

RESEARCH ARTICLE

LY294002 Induces G0/G1 Cell Cycle Arrest and Apoptosis of Cancer Stem-like Cells from Human Osteosarcoma Via Down-regulation of PI3K Activity

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Abstract

Osteosarcoma, the most common primary mesenchymal malignant tumor, usually has bad prognosis in man, with cancer stem-like cells (CSCs) considered to play a critical role in tumorigenesis and drug-resistance. It is known that phosphatidylinositol 3-kinase (PI3K) is involved in regulation of tumor cell fates, such as proliferation, cell cycling, survival and apoptosis. Whether and how PI3K and inhibitors might cooperate in human osteosarcoma CSCs is still unknown. We therefore evaluated the effects of LY294002, a PI3K inhibitor, on the cell cycle and apoptosis of osteosarcoma CSCs in vitro. LY294002 prevented phosphorylation of protein kinase B (PKB/Akt) by inhibition of PI3K phosphorylation activity, thereby inducing G0/G1 cell cycle arrest and apoptosis in osteosarcoma CSCs. Further studies also demonstrated that apoptosis induction by LY294002 is accompanied by activation of caspase-9, caspase-3 and PARP, which are involved in the mitochondrial apoptosis pathway. Therefore, our results indicate PI3K inhibitors may represent a potential strategy for managing human osteosarcoma via affecting CSCs.

Keywords: PI3K/Akt pathway - osteosarcoma - cancer stem-like cells - cell cycle - apoptosis

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Introduction

Osteosarcoma is the most common primary mesenchymal malignant tumor of bone tissue in human, especially in children and adolescents, which tends to metastasize early with serious outcome and is more often in the metaphyseal areas of long bone such as distal femur, proximal tibia and humerus. Long-term survival rate increases from 25% to 65% with advances in surgical techniques and chemotherapy, but this figure has no obvious improvement in many years. Investigation of its reason is many sided and the main aspect is drug-resistance during chemotherapy (Meyers et al., 2005).

The tumorigenic mechanism of osteosarcoma and its respective drug-resistance are barely understood. In the past one to two decades, more and more studies suggest that some tumors are hierarchically organized, which means that these tumor cells maintains differences in their morphology, function and differentiation level (Hanahan and Weinberg, 2000; Dick, 2008). Based on the similarity shared between these tumors, many researchers have turned to a new theory, the CSCs theory, which could help people explain tumorigenesis and drug-resistance better (Dick, 2008; Visvader and Lindeman, 2008). The CSCs theory believes that CSCs locate at the top of hierarchical pyramid and play a key role in tumorigenesis and drug-

resistance (Visvader and Lindeman, 2008). Therefore, the study of CSCs-target treatment may become a new hotspot in searching anticancer drugs.

Gibbs and Wilson firstly isolated the CSCs from human and canine osteosarcoma cell lines, and identified their stemness by studying biological properties and applying respective surface markers (Gibbs et al., 2005; Wilson et al., 2008). The CSCs from osteosarcoma demonstrated more active features in proliferation and self-renewal than normal cancer cell, which also expressed many stem cell markers such as CD44, CD105, Oct-3/4 and Nanog, etc (Gibbs et al., 2005; Wilson et al., 2008). Recently, Tirion and Veselska isolated CD133+ population from human osteosarcoma cell lines MG-63, Saos-2 and U2OS, and demonstrated CD133+ cells might be used as markers for identifying osteosarcoma CSCs (Tirino et al., 2008; Veselska et al., 2008).

Recent studies suggest that CSCs share similar growth regulatory mechanisms and signal pathways such as Wnt, Notch and Hedgehog with normal stem cell (Zhao et al., 2009; Liu et al., 2011). Xu et al. found that survival of acute myeloid leukemia cells requires PI3K activation (Xu et al., 2003), while Eyler and his colleagues similarly points out that brain cancer stem cells display preferential sensitivity to Akt inhibition (Eyler et al., 2008). Thus, PI3K-targeted and CSCs-targeted therapeutic approaches

may suggest a potential cancer treatment for human.

Based on these reports, we examined the effects of PI3K inhibitor in human osteosarcoma CSCs on cell cycle and apoptosis. LY294002 and Wortmannin are the commonly used inhibitory agents of PI3K, and prior studies have demonstrated that these agents can specifically inhibit the catalytic role of PI3K. Wortmannin is derived from *Penicillium wortmanni* and proved to inhibit pancreatic cancer cell invasion (Teranishi et al., 2009), but its chemical stability is poor (Walker et al., 2000). Not only does LY294002 have a better chemical stability than Wortmannin, it also has been shown to inhibit cancer cell proliferation and enhance apoptosis. So LY294002 was selected as the object of this study.

Materials and Methods

Cell line and cell culture

Bone osteosarcoma cell line, MG-63, was initially frozen-stored in our laboratory and maintained in DMEM/F12 medium (Hyclone, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, USA), 1×10^5 U/L penicillin (Sigma, USA) and streptomycin (Sigma, USA) at 37 °C in an incubator with 5% CO₂. The cells were digested with 2.5g/L trypsin (Gibco, USA) for 3~5 min at 37 °C and passaged once 5~6 days.

Isolation and passages of osteosarcoma CSCs

The process of osteosarcoma CSCs isolation was mostly carrying out as previously described (Gibbs et al., 2005; Wilson et al., 2008). In brief, cells were harvested by trypsinization and cells number was counted afterwards. The cells were plated in ultra-low-attachment culture plates (Corning, USA) in serum-free medium at a density of 6×10^5 cells per well. This serum-free medium was consist of DMEM/F12 medium, 20 µg/L human epidermal growth factor (EGF) (Peprotech, USA), 20 µg/L human basic fibroblast growth factor (bFGF) (Peprotech, USA), 1xN2 supplement (Invitrogen, USA), 2 mmol/L L-glutamine (Sigma, USA), 4 U/L insulin (Sigma, USA), 1×10^5 U/L penicillin and 1×10^5 U/L streptomycin and adjusted pH value to 7.2~7.5. The CSCs were cultivated at 37 °C and 5% CO₂. Additional fresh serum-free media were added every other day. After osteosarcoma CSCs sphere contained more than 50 cells formed, the CSCs were collected and dissociated by trypsinization, then passaged in serum-free medium at 1:2~1:4.

Identification of osteosarcoma CSCs by Immunocytochemistry

The osteosarcoma CSCs in logarithmic growth phase were collected, resuspended with serum-supplement medium, and inoculated on polylysine-coated glass coverslips (Boster, China) for overnight. The samples were fixed in fresh prepared cold 4 % paraformaldehyde (Boster, China) for 15 min at room temperature after all cells were anchored to coverslips well and blocked with 25% goat serum (Boster, China) for 20 min in order to avoid non-specific antibody binding. Each sample was labeled with rabbit antibody against human CD44 and CD133 (Santa Cruz, USA, 1:200) alone in a volume

of 0.1mL antibody diluent. The following steps were carried out according to recommended protocols of SABC immunocytochemistry kit (Boster, China).

Drug preparation

LY294002 (Beyotime, China) was dissolved in DMSO at concentration of 10 mg/mL and stored at -20 °C and The solutions of LY294002 finally used in experiments were 5 µmol/L, 15 µmol/L and 45 µmol/L. Semba et al. demonstrated that the concentration of DMSO as we used in this study did not affect cell survival and protein phosphorylation (Semba et al., 2002).

Cell cycle analysis

The process of cell cycle analysis was carried out as Semba described and recommended protocols of cell cycle analysis kit (Beyotime, China) (Semba et al., 2002). After treatment with LY294002 for 24 hours, those groups of LY294002-treated CSCs were detached by trypsinization, collected by centrifugation, and then fixed in 70% ethanol at 4 °C for overnight. Cells were washed by PBS, harvested by centrifugation, and resuspended in a volume of 400 µL PBS. Each group containing up to 1×10^5 cells was stained with 500µL propidium iodide solution for 30 min at 37 °C and analyzed by flow cytometer (BD Biosciences).

Cell apoptosis analysis

Annexin V/PI double staining apoptosis detection kit (KeyGEN, China) was used to detect cell apoptosis and its protocol was followed as Jiang et al. and manufacturer's instructions described (Jiang et al., 2010). In brief, cells were harvested by the method described above after presence of LY294002 for 48 hours, washed twice with PBS, and resuspended in a volume of 500µL binding buffer. The cells were stained with Annexin V-FITC (5 µL) and PI (5 µL) for 15~30 min. Analysis was performed on the flow cytometer (BD Biosciences).

Western blot Analysis

After treatment with LY294002 for 24 hours, the cells were washed twice in ice-cold PBS and lysed in the lysis buffer [RIPA lysis buffer (Boster, China) containing 1% protease inhibitor cocktail (Boster, China) and 1% phosphatase inhibitor cocktail (Boster, China)] on ice. Protein concentrations were determined by BCA protein assay kit (Boster, China). The protein samples were denatured at 100 °C for 10 min and then preserved at -20 °C for later use. The proteins were separated by SDS-polyacrylamied gels and transblotted onto PVDF membranes. The PVDF membranes were probed first with primary antibodies (1:1000 dilution) [Akt antibody (Beyotime, China), phosphorylated Akt (Ser473) antibody (Beyotime, China), Caspase-3 antibody (Cell Signaling Technology, USA), Caspase-9 antibody (Bioss, China), cleaved poly ADP-ribose polymerase (PARP) antibody (Cell Signaling Technology, USA), β-actin antibody (Boster, China)] overnight at 4 °C and then reprobed with horseradish peroxidase-conjugated secondary antibody (1:1000 dilution) (Boster, China) for 1.5 hours at room temperature. Finally the results were detected with an

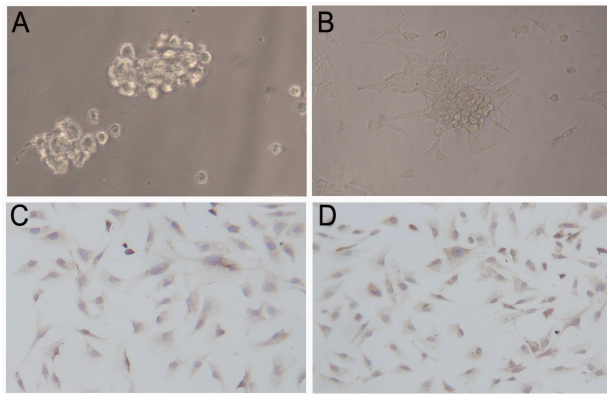


Figure 1. Isolation and Identification of Osteosarcoma CSCs. (A) Sarcosphere clusters formed by MG63 cells in serum-free media after 7 days ($\times 200$); (B) Expanding adherent cells can be observed when sarcosphere was cultured in serum-containing media ($\times 100$); (C) Immunocytochemical analyses on CSCs for CD44 ($\times 100$); (D) Immunocytochemical analyses on osteosarcoma CSCs for CD133 ($\times 100$)

enhanced chemiluminescence (Thermo, USA) kit and exposed to the film (Kodak, USA). The determination of grayscale value was processed by ImageJ 1.43 u.

Results

Isolation of sarcospheres from osteosarcoma cell lines MG-63

As Gibbs and Wilson reported (Gibbs et al., 2005; Wilson et al., 2008), the MG-63 cell line formed a recognized sarcospheres that could be observed microscopically after cultivation in serum-free medium for 7~10 days (Figure 1A). We evaluated the proportion of the cell that can be form sarcosphere in MG-63 cell line preliminarily, which was close to the correlational researches (Gibbs et al., 2005; Meyers et al., 2005; Dick, 2008; Visvader and Lindeman, 2008; Wilson et al., 2008). The cells of sarcospheres could also generate the secondary cell spheres in serum-free medium as Gibbs described (Gibbs et al., 2005). We also found that the cell of each generation sarcospheres could form the expanding adherent osteosarcoma cells in serum-containing media, which were close to the normal MG-63 cells (Figure 1B).

Expressions of CD44 and CD133 in osteosarcoma CSCs

The surface markers of pluripotent stem cell such as CD44, CD133, CD105 and Stro-1 have been proved to become the surface markers of some CSCs such as breast cancer, brain gliosarcoma, colon cancer, Ewing sarcoma and osteosarcoma (Gibbs et al., 2005; Tirino et al., 2008; Veselska et al., 2008; Rosen and Jordan, 2009). We used CD44 and CD133 as cell markers to identify the osteosarcoma CSCs. These assays revealed that the majority of CSCs were identified by the CD44 surface marker (Figure 1C). The osteosarcoma CSCs showed significantly greater expressions of CD133 than MG63 cells (Figure 1D).

Effects of LY294002 on Akt phosphorylation in osteosarcoma CSCs

LY294002 inhibits PI3K activity and then led to

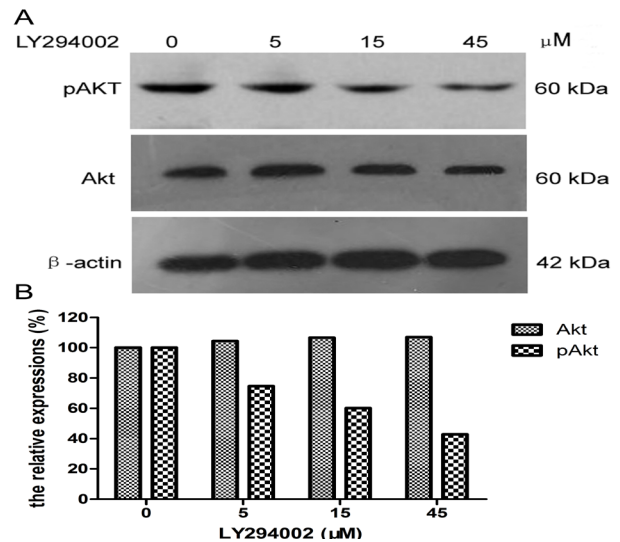


Figure 2. Western Blotting Results Showed that LY294002 Has A Dose-dependent Inhibitory Effect on Phosphorylation of Akt

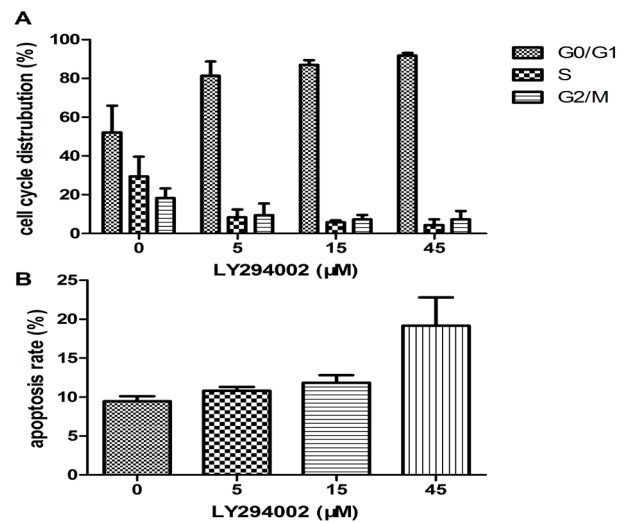


Figure 3. Effect of LY294002 on Cell Cycle Distribution and Apoptosis in Osteosarcoma CSCs. (A) G0/G1 cell cycle arrest was shown after LY294002 treatment.; (B) The percentages of apoptosis cells in osteosarcoma CSCs increased in a dose manner after treatment with different concentrations of LY294002

a decrease phosphorylation of the downstream target such as Akt. We used western blot analysis to determine this effect in osteosarcoma CSCs. When we increased the concentration of supplemental LY294002 in the osteosarcoma CSCs, the levels of Akt phosphorylated at Ser473 were decreased (Figure 2).

Effects of LY294002 on cell cycle of osteosarcoma CSCs

We analyzed the alterations in cell cycle distribution of osteosarcoma CSCs after treatment of LY294002 for 24 hours. As Lee reported, no significant alterations in cell cycle distribution were observed in LY294002 treatment group (Lee et al., 2006). But LY294002 could lead to some effects on cell cycle distribution in osteosarcoma CSCs, which was very different from what Lee reported. As is shown in Figure 3A, LY294002 increased the number of cells in G0/G1 phase after 24hours treatment; a corresponding decrease was shown in S phase.

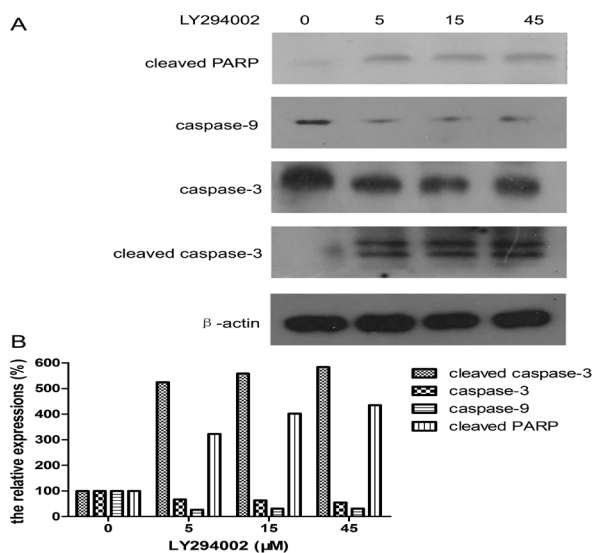


Figure 4. Western Blot Analysis Showed Cleaved Caspase-3 and Cleaved PARP were Highly Expressed after Treated with Different Concentrations of LY294002, but Caspase-3 and Caspase-9 were Opposite

Effects of LY294002 on apoptosis of osteosarcoma CSCs

In order to explore the apoptosis-inducing effect of LY294002 on osteosarcoma CSCs, we analyzed the apoptosis in the CSCs after treatment of LY294002. As is shown in Figure 3B, the decrease of viable cells was corresponding to a significant increase in both early apoptotic and dead cell with different concentrations of LY294002. The apoptosis-inducing effect of LY294002 was significantly dose-dependent.

Then the possible molecular mechanisms related to apoptosis in above alteration were detected by western blot analysis. PARP, caspase-9 and caspase-3 are important apoptosis associated protein. PARP and caspase-3 were critical executioners in the late apoptotic stage. Figure 4 illustrated caspase-3 (35 kDa) was detectable in untreated and treated cells, but the cleaved caspase-3 (17 and 19 kDa) was only observed in groups treated with LY294002. Cleaved caspase-3 and PARP increased with concentration of LY294002 and in contrast to caspase-9.

Discussion

Tumorectomy and chemotherapy are still the most important two approaches to treat solid malignancy such as osteosarcoma, but residual tumor cells may form new tumors at primary and metastatic focus. Recurrence and metastasis in patients with advanced osteosarcoma are the main causes for death. The main explanation for these causes is that subpopulations maintained in osteosarcoma cannot be removed completely.

More and more reports suggested that CSCs, like normal stem cells, were at the top of a hierarchical pyramid of solid tumor, and was identified as the subpopulation of cancer cells to initiate tumorigenesis and metastasis by undergoing self-renewing and differentiation into different classes of cancer cells, but the majority of the cancer cells was more differentiated and lacks these properties (Rosen and Jordan, 2009). Researches later demonstrated that a subset of osteosarcoma cells has the capacity to form

sarcospheres and to self-renew over an extended period of time, and are even able to proliferate and generate rest of the tumor (Gibbs et al., 2005; Tirino et al., 2008; Veselska et al., 2008; Wilson et al., 2008). Recently, Our laboratory has also found knock-down of β -catenin gene, a type of molecule that affects how stem cell differentiation and proliferation, may reduce chemosensitivity to doxorubicin and decrease the invasion ability (Zhang et al., 2011).

In this study, we isolated CSCs from human osteosarcoma carried out by cells sphere culture in serum-free medium according to Gibbs and Wilson's protocols (Gibbs et al., 2005; Wilson et al., 2008). The culture system could promote CSCs survival and proliferation with present of this harsh serum-free medium and only two growth factors. Furthermore, we found that CSCs obtained from human osteosarcoma preferentially expressed the cancer stem cells surface marker such as CD44 or CD133, which is reported to be the markers of cancer stem cells from bladder cancer, brain cancer, breast cancer, colon cancer, pancreatic cancer and osteosarcoma (Tirino et al., 2008; Frank et al., 2010).

Previous studies have shown that CSCs behavior closely mimicked the development paradigms found in normal stem cells systems, a single cells giving rise to a metastatic lesion because of capabilities of self-renewal and form the entire tumor population, which is partially due to the effects of some special signal pathways related to DNA repair, oxidative state and pro-survival such as NF- κ B and PI3K (Rosen and Jordan, 2009). Extensive literature indicated that increased PI3K activity was strongly implicated in cells cycle, proliferation, and resistance to chemotherapy-induced neoplastic apoptosis (Coffey et al., 2005). Given these previous findings regarding to PI3K, we aimed to characterize the role of PI3K/Akt signal pathway in the above-mentioned biological behavior of CSCs, and evaluated the therapeutic effects of LY294002 in vitro.

In order to evaluate the effect of LY294002 on PI3K/Akt pathway, we use western blot to explore the relationship between LY294002 and Akt phosphorylation firstly. Our western blot results revealed the decrease of phosphorylated Akt levels with increasing doses of LY294002. Therefore, our results supported previous studies and suggested that LY294002 dose-dependently inhibits PI3K activity and decreases Akt phosphorylation in osteosarcoma CSCs.

Considering the potential key role of CSCs in the development, progress and metastasis of osteosarcoma, a noteworthy finding in our study was that LY294002, a PI3K-specific inhibitor, showed the inhibitory effect on cell cycle progression in a dose-dependent fashion in vivo, which might lead to an inhibitory effect on proliferation. The primary act of that was G0/G1 arrest, which was similar to Casagrande's results (Casagrande et al., 1998). There were probably two reasons. One was that decreasing of phosphorylated Akt might lead to down-regulation of G1 cycle-dependent kinase, especially cyclin D-CDK4/6, and that of activity and accumulation of its inhibitors, such as p27 and PTEN (Casagrande et al., 1998). Also, LY294002 could inhibit the activity of molecules such as ATM, ATR and DNA-PK, which related to check up and

repair of DNA damage (Lee et al., 2006).

Additionally, we have recently shown that LY294002 significantly increase apoptosis in osteosarcoma CSCs. The CSCs treated with LY294002 showed a significant increase in apoptosis versus the negative control. We presume that the apoptosis pathway might be up-regulated by LY294002, including activation of apoptosis inducing molecule(s) and/or inactivation of apoptosis blocking molecule, and then such pathway would lead osteosarcoma CSCs into apoptosis. In our study, we observed that PARP and Caspase-3 were significantly active in treated groups, whereas the apoptotic rate was higher when cells were treated with LY294002 than that treated with negative control. But PARP and caspase-3 are activated in the apoptotic cells by mitochondrial and non-mitochondrial death pathway, we continued to investigate the changes of caspase-9 in order to reveal which pathway is involved in this apoptotic process. Caspase-9 is regarded as an important activator related to apoptosis induction via mitochondrion-mediated pathway, wherein it subsequently activates caspases-3/6/7 (Luan et al., 2011). The PI3K/Akt pathway conferred resistance by suppressing caspase-9 cascade (Jiang et al., 2010). Earlier study suggested that the amount of caspase-9 (47kD) was significantly reduced in apoptosis (Zhang et al., 2011). Our results also indicated that procaspase-9 (47kD) expression became less with LY294002 treatment, and caspase-9 was the target of PI3K/Akt pathway.

In conclusion, our data demonstrate that LY294002 significantly inhibits growth and induces G0/G1 cell cycle arrest and apoptosis in human osteosarcoma CSCs in vitro. This effect showed a significant dose-dependent effect and relies on mitochondrial apoptotic pathway. This suggests that PI3K/Akt pathway play an important role in the cell cycle and apoptosis of osteosarcoma CSCs, while PI3K-targeted and CSCs-targeted therapeutic treatments may be potentially useful for pharmacological intervention in human osteosarcoma.

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