoptosis assay kit was purchased from BD Biosciences (San Jose, CA, USA). β-catenin inhibitor XAV939 was obtained from Selleck Chemicals (Solon, OH, USA).

**Cell Culture**

U2OS cells were kept in RPMI 1640 medium containing 10% FBS, 2.5 mM L-glutamine and 0.3 mg/ml G418, 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 medium containing 10% FBS, 2.5 mM L-glutamine and 0.3 mg/ml G418, and in an incubator with 5% CO₂ at 37°C. After paving all dishes, cells were passed at 1:4 ratio.

**Induction of U2OS/ADM Resistant Cell Line**

U2OS cells were treated with 0.2 mg/l ADM as the starting concentration. 24 h later, ADM was washed out, for changing fresh medium. When cells status returned to normal, ADM drug concentration gradually reached 2.0 mg/l. Those cells that can normally grow at 2.0 mg/l ADM were named as U2OS/ADM.

**Assay for Drug Sensitivity**

U2OS and U2OS/ADM cells were treated for 48 h with different concentrations of ADM. CCK-8 reagent was then added to observe absorbance values at 450 nm (A45) of each well. Inhibition rate (%) = (1-A450(drug treatment group)) / A450(control group) ×100%. 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.

**Cell Treatment and Grouping**

In vitro cultured U2OS/ADM cells were divided into three groups: si-NC transfection group; si-β-catenin group; and si-β-catenin + XAV939 treatment group. 72 hours after transfection, cells were collected for assay. Sequences were: si-β-catenin sense, 5’-CAGGG GGUUG UGGUU AAGCU CUUdTdT-3’; si-β-catenin anti-sense, 5’-AAGAG CUUAA CCACA ACCCC CUGdT-3’; si-NC sense, 5’-UUCUC CGAAC GUGUC ACGUdT dT-3’; si-NC anti-sense, 5’-ACGUG ACACG UUCGG AGAAdT dT-3’.

**Quantitative Real-time PCR (RT-PCR) for Gene Expression**

PrimeScript RT reagent kit was used to synthesize cDNA by reverse transcription using RNA extracted by Trizol method as the template. Using cDNA as the template, PCR was performed with TaqDNA polymerase. In a 10 μl PCR reaction system, 5.0 μl 2× SYBR Green RT-PCR Master Mix, 0.5 μl of forward/reverse primers, 1 μl Template RNA and ddH₂O, were added. PCR conditions were: 95°C for 5 min pre-denature, followed by 40 cycles each containing 95°C 15 s denature, 58°C 30 s annealing and 72°C 30 s elongation. PCR was performed on ABI ViiA 7 fluoro-
cent quantitative PCR cycler. Primer sequences used were: \( \beta \)-catenin\(_P\)\_F: 5'-AGGAC CACG CATCT CTACA T3'; \( \beta \)-catenin\(_P\)\_R: 5'-GCAGTT TTTGT CAGTT CAGGG A-3'; Bcl-2\_P: 5'-GGTG-GG GGTCA GTGTT GTGGA-3'; Bcl-2\_R: 5'-CG-GTT CAGGT ACTCA GTCAT CC-3'; \( \beta \)-actin\(_P\)\_F: 5'-GAACCC CTAAG GCCAA C-3'; \( \beta \)-actin\(_P\)\_R: 5'-TTCA CGCAC GATTT CC-3'.

**Western Blot**

Cells were collected and extracted for proteins using sodium lauryl sulfate (SDS) lysis buffer. After testing for quality and concentration, 40 μg samples were loaded and separated in 10% sodium lauryl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were then transferred to membrane, which was blocked in 5% defatted milk powder under room temperature incubation. Primary antibody (\( \beta \)-catenin at 1:300, Bcl-2 at 1:200, \( \beta \)-catenin at 1:500) was added for 4°C overnight incubation. After phosphate-buffered saline tween-20 (PBST) rinsing, horse radish peroxidase (HRP) labeled secondary antibody (1:10000 dilution) was added for 1 h incubation. The membrane was rinsed in PBST and developed by enhanced chemiluminescence (ECL) method. After exposure and fixation, the film was scanned and saved for data.

**CCK-8 Assay for Cell Proliferation Activity**

All cells were collected, digested and seeded into 96-well plate, which was incubated for 24, 48 or 72 h. 10 μl CCK-8 was added for 4 h continuous incubation. Absorbance values at 450 nm (A450) were measured. Relative proliferation activity = (A450 of treated cells – A450 of blank group) / (A450 of control group – A450 of blank group) * 100%.

**Cell Apoptosis Assay**

Cells were digested in trypsin and were collected. After re-suspension in Binding Buffer, 5 μl Annexin V-FITC and 5 μl PI were sequentially added. Beckman Coulter Gallios flow cytometry was used to test cell apoptosis.

**Statistical Analysis**

SPSS 18.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Data were presented as mean ± standard deviation (SD). Comparison of measurement data between groups was performed by student t-test. Statistical significance was defined when \( p < 0.05 \).

**Results**

**Elevated \( \beta \)-catenin and Bcl-2 Expression with Potent Proliferation Ability in U2OS Cells**

qRT-PCR results showed that, compared to hFOB1.19 cells, U2OS cells had significantly elevated \( \beta \)-catenin and Bcl-2 mRNA levels (Figure 1A). Western blot results also showed significantly higher \( \beta \)-catenin and Bcl-2 protein levels in U2OS cells compared to hFOB1.19 cells (Figure 1B). CCK-8 assay showed significantly potentiated proliferation potency of U2OS cells than hFOB1.19 cells (Figure 1C). These results showed possible correlation between \( \beta \)-catenin up-regulation and osteosarcoma occurrence.

![Figure 1](image-url)  
**Figure 1.** Elevated \( \beta \)-catenin and Bcl-2 expression with potent proliferation ability in U2OS cells. **(A)** qRT-PCR for gene expression; **(B)** Western Blot for protein expression; **(C)** CCK-8 assay for cell proliferation activity. *, \( p < 0.05 \) comparing between U2OS cells and hFOB1.19 cells. Bcl-2: B-cell lymphoma 2; CCK-8: cell counting kit-8; qRT-PCR : quantitative Real-time PCR.
Correlation between β-catenin and Bcl-2 up-regulation with U2OS Cell Drug Resistance

CCK-8 assay showed significantly lower proliferation activity of U2OS cells under various concentrations of ADM compared to U2OS/ADM cells (Figure 2A). IC50 of U2OS cells was 0.157 mg/l, whilst U2OS/ADM cells had IC50 value at 4.282 mg/l. U2OS/ADM cells had RI of 27.27 compared to parental U2OS cells. Under treatment by 0.157 mg/l ADM, U2OS cells had significantly higher apoptotic rate than U2OS/ADM cells (Figure 2B). qRT-PCR results showed significantly elevated expression of β-catenin and Bcl-2 mRNA in U2OS/ADM cells compared to U2OS cells (Figure 2C). Western blot results showed significantly higher β-catenin and Bcl-2 protein expression in U2OS/ADM cells compared to U2OS cells (Figure 2D).

Suppression of β-catenin Expression Enhanced ADM Sensitivity of U2OS/ADM Cells

Setting ADM concentration at 2.0 mg/l, we observed change of ADM resistance of U2OS/ADM cells after inhibition of β-catenin expression. Results showed that after siRNA silencing of β-catenin expression, U2OS/ADM cells had decreased Bcl-2 expression (Figure 3A and 3B). Under 2.0 mg/l ADM, cells had weakened proliferation

Figure 2. Correlation between β-catenin and Bcl-2 up-regulation with U2OS cell drug resistance. (A) CCK-8 assay for cell proliferation activity; (B) Flow cytometry for cell apoptosis; (C) qRT-PCR for gene expression; (D) Western Blot for protein expression. *, p < 0.05 comparing between U2OS cells and hFOB1.19 cells. Bcl-2: B-cell lymphoma 2; CCK-8: cell counting kit-8; qRT-PCR: quantitative Real-time PCR.
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potency (Figure 3C), plus significantly enhanced cell apoptosis (Figure 3D). After silencing β-catenin expression, β-catenin inhibitor XAV939 was further added to suppress Bcl-2 activity (Figure 3A and 3B). Those cells had more apoptosis after ADM induction, and more potent inhibitory effects on proliferation (Figure 3C).

Discussion

Wnt signal pathway is a highly conserved signal pathway in evolution, and consists of canonical and non-canonical signal pathway. Canonical Wnt signal pathway is closely correlated with tumor onset, progression and metastasis, and is the most widely studied signal pathway so far7. β-catenin protein is firstly identified as cell adhesion protein molecule. Expression level of β-catenin largely determines Wnt/β-catenin signal activity, and is the core regulatory factor in canonical Wnt signal pathway9. Without signal stimuli, Wnt/β-catenin signal pathway is at test phase, and β-catenin can be degraded to keep relatively lower cytoplasmic level9. When Wnt signal pathway is activated, Wnt protein can pass the signal to degradation complex via a series of downstream proteins, impeding degradation complex function and phosphorylated degradation of β-catenin, therefore elevating its cytoplasmic expression. β-catenin further enters into cell nucleus, where it can form transcription complex with T-cell factor/lymphoid enhancing factor (TCF/LEF) to initiate transcription and

Figure 3. Suppression of β-catenin expression enhanced ADM sensitivity of U2OS/ADM cells. (A) qRT-PCR for gene expression; (B) Western Blot for protein expression; (C) CCK-8 assay for cell proliferation activity; (D) Flow cytometry for cell apoptosis. *, p < 0.05 comparing between si-β-catenin and si-NC group; #, p < 0.05 comparing between si-β-catenin + XAV939 and si-β-catenin group. Bcl-2: B-cell lymphoma 2; CCK-8: cell counting kit-8; ADM: adriamycin; qRT-PCR: quantitative Real-time PCR.
expression of multiple downstream target genes, thus modulating cell growth, proliferation, apoptosis, differentiation and invasion. Bcl-2 protein family can be classified into anti-apoptotic factors and pro-apoptotic factors based on differential functions. B-cell lymphoma 2 (Bcl-2) is the most important and widely studied in Bcl-2 family, and can inhibit mitochondrial dependent cell apoptosis pathway. Previous studies have shown important regulatory functions of Bcl-2 induced cell apoptosis in acquisition of drug resistance in osteosarcoma cells. In a comparison observation showing elevation of both β-catenin expression and Bcl-2 in osteosarcoma cells. In osteosarcoma MG63 cell apoptosis, as consistent with our study, revealed the role of Wnt/β-catenin signal pathway activation in up-regulating anti-apoptotic factor Bcl-2 expression and decreasing cell apoptosis. This study generated an ADM-resistant cell model by drug treatment, and intervened Wnt/β-catenin signal pathway activity, to investigate its role in drug resistance of osteosarcoma cells. Results of this study showed that compared to normal human osteoblast cells hFOB1.19, osteosarcoma cells U2OS showed significantly elevated β-catenin mRNA and protein expression. Bao et al showed significantly elevated β-catenin expression in tumor tissues of osteosarcoma patients, and its close correlation with distal metastasis and histo-pathology grading. β-catenin expression level can also work as a predictive index for patient’s survival and prognosis. Lu et al also showed abnormally elevated β-catenin expression in osteosarcoma tissues, and its close correlation with distal metastasis, clinical stage, and patient’s survival or prognosis. In this paper, osteosarcoma cells had abnormally elevated β-catenin expression, indicating the role of β-catenin up-regulation in pathogenesis mechanism of osteosarcoma, as similar with Bao et al and Lu et al, who revealed the correlation between β-catenin up-regulation and osteosarcoma occurrence. Moreover, this study also showed significantly higher Bcl-2 expression in osteosarcoma U2OS cells than that in hFOB1.19 cells, as consistent with β-catenin expression pattern. These results indicated the role of β-catenin up-regulation in enhancing Bcl-2 expression. Xu et al revealed the role of β-catenin up-regulation in increasing anti-apoptotic factor Bcl-2 expression and inhibiting osteosarcoma MG63 cell apoptosis, as consistent with our observation showing elevation of both β-catenin and Bcl-2 in osteosarcoma cells. In a comparison study between parental cells and drug resistant cells, we found significantly higher β-catenin and Bcl-2 expression in U2OS/ADM cells compared to their parent drug sensitive cells U2OS. Under IC50 concentration of U2OS cells of ADM, apoptotic rate of U2OS was about 40%, but with minor effects on apoptosis of U2OS/ADM cells. Results showed the correlation between β-catenin and Bcl-2 up-regulation with not only osteosarcoma pathogenesis, but also drug resistance of osteosarcoma cells. Martins-Neves et al showed that chemotherapy drug treatment significantly enhanced transduction activity of Wnt/β-catenin signal pathway inside osteosarcoma cells MNNG-HOS, and up-regulated expression of drug resistant protein ABCG, thus making chemotherapy failure, and enhancing expression of stem transcription factor inside cells to endow their features of tumor stem cells. In this study, chemotherapy reagent treatment further elevated β-catenin expression in osteosarcoma cells, as consistent with Martins-Neves et al. Further results showed that, after suppression of β-catenin expression by siRNA, drug resistant cells U2OS/ADM showed significantly lower Bcl-2 expression and weakened proliferation ability, plus higher sensitivity for ADM-induced cell apoptosis. The combination of β-catenin inhibitor XAV939 treatment further suppressed Bcl-2 expression, weakened proliferation ability of U2OS/ADM cells, and enhanced apoptosis. These results showed that long-term of ADM treatment up-regulated expression of β-catenin and downstream anti-apoptotic factor Bcl-2 in osteosarcoma cells, thus enhancing ADM resistance of cells. On the other hand, inhibition of β-catenin expression or activity can somehow weaken ADM resistance of osteosarcoma cells. Zhang et al showed that inhibition of β-catenin expression significantly weakened malignant biological features of sarcoma MG63 cells including migration and motility. Ma et al showed the correlation between β-catenin up-regulation plus enhanced activity of Wnt/β-catenin signal pathway and potentiated proliferation and invasion potency of osteosarcoma cells. The inhibition of Wnt/β-catenin signal pathway activity or β-catenin expression could weaken proliferation or invasion potency of osteosarcoma cells. Liu et al found that interference of β-catenin expression by siRNA significantly suppressed proliferation or migration of osteosarcoma U2OS and MG63 cells. Xu et al found that after inhibition of β-catenin expression or activity using Polydatin, osteosarcoma MG63 cells showed significantly increased apoptosis. Li et al showed that over-expression of metalloprotease TIK12 for suppressing Wnt/β-catenin
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activity played an important role in inhibiting osteosarcoma cell proliferation, clonal formation or migration. Martins-Neves et al.\textsuperscript{27} found that the inhibition of β-catenin expression or activity significantly weakened acquisition of stem property of MNNG-HOS cells after chemotherapy, or their transformation towards tumor stem cells with lower drug sensitivity. This study supported Martins-Neves et al.\textsuperscript{27} results from another perspective. Li et al.\textsuperscript{12} also showed the role of β-catenin over-expression in enhancing Bcl-2 expression and enhancing chemotherapy drug resistance of tumor cells. This study revealed the role of β-catenin in down-regulating Bcl-2 expression and weakening chemotherapy resistance of tumor cells, further supporting Li et al.\textsuperscript{12} study.

Conclusions

Up-regulation of β-catenin plays a role in enhancing expression of downstream anti-apoptotic factor Bcl-2, potentiating ADM resistance of osteosarcoma U2OS cells.

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Conflict of Interest

The Authors declare that they have no conflict of interests.

References


