European Review for Medical and Pharmacological Sciences 2015; 19: 1182-1190

LY2109761 inhibits metastasis and enhances chemosensitivity in osteosarcoma MG-63 cells

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Abstract. – OBJECTIVE: Studies have shown that transforming growth factor-beta (TGF-β) is associated with metastasis and chemoresistance of osteosarcoma. The TGF-β kinase inhibitor LY2109761 could inhibits metastasis and enhances chemosensitivity in several cancers, but its role and mechanisms in osteosarcoma (OS) is unclear. Here, we investigated the role and mechanism of LY2109761 on metastasis and chemosensitivity of OS MG-63 cells.

MATERIALS AND METHODS: MG-63 cells were treated with LY2109761 or/and cisplatin. The cell viability and apoptosis of MG-63 cells were detected by MTT and ELISA. Matrigel invasion assay was used to detect cell invasion *in vitro*. pSMAD2 and S100A4 was detected by western blot assay. Furthermore, the efficacy of LY2109761 combined with S100A4 cDNA plaismid transfection on cell viability, apoptosis and chemosensitivity to cisplatin in OS MG-63 cells was further examined.

RESULTS: LY2109761 was sufficient to induce apoptosis and inhibited growth of MG-63 cells *in vitro*. Combination with LY2109761 significantly augmented the cytotoxicity of cisplatin in MG-63 cells. LY2109761 significantly inhibited invasion of MG-63 cells *in vitro*. The LY2109761-induced increase in cell apoptosis and the cytotoxicity of cisplatin, and decrease in cell invasion was blocked completely when S100A4 expression was restored in the MG-63 cells by S100A4 cDNA plasmid transfection.

CONCLUSIONS: Our data indicate that LY2109761 suppresses OS metastasis and enhanced chemosensitivity by targeting S100A4. LY2109761 may have important implications for the development of strategies for inhibiting metastasis and overcoming OS cell resistance to chemotherapy.

Key Words:
Osteosarcoma, Metastasis, Chemotherapy, Transforming growth factor-beta, LY2109761.

Introduction

Osteosarcoma is one of the most common primary bone tumors in children and adolescents and the focus is most often localized in the metaphysis of the adolescent long bones[1]. Osteosarcoma is characterized by a high propensity for metastasis, especially in lung, which is the main cause of death[2]. Surgery and neoadjuvant chemotherapy has been the standard treatment for osteosarcoma all the time[3]. Chemotherapy has significantly improved the survival rate from 11% with surgery alone to 60-70% when surgery is combined with chemotherapy[3,4]. Patients with advanced osteosarcoma after first-line chemotherapy usually receive further treatment with additional chemotherapy, which may be considered toxic[5]. Unfortunately, not much progress has been made on improving survival over the past 20 years with regard to the treatment of osteosarcoma. Therefore, understanding the mechanisms underlying OS as well as identifications of new molecular targets are of great importance.

TGF-β is a family of polypeptides that regulates a wide variety of biologic functions including cell proliferation, migration, survival, angiogenesis, immunosurveillance, and embryonic stem cell maintenance and differentiation. The multifunctional effects of TGF-β are elicited through dimerization of the type I (TGF-βR-I) and type II (TGF-βR-II) serine/threonine kinase receptors. Upon TGF-β binding, the receptor complex phosphorylates the transcription factors Smad2 and Smad3, which then binds to Smad4 and translocates to the nucleus, where they regulate transcription of various target genes[6].

S100A4 is a ubiquitous small, calcium-binding protein that enables cell migration and invasion to increase cell motility[7].

S100A4 is overexpressed in many cancers and plays a pivotal role in tumor proliferation, invasion, metastasis and angiogenesis[8-12], and downregulation of S100A4 could inhibit the effect above[13-21]. Recently, it has found S100A4 knock-

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down leads to p53-dependent cell cycle arrest and increased cisplatin-induced apoptosis[22]. The expressions of S100A4 was significant predictive factors of relapse in gastric cancer after curative resection and adjuvant chemotherapy[23]. Liang et al[20] has found overexpression of S100A4 may be associated with the resistance to cisplatin of laryngeal carcinoma Hep-2 cells. Knockdown of S100A4 enhances the sensitivity to cisplatin of laryngeal carcinoma cells.

Xue et al[24] has found S100A4 is a critical mediator of invasion in endometrial cancer and is upregulated by the TGF-beta1 signaling pathway. Matsuura et al[25] has found S100A4 can physically and functionally interact with Smad3, an important mediator of TGF-beta signaling and TGF-beta increases cell invasion ability induced by S100A4 in MCF10CA1a.cl1 cells.

Wang et al[26] has found that blocking TGF-β inhibits breast cancer cell invasiveness, migration and angiogenesis via ERK/S100A4 signalling.

LY2109761, a novel TβRI/II kinase inhibitor, has shown a Smad2-selective inhibitory profile with antitumor activity in various tumor models such as colorectal cancer[27], pancreatic cancer[28], and hepatocellular carcinoma[29]. LY2109761 has been also as radiosensitizers to improve the treatment of glioblastoma[30].

In the present study, we investigated the effect of LY2109761 on tumor proliferation, apoptosis and invasion *in vitro*. Furthermore, we investigated the combination effects of cisplatin and LY2109761 in established human OS cell lines *in vitro*. We then investigated the molecular mechanism for the effect of LY2109761.

Materials and Methods

Cell Culture

Osteosarcoma cell line MG-63 was obtained from the ATCC (Shanghai, China), and incubated in RPMI 1640 medium containing 10% fetal calf serum (FCS, Gibco, Grand Island, NY, USA) and 1% antibiotics (P/S, penicillin 10.000 U/ml and streptomycin 10.000 mg/ml, in 75 cm^2 culture flasks (Falcon, Mountain View, CA, USA) until they had formed a confluent monolayer. LY2109761 is an orally active TβRI/II kinase dual inhibitor, which was purchased from Selleck (Houston, TX, USA). Test compounds were dissolved in dimethyl sulfoxide (DMSO) and diluted with culture medium [DMSO final concentration, 0.1% (v/v)]. Stock DDP solution was prepared in DMSO (330 mM), stored as aliquots at 20°C, and used within 2 weeks. DDP was further diluted in medium before adding to the cells.

pcDNA3.1-S100A4 cDNA Plasmid and Transfection

The pcDNA3.1-S100A4 cDNA plasmid and its control pcDNA3.1 plasmid was kindly gifted by Doct W Jia, Department of Hepatobiliary and Pancreatic Surgery, Huaxi Hispital Sichuan University, Chengdu, Sichuan, China[14]. For transfection studies, MG-63 cells were plated at a density of 1×10^6 cells per well in six-well plates and incubated for 24 h in complete medium. The cells were then transfected with 4 ug of the S100A4 construct by using an Amaxa transfection kit (Gaithersburg, MD, USA) for 48 hs. For controls, the same amount of empty vector pcDNA3.1 was also transfected.

Tumor Invasion Assay

MG-63 cells were pretreated with 10 M LY2109761 for 24 hs, then transfected with pcDNA3.1-S100A4 cDNA plasmid or pcDNA3.1 plasmid for 48 hs. MG-63, MG-63/LY2109761, MG-63/LY2109761/pcDNA3.1-S100A4cDNA and MG-63/LY2109761/pcDNA3.1 cells were resuspended in fresh culture medium and incubated in chemoinvasion chamber containing polycarbonate filter coated with Matrigel (Chemicon International, Temecula, CA, USA) for 24 h. In the upper chamber, 30,000 cells were seeded in fetal bovine serum-free culture media and the lower chamber contained culture media containing 10% fetal bovine serum (FBS) as a chemoattractant. After 24 h, cells migrated into the lower chamber were stained and counted. Experiments were carried out in triplicate and repeated twice.

MTT Assay

MG-63, MG-63/pcDNA3.1-S100A4cDNA and MG-63 /pcDNA3.1 cells were seeded at a density of 3.5×10^3 per well in 96-well microtiter culture plates. After overnight incubation, medium was removed and replaced with fresh medium containing different concentrations of LY2109761 (10 μM) for 72 hs. Cell growth inhibition was detected using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

To determine the effect of drug combination, MG-63, MG-63/pcDNA3.1-S100A4cDNA and MG-63 /pcDNA3.1cells were plated onto 96-well

plates. After 72 h treatment with trans-diammineplatinum (DDP) (3.0 μM) and/or LY2109761(10 μM), cell viabilities were measured using MTT assays.

Detection of Apoptosis by ELISA

The cell death detection ELISA kit was used for assessing apoptosis according to the manufacturer's protocol. Briefly, MG-63, MG-63/pcDNA3.1-S100A4cDNA and MG-63/pcDNA3.1 cells were treated with LY2109761 or/and DDP for different periods of time. After treatment, the cells were lysed and the cell lysates were overlaid and incubated in microtiter plate modules coated with anti-histone antibody for detection of apoptosis.

Western Blotting

Western blot was used to measure the expression levels of proteins. Cells cultured with LY2109761 were harvested and the proteins in total cell extracts were generated using radioimmunoprecipitation assay (RIPA) buffer supplemented with protease inhibitors. LY2109761 treated cells were prepared using protein Extraction Kit (Affymetrix, Santa Clara, Guangzhou, China) according to the manufacturer's protocol. Protein samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) then transferred to polyvinylidene fluoride (PVDF) membrane. Anti-phospho (p)-Smad2 and total Smad2 (Cell Signaling, Danvers, MA, USA) were used as primary antibodies. An anti-β-actin monoclonal antibody (Sigma, St. Louis, MO, USA) as internal loading control. Anti-rabbit IgG peroxidase antibodies (Sigma, St. Louis, MO, USA) were used for secondary antibody and enhanced chemiluminescence (ECL) solution (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) was used for detection.

Statistical Analysis

Results consisting of three or more groups were analyzed using single-factor ANOVA. Analysis of results containing two groups was done using the Student's *t* test, assuming unequal variance. Values of $p < 0.05$ were considered statistically significant.

Results

Effects of LY2109761 on p-SMAD2 and S100A4 Expression

We first examined the effect of LY2109761, a Smad2-selective inhibitory profile on phospho (p)-Smad2 (p-Smad2) and total Smad2 in MG-63 cells. MG-63 cells were treated with LY2109761 at concentration of 10 μM for 24 hours. p-Smad2 and total Smad2 was detected by western blot assay. The results showed that p-Smad2 protein expression was completely inhibited (Figure 1A). LY2109761 did not have significant effect on total Smad2 (data not shown).

The baseline expression of S100A4 was determined in MG-63 cells. After treatment with LY2109761 at concentration of 10 μM for 24 hours, S100A4 protein was completely inhibited (Figure 1B).

Effects of LY2109761 on Cell Growth

The results showed that p-Smad2 and S100A4 was overexpressed in the MG-63 cells (Figure 1). Next, we examined the growth inhibitory effects of LY2109761 using the MTT assay in MG-63 cells. The treatment of MG-63 cells for 1-3 days with 10 μM of LY2109761 resulted in cell growth inhibition significantly (Figure 2A).

Next, we examined whether the inhibition of cell growth was also accompanied by the induction of apoptosis induced by LY2109761. ELISA analysis was employed to investigate the degree of apoptosis induced by LY2109761.

Effects of LY2109761 on Cell Apoptosis

MG-63 cells were treated with 10 μM LY2109761 for 24-72 hr. After treatment, the degree of apoptosis was measured in MG-63 cells. The induction of apoptosis was found to be as the same as MTT (Figure 2B). These results provided convincing data showing that LY2109761 could induce apoptosis in MG-63 cells.

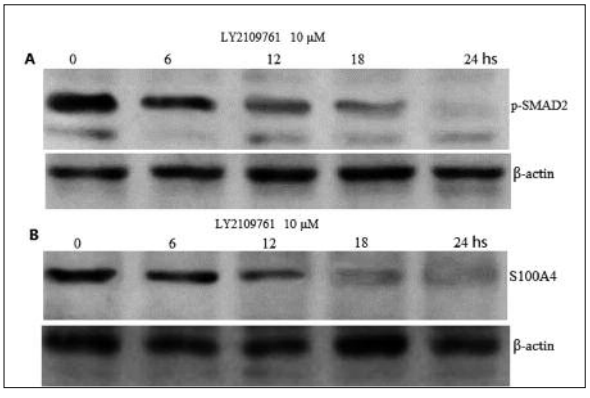

Figure 1. Western blot assay of LY2109761 on *p*-Smad2 and S100A4 expression. ***A,*** *p*-Smad2; ***B,*** S100A4.

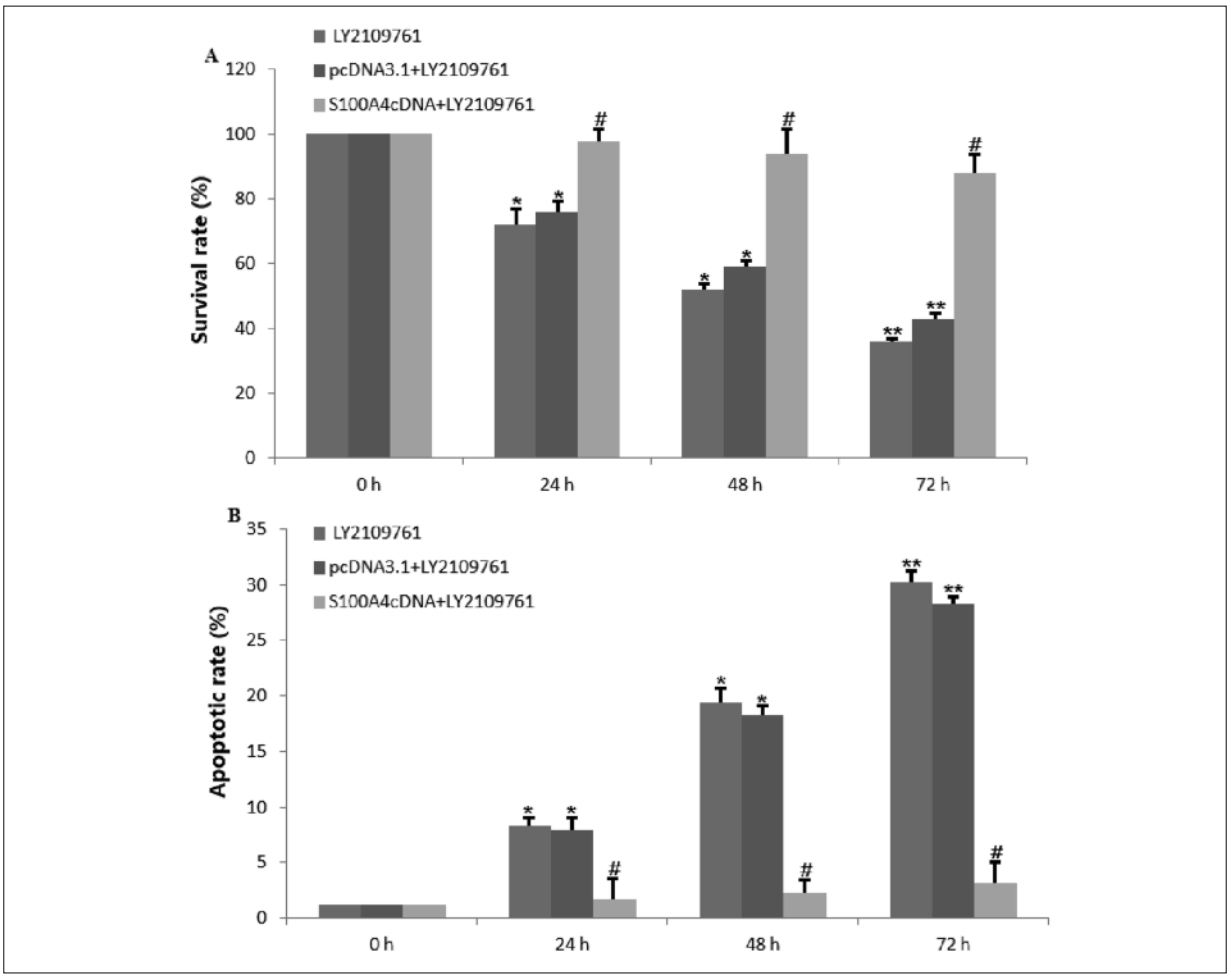

Figure 2. Effects of LY2109761 on cell growth and apoptosis. MG-63 cells were transfected with pcDNA3.1-S100A4 cDNA or pcDNA3.1, then treated with 10 μM of LY2109761 for 1-3 days. The growth inhibitory effects of LY2109761 on cells by MTT assay ***(A)***. The apoptosis effects of LY2109761 on cells by ELISA assay ***(B)***. Vs control; $p^* < 0.05$, $^{**}p < 0.01$, $^{\#}p > 0.05$

Effects of LY2109761 on Cell Growth and Apoptosis via Downregulation of S100A4

MG-63 cells were transfected with pcDNA3.1-S100A4 cDNA plasmid or pcDNA3.1 plasmid for 48 hs, then treated with 10 μM LY2109761 for 24-72 hr. After treatment, the degree of apoptosis and growth was measured in MG-63 cells. After transfection with pcDNA3.1-S100A4 cDNA, LY2109761 did not significantly enhance apoptosis (Figure 2B) and inhibit growth of MG-63 cells (Figure 2A).

LY2109761 Enhance the Cytotoxicity of DDP

In order to evaluate the combinatorial effect of LY2109761 with DDP, we measured cell viability after treatment of MG-63 cells with DDP and LY2109761. Combined treatment of DDP and LY2109761 significantly reduced cell viability of MG-63 cells (Figure 3A). Similarly, combined treatment of LY2109761 with DDP also effectively promoted apoptosis of MG-63 cells compared to single treatment with DDP or LY2109761 (Figure 3B).

LY2109761 Enhance the Cytotoxicity of DDP via Downregulation of S100A4

MG-63 cells were transfected with pcDNA3.1-S100A4 cDNA plasmid or pcDNA3.1 plasmid for 48 hs, then treated with 10 μM LY2109761 in combination with DDP for 72 h. After treatment, the degree of apoptosis and growth was measured in MG-63 cells. The results showed transfection with pcDNA3.1-S100A4 cDNA, LY2109761 in combination with DDP did not significantly enhance apoptosis and inhibit growth of MG-63 cells (Figure 3).

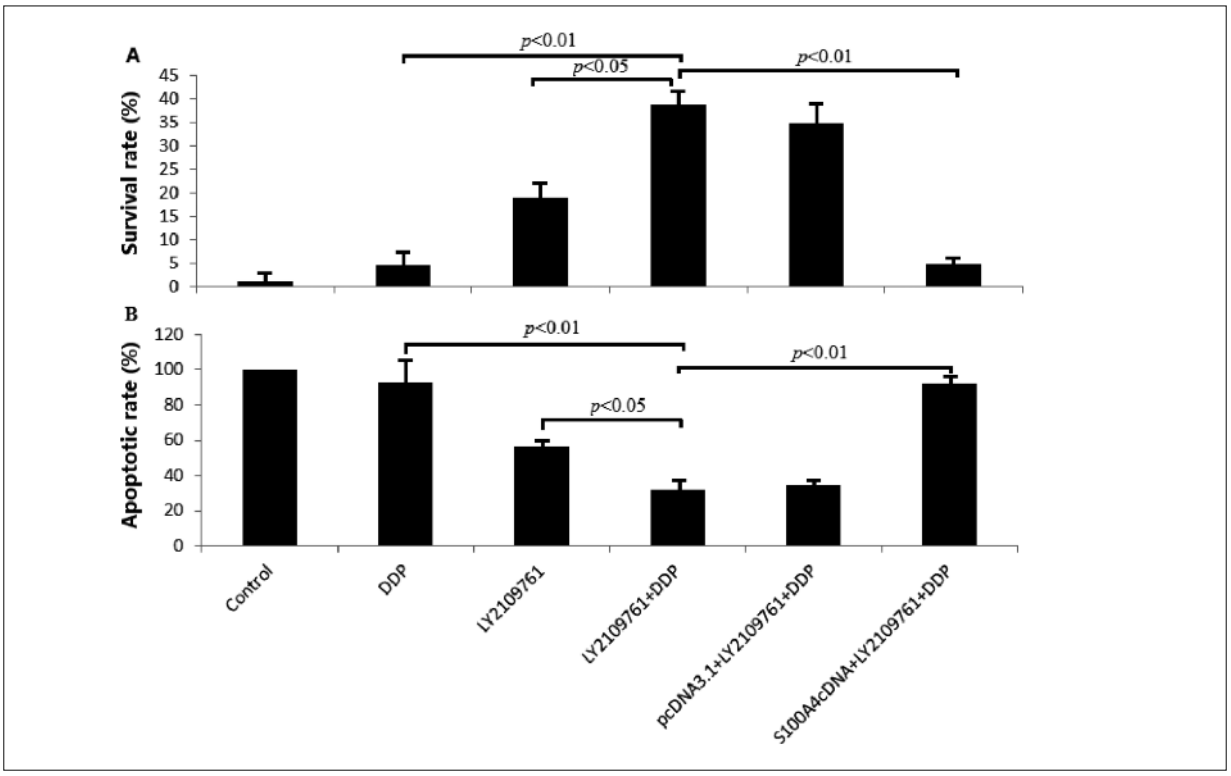

Figure 3. Combination of LY2109761 with DDP efficiently reduces the viability and increases the apoptosis of MG-63 cells. MG-63 cells were transfected with pcDNA3.1-S100A4 cDNA, then, treated with 10 μM of LY2109761 in combination with DDP for 72 h then cell viability was determined by MTT assay and ELISA assay as described in Materials and Methods. Data are expressed as mean ± SD. Student's *t*-test was applied for statistical analysis for comparison between DDP treatment and combined treatment

LY2109761 Inhibits Invasion of MG-63 Cells via Downregulation of S100A4 in vitro

In this study, we assessed the ability of LY2109761 to inhibit invasion of MG-63 cells in vitro. MG-63 cells were seeded in Matrigel-coated invasion chambers in the absence or presence of LY2109761 (10 μM). After 24 h, cells that migrated through the Matrigel barrier were stained and counted. Invasion was significantly inhibited by LY2109761 in MG-63 cells (Figure 4). However, when the MG-63 cells were transfected with pcDNA3.1-S100A4 cDNA, invasive ability was restored in the LY2109761 treated MG-63 cells (Figure 4).

Discussion

TGF-β overproduction is a universal event in cancer cells and is a poor prognostic marker[31-38]. TGF-β initiates cell signaling by dimerizing the TGF-β type I (TβRI) and type II (TβRII) serine/threonine kinase receptors. Many studies have reported that overexpression of TGF-β signal increased the chemoresistance in cancer cells[39-41], and vice versa[27,30]. LY2109761, TβRI/II kinase inhibitor reduced clonogenicity and increased radiosensitivity in glioblastoma (GBM) cell lines and cancer stem-like cells, augmenting the tumor growth delay produced by fractionated radiotherapy in a supra-additive manner *in vivo*. In an orthotopic intracranial model, LY2109761 significantly reduced tumor growth, prolonged survival, and extended the prolongation of survival induced by radiation treatment[30]. In our study, we found that LY2109761 treatment promoted apoptosis in MG-63 cells. Our observation on potentiation of growth inhibition was consistent with the induction of apoptosis as in MG-63 cells.

Many studies on clinical specimens have shown that TGF-β expression is associated with resistance to chemotherapy or radiation

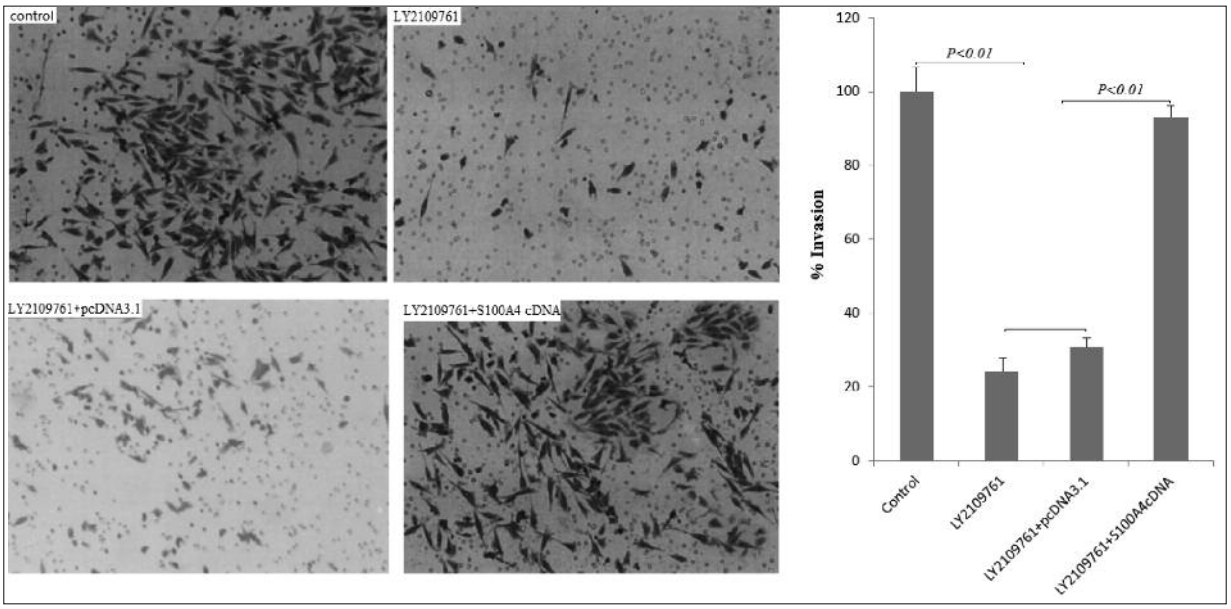

Figure 4. LY2109761 treatment inhibits invasion of MG-63 cells in Matrigel-coated invasion chambers. MG-63 cells were seeded in the upper chamber in medium supplemented with 5% FCS, treated with LY2109761 (10 μM). The LY2109761 treated MG-63 cells were transiently transfected with pcDNA3.1-S100A4 cDNA or pcDNA3.1, then seeded in the upper chamber in medium supplemented with 5% FCS. After 24h, cells migrated in the lower chamber were stained and counted. In the lower chamber, medium supplemented with 10% FCS was used as chemoattractant. Invasion of the untreated cells was set to 100. Results are reported as percent migration ± SD compared with untreated cells. Experiments were carried out twice in triplicate.

therapy[37-39], and linked to poor prognosis[40-41], suggesting that cancer cells survive with TGF-β.

In the current study, the anticancer drug cisplatin did not up or down-regulation of TGF-β signals in MG-63 cells (data not shown), which suggested that TGF-β signals was not associated with the acquired cisplatin drug resistance in the MG-63 cells. It is associated with the endogenous drug resistance in the MG-63 cells. In this study, we observed that inhibition of TβRI/II kinase receptors by LY2109761 combined with cisplatin is much more superior than the single agents *in vitro*. In this study, we also show that inhibition of TβRI/II kinase receptors by LY2109761 inhibited MG-63 cell migration by migration assay, which suggested that the TGF-β gene may be associated with proliferation and invasion of cancerous cells during progression of human osteosarcoma *in vitro* and LY2109761 could be useful for potentiating the anti-tumor activity *in vitro*. However, the mechanisms by which LY2109761 participates in the regulation of above are mostly unknown.

It has found that both extracellular and intracellular S100A4 participates in the regulation of cell death. Prosurvival functions have been described both in malignant[42] and nonmalignant cell systems[43], whereas osteosarcoma cells were sensitized to apoptosis on treatment with extracellular S100A4[44]. Overexpression of S100A4 in a benign rat mammary epithelial cell line was shown to promote subcutaneous tumor growth and metastasis to the lungs and lymph nodes[45-46]. Furthermore, decreased expression of S100A4 in highly metastatic human osteosarcoma cells produced a significant suppression of experimental metastasis formation after intracardial injection in rats and S100A4 antisense-transfected Lewis lung carcinoma cells displayed reduced metastatic capacity upon tail vein injection in syngeneic mice[47,48].

In this study, we show that LY2109761 could inhibit S100A4 expression, followed by decreased invasiveness, increased apoptosis and chemosensitivity to cisplatin in MG-63 cells *in vitro*. We also show that overexpression of S100A4 gene could reverse of LY2109761-induced effect. Altogether, the above-mentioned studies provide compelling evidence that LY2109761, TβRI/II kinase inhibitor is directly involved in the regulation of apoptosis, growth, invasion and chemosensitivity to cisplatin in MG-63 cells *in vitro* via S100A4 regulation.

Conclusions

The inhibition of TGF-β signals by LY2109761 could be useful for suppression OS metastasis and enhanced chemosensitivity. LY2109761may have important implications for the development of strategies for inhibiting metastasis and overcoming OS cell resistance to chemotherapy. The mechanisms by which LY2109761 exerts these functions might be through S100A4 upregulation.

Acknowledgements

This reasch was supported by grants from Department of Sience and Technology of Shandong Province (Grant No. BS2011YY063), and Department of Health of Shandong Province (Grant No. 2011QZ030).

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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