RESEARCH COMMUNICATION

SiRNA-mediated Silencing of β-catenin Suppresses Invasion and Chemosensitivity to Doxorubicin in MG-63 Osteosarcoma Cells

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Abstract

Purpose: β-catenin, the chief oncogenic component of the canonical Wnt pathway, is known to be involved in development of a variety of cancers, but its role in human osteosarcomas is not fully understood. Here we investigate the effect of small interfering RNA-mediated β-catenin knockdown on the survival, invasion and chemosensitivity of a human osteosarcoma cell line. Methods: A siRNA against β-catenin was constructed and transfected into MG-63 cells. Expression of β-catenin was determined by qRT-PCR and Western blotting. Cell growth and apoptosis were assessed in the presence or absence of doxorubicin by MTT and flow cytometry, respectively, cell invasion by transwell assay, and XIAP, Bclxl, nulear P65 and MT1-MMP expression by western blot and real-time PCR. Results: Transfection of β-catenin siRNA resulted in decreased expression of β-catenin, suppression of invasion and motility of MG-63 cells and reduced chemosensitivity to doxorubicin in vitro, but little change in cell growth and apoptosis. At the same time, down-regulation of MT1-MMP and up-regulation of NF-kappaB activation were observed. Conclusion: Knock-down of β-catenin gene may decrease the invasion ability through down-regulation of MT1-MMP expression and enhance the chemoresistance to doxorubicin via the NF-kappaB pathway. In contrast to other tumors, β-catenin may not play an oncogenic role in osteosarcoma cells.

Keywords: β-catenin - osteosarcoma cell line - siRNA - gene therapy

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Introduction

Osteosarcoma (OS) is a primary malignancy of bone with a high tendency for early metastasis. Due to the poor response to drug and radiation therapies, the disease is highly lethal (Jawad et al., 2010). The prognosis of OS is very poor, especially in patients with clinically detectable metastasis at diagnosis or relapsed disease (Bielack et al., 2002). Molecular mechanisms underlying disease progression currently are largely unknown, however more evidence suggests that epigenetic processes are important (Thomas et al., 2006). So better understanding of the underlying mechanisms of the tumor progression, before more targeted therapies can be realized, should be much required.

The Wnt/β-catenin pathway plays important roles in multiple cellular processes during development, including cell differentiation, migration and proliferation (Peifer et al., 2000). Aberrant signaling involving the stabilization and nuclear translocation of β-catenin has been observed in many types of cancers, however, involvement of the Wnt pathway in disease progression has not been clearly established. Several groups have reported that LRP5 expression, a Wnt signaling receptor, predicted progression in high-grade osteosarcoma (Hoang et al., 2004) and that blockade of Wnt/LRP5 signaling inhibited met and metalloproteinase expression and decreased tumorigenicity and metastases in animal models (Guo et al., 2007; 2008). Moreover, a study published by Haydon et al has reported that both nuclear and cytoplasmic β-catenin have been associated with osteosarcoma development (Haydon et al., 2002). Further more accumulative β-catenin expression correlate with metastasis(Iwoa et al., 1999; Iwaya et al., 2003; Yang et al., 2006). Interestingly Cai et al reported that the canonical Wnt pathway seems to be inactive in osteosarcoma and suggest that β-catenin signaling may contribute to decreased tumourigenesis (Cai et al., 2010). Therefore, the role of β-catenin in osteosarcoma remains unclear.

In the present report we investigate the role of β-catenin in the pathobiology of OS and show that downregulation of this protein can effectively reduce invasion of OS cells by inhibition of β-catenin target gene MT1-MMP expression. But in this study, we found that SiRNA-mediated silencing of β-catenin causes enhanced chemoresistance to Doxorubicin in osteosarcoma MG-63 cells. Previous work has demonstrated that NF-kappaB is activated in response to doxorubicin (Ashikawa et

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al., 2004; Brian et al., 2009) and β-catenin can inhibit NF-kappaB transactivation, DNA binding activity, and target gene expression (Deng et al., 2002). Thus, we hypothesized that downregulation of β-catenin induces increased chemoresistance to Doxorubicin through NF-kappaB pathway in osteosarcoma. Taken together, these findings provide the evidence linking Wnt/β-catenin signaling to tumour progression in human OS.

Materials and Methods

Cell line and cell culture

The human osteosarcoma cell line MG-63 was purchased from the China Center for Type Culture Collection (Wuhan.China). Cell line was cultured in DMED/F12 (Invitrogen, USA) medium supplemented with 10% fetal bovine serum (Gibco, AUS) at 37°C in an atmosphere of 5% CO_2 with humidity..

β-catenin-siRNA transfection

Small interfering RNA (siRNA) targeted against β-catenin was designed according to the protocol developed by Reynolds et al(Reynolds et al., 2004). The sense and antisense sequences were 5/-UGGUUGCCUUGCUCAACAAdTdT-3/ and 5/-UUGUUGAGCAAGGCAACCAdTdT-3/ and synthesized by Qiagen (Germany). Non-target-directed siRNA used as a negative control was also purchased from Qiagen. The negative control siRNA exhibits no target in the human transcriptome, whereas β-catenin siRNA is directed against the region 842-860 of the human β-catenin mRNA(NM_001904).

The transfection procedure was performed using Lipofectamine 2000(Invitrogen, USA) according to the manufacturer's recommendations. Briefly, one day before siRNA transfection, MG-63 cells were seeded in six-well plates at a density of 5×10^5 cells/well, resulting in 40% confluence after 24 hours of incubation. For preparing the transfection mixtures, the respective siRNAs were added to 250μL of serum-free DMEM in a final concentration of 100 nM. In a separate tube, 5μL of Lipofectamine 2000 was diluted in 250μL of serum-free DMEM. After unifying both solutions, the final transfection mixture was incubated for 20 minutes at room temperature. NaCl solution (NS) was used as control agent. The above agents were applied to the cells and incubated for 4h, and then the medium was replaced with fresh growth medium.

Real-time PCR analysis

Total RNA was isolated from cultured cells using the Trizol reagent(Invitrogen , USA). The first strand cDNA synthesis was performed from 100-500 ng of total RNA using the ReverTra Ace qPCR RT Kit(TOYOBO, Japan). Real-time PCR was carried out on a LightCycler(Roche) using SYBR Green Realtime PCR Master Mix-Plus(TOYOBO, Japan). Thermocycling was performed as follows: 95°C for 10min; 40 cycles of 95°C for 10s and 60°C for 30s; 72°C for 10s. The 5' forward and 3' reverse complement PCR primers for amplification of β-catenin were TGAGGACAAGCCACAAGATTAC and TCCACCAGAGTGAAAAGAACG, respectively. For XIAP, PCR primers were respectively CCGTGCGGTGCTTTAGTTGT and TTCCTCGGTATATGGTGTCTGAT, For Bclxl, PCR primers were respectively TTACCTGAATGACCACCTA and ATTTCCGACTGAAGAGTGA. For GAPDH, PCR primers were respectively GAAGGTGAAGGTCGGAGTC and GAAGATGGTGATGGGATTTC. To quantify gene expression changes, the Ct method was used to calculate the relative fold-changes normalized against GAPDH.

Western blot analysis

After transfection with siRNA, cells were harvested and total proteins were extracted using RIPA Extraction Reagents (ProMab Biotechnology, CA). Protein concentration was determined by Bradford method. 40μg of protein lysates from each sample was subjected to SDS-PAGE on 10% SDS-polyacrylamide gel. Separated proteins were transferred to a PVDF membrane. The membrane was incubated with primary antibodies against β-catenin (Proteintech Group, USA; 1:600 dilution), p65, MT1-MMP, XIAP and Bclxl(Santa Cruz, USA; 1:500 dilution), followed by HRP-conjugated secondary protein (KPL, USA; 1:3000 dilution). The specific protein was detected using a SuperSignal protein detection kit (Pierce, USA). The band density of specific proteins was quantified after normalization with the density of β-actin. All of the Western blots were performed at least three times.

Flow cytometry for apoptosis

Detection of apoptosis by flow cytometry was performed using the Annexin V-FITC-PI kit (Becton, USA). The transfected cells were harvested with trypsinization, fixed with cold 80% ethanol at 4°C for 24 hours. The staining was performed according to the producer's manual. Flow cytometry was performed immediately. Each groups detected in triplicate experiments and mean were calculated. For Doxorubicin treatment experiments, 1×10^6 cells were seeded in 100-mm culture dishes and allowed to adhere overnight. Then, cells were treated with doxorubicin at 1μM. After 24h, adherent and floating cells were combined and processed for flow cytometry as described previously.

MTT assay

The viability of control and siRNA-transfected MG-63 cells were measured by MTT (3-(4,5-dimethylthiazol-2)-2,5-diphenyltetrazolium bromide) assay. Briefly, 4×10^3 cells were plated into each well of a 96-well plate. At 48, 72, 96 h and 120h after plating, 20μL of MTT (5 mg/ml) was added to each well and incubated for additional 4h at 37°C. The reaction was stopped by lysing the cells with 200ml of DMSO for 5 min. The absorbance of the wells was measured at a wavelength of 490 nm. All experiments were performed in triplicate. The cell proliferation curve was plotted using the absorbance at each time point.

In vitro motility and invasion assays

Invasion assays were performed using 24-well invasion chamber system(Corning Inc, USA). Cells were trypsinized and counted with a hemocytometer using trypan blue, and viable cells were seeded in the upper

chamber at 3×10^4 cells/well in serum-free medium. The lower chamber received 500 μL of 10% FBS-containing medium. Incubation was carried out for 36 h at 37°C in humidified air with 5% CO_2. Nonmigratory cells in the upper chamber then were removed with a cotton-tip applicator. Migrated cells on the lower surface were fixed with methanol and stained with Giemsa. The number of migrating cells was determined by counting five high-powered fields (200×) on each membrane. An invasion index, corrected for cell motility, was calculated using the following formula:

No. of cells invaded through a Matrigel-coated membrane / No. of cells migrated through an uncoated (control) membrane.

Motility assay is a modification of the invasion assay described previously. A total of 3×10^4 cells were placed in the upper chamber in serum-free medium. Medium containing 10% FBS placed in the lower chamber as a source of chemoattractant. Cells were allowed to migrate through a porous, uncoated membrane for 12 h at 37°C. The membrane was processed as described for the invasion assay. The number of migrating cells was determined by counting five high-powered fields (200×) on each membrane and calculated as mean number of cells/field. Each test group was assayed in triplicate

Drug cytotoxicity assay

Subconfluent cells were harvested and plated in 96-well culture plates (2×10^3 cells/well). After incubation at 37°C overnight to allow attachment, cells were treated with doxorubicin at several dilutions. After 24h, the percentage of viable cells relative to untreated controls was determined by MTT method as described earlier.

Statistical analysis

All data are expressed as mean±SEM of three independent experiments. Student's t test was used to compare the difference between means. P<0.05 was considered to be statistically significant.

Results

siRNA targeting of β-catenin in MG-63 cells

MG-63 cells were transfected with siRNA against β-catenin. The knockdown efficiency was examined on mRNA and protein level by quantitative RT-PCR and semiquantitative Western blot analysis, respectively. On mRNA level knockdown efficiencies of 75%-85% for β-catenin were observed during a time period of 5 days after transfection as compared with control cells treated with NS, while negative control showed no effects (Figure 1). Consistently, the amounts of proteins were also significantly reduced under specific knockdown conditions in comparison with the controls (Figure 1). Interestingly, the reduction of β-catenin on mRNA level was already observed at day 1 after siRNA transfection, whereas the knockdown on protein level became most obvious on days 3 and 5 in the case of β-catenin.

Cell proliferation assay

To examine the role of β-catenin on osteosarcoma

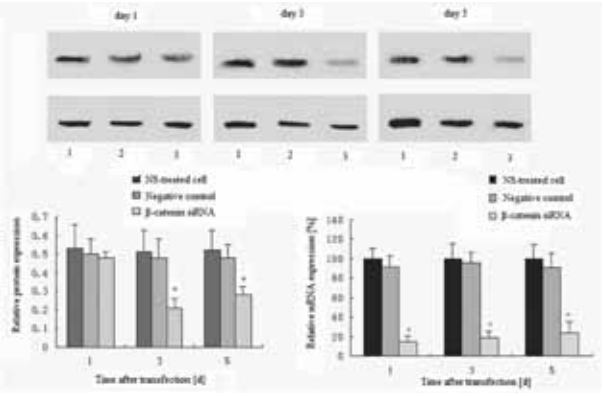

Figure 1. siRNA-mediated Knockdown of β-catenin in MG-63 Cells on Transcriptional and Protein Level. β-catenin mRNA and protein levels were quantified by real-time PCR and western blotting, with β-actin expression as an internal standard *P<0.05 versus NS-treated cells

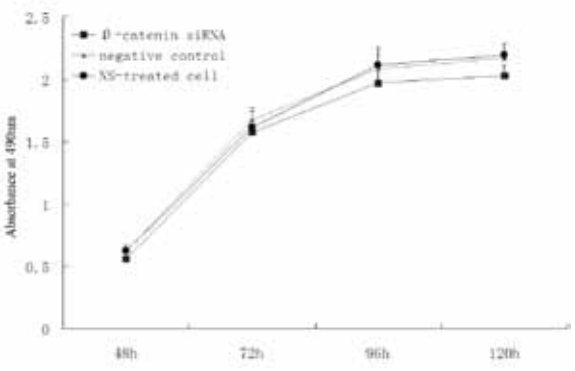

Figure 2. Cell Viability Evaluated by MTT Assay. Note slight decrease in the number of cells with the siRNA (p>0.05)

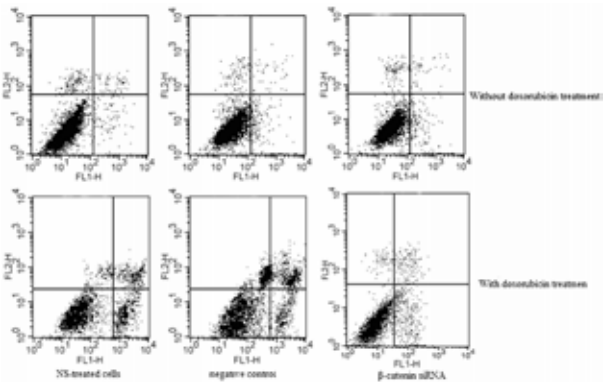

Figure 3. Flow Cytometry Analysis of Three Groups in the Presence or Absence of Doxorubicin. β-catenin siRNA transfection reduced apoptosis only in the presence of the drug (p<0.05)

MG-63 cell proliferation, we examined the effect of β-catenin siRNA on osteosarcoma cell growth. As shown in Fig.2, β-catenin-siRNA slightly decreased the growth rate of MG-63 cells as compared to the NS-treated cells at different time points (p>0.05).

β-catenin silencing does not promote apoptosis but promotes survival with Doxorubicin treatment

Next, we examined whether β-catenin-siRNA induced apoptosis in MG-63 cells. With flow cytometry, average cellular apoptosis rate was 5.41±2.25%, 4.6±2.07% and 3.43±2.14% in β-catenin-siRNA group, negative control group and control group, respectively (Figure 3,

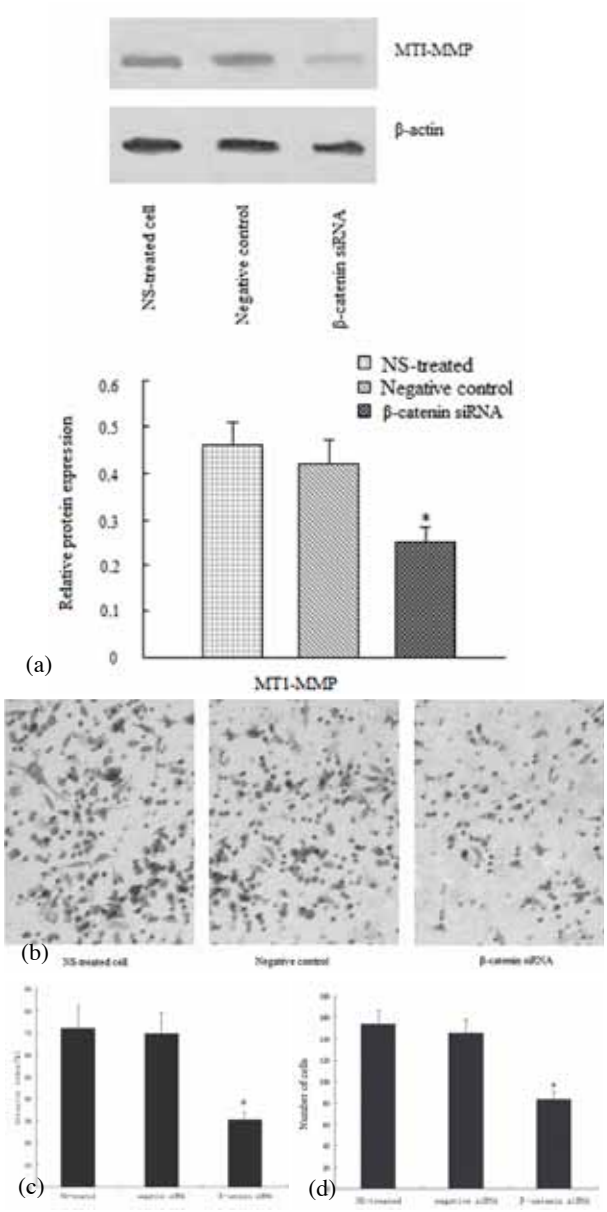

Figure 4. β-catenin Knockdown Suppresses the Expression of MT1-MMP and Leads to Decrease in Invasion and Motility. a) Expression level of MT1-MMP protein detected by Western blotting with β-actin as an internal control for loading. Compared with NS-treated or negative control, note significant decrease with β-catenin siRNA (*P<0.05); b) typical invasion photographs; c) β-catenin siRNA transfected cells exhibited reduced invasiveness across Matrigel when compared with controls (*P<0.05); d) migration across a microporous membrane, with decrease in number of β-catenin siRNA transfected cells(*P<0.05)

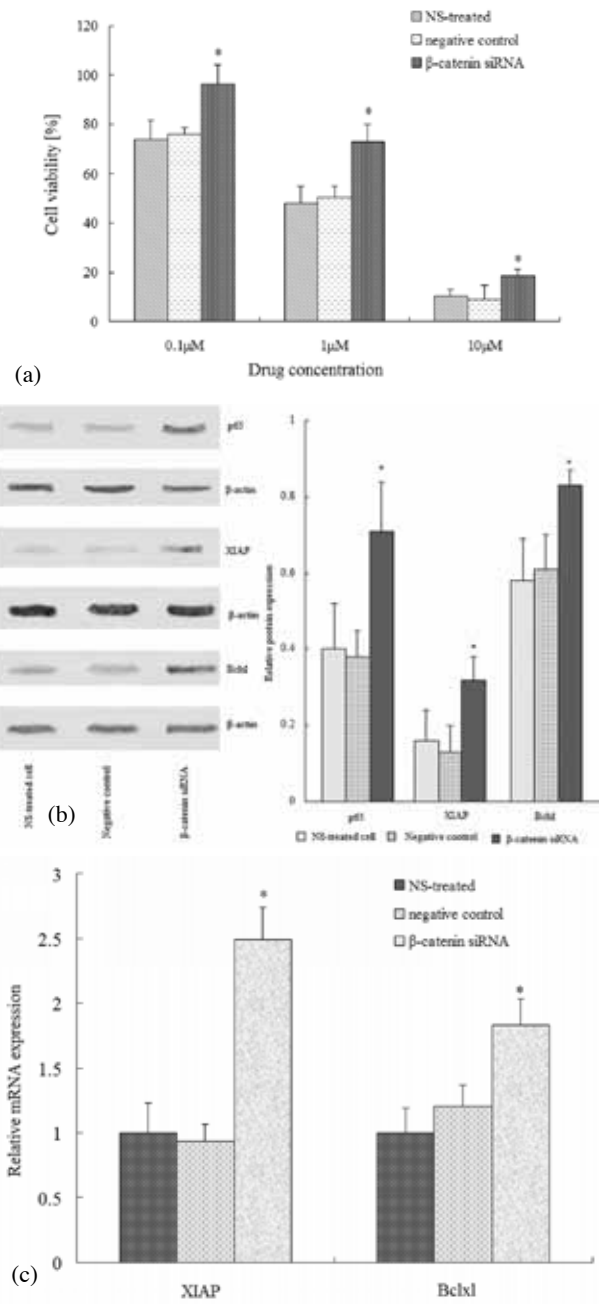

Figure 5. β-catenin Knockdown Reduces Chemosensitivity to Doxorubicin via NF-kappaB Pathway. a) MG-63 cells were exposed to doxorubicin for 24h and cell viability was determined by MTT assay. β-catenin siRNA transfected cells showed more viability than NS-treated or negative control cells (*p<0.05); b) Western blot analysis of nuclear p65 and NF-kappaB target genes, XIAP and Bclxl in MG-63 cells after exposure to doxorubicin for 24h with β-actin as loading control; c) mRNA level was measured by real-time RT-PCR and displayed as relative gene expression compared to control (NS-treated), significant increase being observed for Bclxl and XIAP (p<0.05)

P>0.05). β-catenin-siRNA transfection did not induce any significant increase in apoptosis compared with control group. However, when exposed to doxorubicin, β-catenin-siRNA transfected cells were much more resistant to apoptosis. The cellular apoptosis rate was 13.1±1.34%*, 27.7±2.52% and 29.6±1.63% in β-catenin-siRNA group, negative control group and control group, respectively (Figure 3, *P<0.05).

β-catenin siRNA suppresses MT1-MMP with decrease in invasion and motility

To examine whether the down-regulation of β-catenin affected its invasive ability, we analysed β-catenin silencing on the expression of Wnt signaling target gene MT1-MMP using western blot and performed an in vitro Matrigel Transwell analysis. As shown in Figure 4a, compared with control group, the expression of MT1-MMP were found to be reduced by 44.4% (p<0.05). There was no significant difference between negative control and control group.

We examined the in vitro capacity of these cells to invade through a Matrigel-coated membrane. Invasion through Matrigel has been reported to mimic the three-step (Albini et al., 1998): (a) adhesion to a substrate; (b) dissolution of the extracellular matrix; and (c) migration. Using this assay, we observed a significant decrease in invading capacity (expressed as percent invasion) of β-catenin siRNA -transfected cells. As seen in Figure 4b and 4c, The number of β-catenin siRNA transfected cells (25.5±4.28, $p<0.05$) passing through the Matrigel was markedly lower than the numbers of negative control (101.6±15.0) and control (110.2±14.4) cells.

Motility is a component of the invasion process of tumor cells. We examined whether the inhibition of β-catenin also affected cell motility. As seen in Figure 4d, β-catenin siRNA transfected cells were significantly less motile than negetive control and control cells.

β-catenin knockdown reduces chemosensitivity to Doxorubicin via NF-kappaB pathway

Using MTT assay, the growth of cells was measured after 24h of exposure to a range of concentrations of doxorubicin. As shown in Figure 5a, doxorubicin demonstrated a dose-dependent decrease in cell proliferation and cells transfected with β-catenin siRNA were less sensitive to doxorubicin than controls. Next, we examined whether β-catenin knockdown reduces chemosensitivity to Doxorubicin via NF-kappaB pathway. To test this, we evaluated the effects of doxorubicin treatment on the level of nuclear p65. We found that treatment with doxorubicin results in a increase in nuclear p65 expression compared to negative control or NS-treated control (Figure 5b). We also examined the expression of NF-kappaB target gene, Bclxl and xIAP. As shown in Figure 5c, the mRNA level of Bclxl and XIAP were significantly inreased. And western blot analysis also demonstrated significant increase in β-catenin siRNA treated cells compared to control cells (Figure 5b).

Discussion

Although the role of Wnt/β-catenin signaling has been well studied in many types of human cancers, its biological significance in osteosarcoma has not been determined clearly. Previous works have reported that the excessive expression of stabilized β-catenin leads to tumorigenesis in the bone, central nervous system, colorectum and other tissues (Polakis et al., 2000; Hajra et al., 2002; Haydon et al., 2002). So, β-catenin has attracted much attention as gene therapy target for carcinomas. β-catenin has been successfully down-regulated by RNAi in some previous study (Verma et al., 2003; Zeng et al., 2007; Pu et al., 2009) and all of the studies have succeeded in decreasing growth and survival of tumors. However, the down-regulation of β-catenin expression in osteosarcoma cells has not been reported before. In our study, we used siRNA transfection approach, which enables a highly efficient RNA interference. RNAi attempts resulted in a prominent knockdown of β-catenin on mRNA and protein levels. However, in contrast to some previous studies, which have reported and shown in many other cancer types (Klaus et al., 2008), the knockdown of β-catenin resulted in a slightly diminished proliferation rate as well as in slightly increased apoptotic rate. A recent study (Cai et al., 2010) reported that the canonical Wnt pathway seemed to be inactive in osteosarcoma and the authors found that β-catenin expression is localized to the cytoplasm and plasma membrane, rather than the nuleus. Furthermore, the authors inhibited the growth of osteosarcoma cell line by GSK3β inhibitor, GIN. Therefore, our findings support that Wnt signaling may not play an oncogenic role in osteosarcoma, which is in contrast with its role in other tumors.

Increasing evidence indicates that Wnt signaling may play a potential role in invasiveness of OS. It has been reported that Wnt10b expression was found to correlate with poorer survival and activated chemotaxis of metastatic osteosarcoma cells (Chen et al., 2008). Human OS cells transfected with dominant negative LRP and Dkk3 showed decreased invasiveness and motility (Hoang et al., 2004a; Hoang et al., 2004b). Our data indicated that downregulation of β-catenin by siRNA reduced the MG-63 osteosarcoma cell invasion and motility. In addition, inhibition of β-catenin resulted in the downregulation of MT1-MMP expression, suggesting that this canonical Wnt signaling target gene plays a potential role in osteosarcoma invasion.

With respect to the regulation of OS cells migration/invasion by Wnt signaling, it is of particular importance that the variety of Wnt target genes encompasses several members being involved in the process of cancer cell invasion, such as urokinase-type plasminogen activator (uPA) (Hiendlmeyer et al., 2004), uPA receptor (Mann et al., 1999) and MT1-MMP (Takahashi., et al 2002). Particularly, MT1-MMP is highly expressed in different cancers and promotes migration, invasion and metastasis of cancer cells in vitro as well in vivo (Seiki et al., 2003). MT1-MMP degrades various extracellular matrix (ECM) macromolecules including collagen II, fibronectin and aggrecan et al (Zucker et al., 2003). On the cell surface, MT1-MMP also activates other members of the MMP family, for example, MMP-2 and MMP-13, creating a wider proteolytic repertoire on the cell surface. Thus, it is conceivable that β-catenin downregulation may suppress OS invasiveness by affecting the expression of the MT1-MMP.

The observation that downregulation of β-catenin promotes survival of MG-63 cells during doxorubicin treatment is intriguing. Doxorubicin, an anthracycline antibiotic, is widely used for the treatment of a wide variety of different cancers and previous works have demonstrated that NF-kappaB is activated in response to a number of chemotherapeutic agents (Wang et al., 1996), including doxorubicin. A large number of NF-kappaB-regulated genes that can abrogate apoptosis including TRAF1, TRAF2, cIAP1, cIAP2, XIAP, Bclxl, and survivin have been identified (Wang et al., 1998). In addition, the activation of these anti-apoptotic gene transcriptions by NF-kappaB has been linked to the ability of malignancies to resist the cytotoxic effects of chemotherapeutics. In the present work, when β-catenin expression was down-regulated, we found that the expression of NF-

kappaB pathway was also influenced. The expression of Nuclear P65 and anti-apoptosis gene, XIAP and Bclxl, was significantly increased. The findings support previous report (Deng et al., 2002), which demonstrated that β-catenin can physically interact with NF-kappaB and inhibit its activity. In addition, high levels of NF-kappaB activity, which are associated with increased resistance to apoptosis induced by a variety of anticancer reagents, have been shown in diverse solid cell line (Karin, 2006). Therefore, the present study indicated that silencing of β-catenin enhances the NF-kappaB-mediated chemoresistance in MG-63 osteosarcoma cells.

However, the mechanism underlying the biological function of β-catenin in OS cells remains elusive. More recently, it was reported that the Wnt pathway is transcriptionally active in radiation-induced rat osteosarcomas (Daino et al., 2009) and Wnt/β-catenin pathway antagonists curcumin and PKF118-310 demonstrated anti-tumour activity against human osteosarcoma cells (Leow et al., 2009). In addition, the Wnt inhibitory factor 1 (Wif1) is silenced in human osteosarcomas and accelerates radiation-induced osteosarcoma formation when it is deleted in mice (Kansara et al., 2009). All these studies are suggestive of the complexity of Wnt pathway involving a number of receptors, ligands and inhibitors. Within the limitations of an in vitro model system, our findings indicate involvement of Wnt/β-catenin signalling in inhibiting invasion and chemosensitivity in MG-63 cells. Therefore, we must take effect of chemical inhibitor with a grain of salt, since β-catenin plays so many roles in cellular processes.

In summary, the data suggest that siRNA mediated downregulation of β-catenin induces no significant difference in growth and apoptosis in osteosarcoma MG-63 cells. Additionally, we also demonstrate that decreased β-catenin expression can suppress MT1-MMP expression and enhance XIAP, Bclxl and nuclear p65 expression, thereby resulting in suppression of invasion and motility of MG-63 cells and reduction of chemosensitivity to doxorubicin in vitro. Thereby, combination with NF-kappaB inhibitor is a promising new approach to the treatment of osteosarcoma. However, further work is needed to explore the effects of combination therapies incorporating NF-kappaB inhibitor together with β-catenin inhibitor in the future.

Acknowledgments

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