Int J Clin Exp Med 2017;10(1):944-950
www.ijcem.com /ISSN:1940-5901/IJCEM0037285

Original Article

Effect of PI3K-mediated autophagy in human osteosarcoma MG63 cells on sensitivity to chemotherapy with Gefitinib

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Received July 28, 2016; Accepted September 29, 2016; Epub January 15, 2017; Published January 30, 2017

Abstract: Background: Osteosarcoma is one of the most common malignant bone tumor in adolescent. With high malignant degree and poorer prognosis, osteosarcoma seriously influences the prognostic survival of patients. Autophagy which is relatively conservative, is the main means of degrading autologous organelles or proteins in the evolutionary of eukaryocyte, regulating and controlling the cell proliferation and function. Objective: To explore the effect of PI3K-mediated autophagy in human osteosarcoma MG63 cells on sensitivity to chemotherapy with Gefitinib. Methods: MTT and flow cytometry were employed to measure cell proliferation and cell apoptosis of human osteosarcoma MG63 cells treated with Gefitinib combined with autophagy inhibitor 3-methyladenine (3-MA), autophagy promoter rapamycin, PI3K inhibitor LY294002. The expression of intracellular protein and its total mRNA was detected by Western blot and RT-PCR after different drugs treated on MG63 cells. Results and conclusion: Western blot showed that basic autophagy level of MG63 cells was significantly lower than that of osteoblast hFOB cells. MTT, western blot and RT-PCR analysis revealed that the cell proliferation inhibition rate of MG63 cells treated with Gefitinib combined with 3-MA, RAPA, LY294002 were significantly inhibited. Besides, the expressions of autophagy associated proteins and total mRNA in human osteosarcoma cells were enhanced. In addition, the result of flow cytometry demonstrated that Gefitinib alone or combined with other drugs could increase the cell numbers in G1 phase and reduce the cell numbers in S phase. Taken together, our data showed that upregulating the autophagy significantly reduced the sensitivity of MG63 cells to chemotherapy with DDP and DDP induced autophagy of MG63 cells by blocking the cell cycle at G1 phase.

Keywords: Osteosarcoma, autophagy, PI3K, Bcl-2, Beclin-1, LC-3

Introduction

Osteosarcoma is one of the most common malignant bone tumor in adolescent [1]. With high malignant degree and poorer prognosis, osteosarcoma seriously influences the prognostic survival of patients [2]. Although current clinical surgery combined with adjuvant chemotherapy can significantly improve the life quality and survival rate of patients, the long-term survival rate of patients is still seriously threatened by recurrence, metastasis and chemotherapy resistance [3, 4]. Therefore, it is very urgent to enhance the sensitivity of osteosarcoma chemotherapy and study its molecular mechanism in clinical treatment [5].

The inhibitory and protective effects of autophagy need to be further explained and the role of autophagy remains controversial in tumor-therapy [6]. In this study, we explored the changes of autophagy activity and the molecular mechanism of PI3K/Akt pathway in osteosarcoma chemotherapy autophagy to provide evidence for clinical treatment of osteosarcoma. Cell proliferation, cell cycle and the changes of autophagy in the molecular level were detected by methods of MTT, flow cytometry, RT-PCR and western blot after treated with Gefitinib combined with 3-MA, RAPA and LY294002 in human osteosarcoma MG63 cells.

Materials and methods

Cells culture

Human osteosarcoma cell line MG63 and human normal osteoblast hFOB were purchased

Table 1. Groups of Experiments

Groups	Ingredients
Control	0.1% DMSO
Gefitinib (GFT)	10 μM Gefitinib
PI3K (PI)	5 μM LY294002
RAPA	10 μM rapamycin
3-MA	5 mM 3-MA
GFT + PI	5 μM LY294002 + 10 μM Gefitinib
GFT + RAPA	10 μM rapamycin + 10 μM Gefitinib
GFT + 3-MA	5 mM 3-MA + 10 μM Gefitinib

Concentration above is the final concentration.

from the Cell Resource Center of Shanghai Institutes for Biological Sciences, the Chinese Academy of Sciences. Cells were cultured in the DMEM complete medium (Gibco) with 10% fetal bovine serum, 100 IU/mL penicillin and 100 μg/mL streptomycin at 37°C in 5% CO_2.

MTT method and cell proliferation activity

Cells were seeded in 96-well plates (3×10^3/well). After cellular adhesion, freshly prepared drugs (Gefitinib, LY294002, Rapamycin, 3-MA, purchased from Shanghai source biological technology co., LTD) were added as shown in **Table 1**. Forty-eight hours after the addition of drugs, 20 μl MTT (5 mg/ml) was added in each well. After cultivating for 4 h, the supernatant was discarded and each well was dissolved with 150 μl DMSO and shake for 10 min. The absorbance value was detected at 490 nm wavelength using Microplate Reader (Tecan GENIOS, Sweden) and the cell proliferation inhibition rate and median inhibitory dose (IC50) were calculated.

Flow cytometry and cell cycle

Cells were plated in 6-well plates (6×10^5 cells/well). After cellular adhesion, freshly prepared drugs (Gefitinib, LY294002, Rapamycin, 3-MA, purchased from Shanghai source biological technology co., LTD) were added as shown in **Table 1**. Twenty-four hours later, flow cytometry was used to detect cell cycle. The results were analysed with CellQuest and Modfit software.

Real-time PCR (RT-PCR)

After 24 h treated with different drugs, total RNA was extracted using Trizol reagent following the manufacturer's instructions. Then 1 μg RNA was added to the 25 μl reverse transcription system, and reversed transcription at the conditions of 42°C for 60 min, 75°C for 10 min. 1 μl reverse transcription samples were taken as the template and PCR was carried out by SYBR® Premix Ex Taq™II. Conditions for PCR were as follows: denaturation at 95°C for 3 min, annealing 95°C for 15 sec, extension at 59°C for 30 sec, in 40 cycles. The relative expression of target mRNA was calculated with the $2^{-\Delta\Delta Ct}$. The primer sequences were as **Table 2**.

Intracellular expression of GFP-LC3 fusion protein

GFP-LC3 plasmid was transfected into MG63 cells using Lipofectmine 2000 following the manufacturer's instructions. The transfected cells were treated with different drugs for 24 h after stable transfection of GFP-LC3 fusion protein. Cells were collected and determined by flow cytometer.

Western blot

After treated with different drugs for 24 h, cells were harvested and homogenized with lysis buffer. Total protein was separated by denaturing 10% SDS-polyacrylamide gel electrophoresis. The primary antibodies for GAPDH, Beclin-1, PI3K, Bcl-2 and LC-3 I/LC-3 II were purchased from Cell Signaling Technology. Protein levels were normalized to GAPDH

Statistical analysis

Each experiment was repeated at least 3 times. Data were presented as mean ± SD. The difference between means was analyzed with Student's t test. All statistical analyses were performed using SPSS11.0 software (Chicago, IL). Differences were considered significant when $P<0.05$.

Results

The expression of autophagy-related proteins

The expression of autophagy-related proteins, PI3K, Bcl-2, Beclin-1 and LC-3 (LC-3 I/LC-3 II), of MG63 cells and hFOB cells were detected by western blot. As show in **Figure 1** and **Table 3**, the expression of autophagy-related proteins at MG63 cells, LC-3 I/LC-3 II and Beclin-1, were significantly lower than normal osteoblasts ($P<0.01$) while the anti-apoptotic protein Bcl-2

Table 2. The primer sequences

Primer	Sense (5-3)	Antisense (5-3)
GAPDH	CATCTTCTTTTGCGTCGCCA	TTAAAAGCAGCCCTGGTGACC
PI3K	CATCACTTCCTCCTGCTCTAT	CAGTTGTTGGCAATCTTCTTC
Bcl-2	CGACGACTTCTCCCGCCGCTACCGC	CCGCATGCTGGGGCCGTACAGTTCC
Beclin-1	AAGAGAGAGCGATGGTAG	CTGGGCTGTGGTAAGTAA

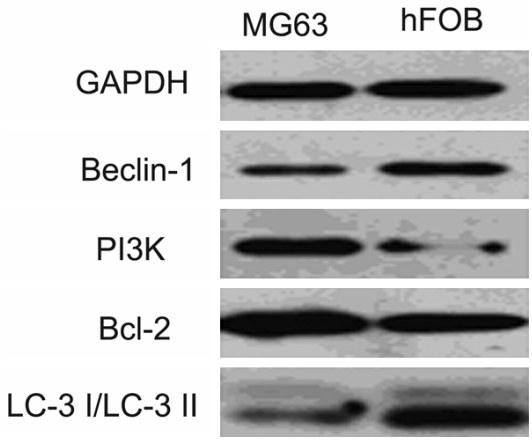

Figure 1. The expression of autophagy related protein at MG63 cells and hFOB cells.

and PI3K were significantly increased ($P<0.01$). Those results showed that the autophagy activity in human osteosarcoma MG63 cells was lower than the normal osteoblasts, and decreased autophagy could inhibit cell apoptosis. Meanwhile, abnormal expression of PI3K may play an important role in the occurrence and development of osteosarcoma.

Effect of autophagy in MG63 cells on sensitivity with Gefitinib

Effect of autophagy in MG63 cells on sensitivity with Gefitinib was detected by the MTT with the drugs of Gefitinib (10 μM), 3-MA (1 mM, 5 mM, 50 mM) and RAPA (1 mM, 5 mM, 50 mM) in MG63 cells for 24 h, respectively. As show in **Figure 2**, results showed that the sensitivity in MG63 cells was weak and the inhibition rate was low with RAPA at the concentration of 1 μM and 10 μM. We selected 10 μM for the subsequent experimental study as the opposite effect occurred when the concentration of RAPA was 100 μM. The inhibitory effect of 3-MA was dose-dependent at the concentration of 1 mM-50 mM and it reached about 80% at 5 mM. Therefore, we selected 5 mM for the subsequent experimental dosage as the cells are difficult to survive at 50 mM. Thus, Inhibition of autophagy may promote the inhibition of cell proliferation.

Role of PI3K in cells autophagy and chemotherapy sensitivity of MG63 cells

The cell proliferation activity was detected by MTT assay after treated with different drugs for 24 h in MG63 cells. As show in **Figure 3**, results indicated that 3-MA can enhance the inhibitory effect of Gefitinib when the autophagy was blocked and LY294002 can reduce the inhibitory effect of Gefitinib when PI3K was inhibited.

Effect on autophagy-related proteins of MG63 cells with Gefitinib

The expression of autophagy-related proteins, PI3K, Bcl-2, Beclin-1 and their mRNA, were detected by the means of RT-PCR and western blot. As show in **Tables 4** and **5**, Gefitinib can significantly inhibit the expression of PI3K, Bcl-2 and enhance the expression of Beclin-1. The expression of PI3K and Beclin-1 increased when Gefitinib combined with 3-MA. In contrast, the expression of PI3K decreased when Gefitinib combined with RAPA. LY294002 and PI3K inhibitor can significantly enhance the inhibition of Gefitinib on the expression of PI3K and Bcl-2 and promote the expression of Beclin-1.

Effect on autophagy in MG63 cells with Gefitinib

The autophagy of GFP-LC3 fusion protein in MG63 cells with different drugs was detected by flow cytometry. PI3K inhibitors can enhance autophagy of MG63 cells and RAPA was also able to promote cell autophagy (**Table 6**). Thus, PI3K inhibitors and RAPA could enhance the proliferation inhibitory effect on MG63 cells with Gefitinib.

Effect of MG63 cell cycle with Gefitinib

The effect of different drugs on the cell cycle of MG63 cells was detected by flow cytometry. As show in **Table 7**, Gefitinib could increase the proportion of cells in G1 phase and reduce the

Table 3. The expression of autophagy related protein at MG63 cells and hFOB cells

	Beclin-1	PI3K	Bcl-2	LC-3 II/LC-3 I
MG63	21.32±4.78	52.69±5.43**	67.19±6.21**	16.59±4.98
hFOB	43.64±5.36**	12.83±5.01	31.28±6.14	51.28±6.47**

Compared with the hFOB cells, **P<0.01.

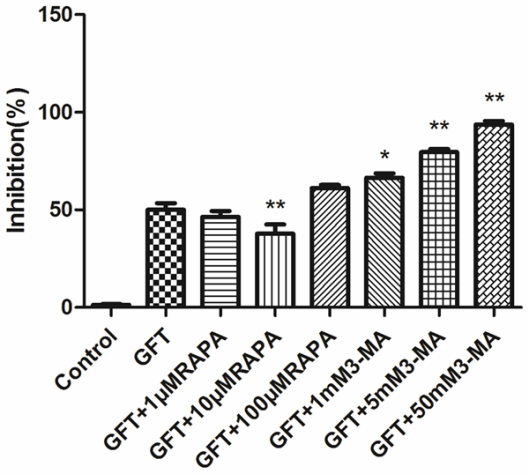

Figure 2. Effect of autophagy in MG63 cells on sensitivity with Gefitinib.

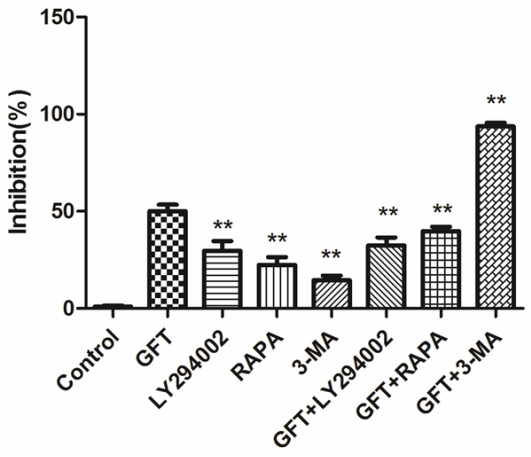

Figure 3. Proliferation Inhibitory effects of different drugs on MG63 cells.

cells number of S phase in MG63. Consequently, MG63 cells were blocked in G1 phase by Gefitinib and the inhibition of PI3K can increase the inhibitory effect of Gefitinib.

Discussion

The mechanism of the occurrence and development of malignant tumors is more complicated and has not been clarified [7]. Surgery combined with adjuvant chemotherapy can significantly improve the prognosis of osteosarcoma [8]. The resistance has a serious impact on the effect of chemotherapy drugs [9]. Therefore, it is important to find effective chemotherapy drugs with high sensitivity in the treatment of cancer [10]. Chemotherapy drugs can induce tumor cells death by autophagy, necrosis, apoptosis and the cells mitotic mutation [11]. What's more, chemotherapy drugs can induce autophagy in tumor cells, in other words, it can induce cell apoptosis and it is also a self-protection mechanism of tumor cells survival in the adverse environment [12, 13]. Adjuvant chemotherapy is of great significance in the treatment of osteosarcoma, and the sensitivity of patients to chemotherapy drugs is the key factor to the prognosis [14].

Autophagy is the main means relatively-conservative to reduce the autologous organelles or proteins in eukaryotic cells, which can regulate and control cell proliferation and function [15]. Studies indicated that the autophagy played an important role in the occurrence and development of malignant tumors [16]. LC3, a specific marker for autophagy, is located on the cell-membrane surface of the pre-autophagy and autophagy [17]. As a major regulator of regulating autophagy, Beclin-1 was also thought to be a marker of autophagy, which inhibited the growth of tumor by enhancing autophagy, and acted as an important autophagy-related suppressor gene [18, 19]. Current study showed that LC3 and Beclin-1 are the important factors in the regulation of autophagy, among which LC-3 is a homologues of yeast and also a marker of Autophagy-membrane [20]. The expression of LC-3 I and LC-3 II protein was decreased and increased respectively when autophagy was enhanced [21]. Beclin-1, a yeast homologues, can promote the formation of autophagy bodies, and the level of its expression reflects the degree of autophagy [22]. LC3 is expressed as LC3-I and LC3-II in the cytoplasm, but LC3-I is transformed into LC3-II when autophagy occurs [23]. LC3-II is an important protein of autophagy, which is thought to be the molecular marker of autophagy, so the ratio of LC3-I/LC3-II reflects the

Table 4. Expression of autophagy-related protein of MG63 cells

	PI3K	Beclin-1	Bcl-2
Control	42.45±2.03	39.84±1.98	36.49±2.38
GFT	16.73±4.62**	56.17±5.01*	23.52±4.66*
LY294002	21.04±6.14*	54.83±4.78*	21.43±4.59*
RAPA	29.38±4.11*	69.81±5.32**	39.16±6.41
3-MA	56.79±3.98	11.27±6.31**	34.23±5.84
GFT + LY294002	9.78±3.75**	34.56±4.16	18.64±4.99*
GFT + RAPA	11.67±5.77**	36.64±3.91	35.22±5.94
GFT + 3-MA	22.03±6.71*	29.85±4.23	11.09±6.44*

Compared with the control group, *P<0.05, **P<0.01.

Table 5. Expression of mRNA of autophagy-related protein in MG63 cells

	PI3K	Beclin-1	Bcl-2
Control	100	100	100
GFT	35.67±6.07**	221.34±18.39*	186.54±12.31*
LY294002	41.82±4.98*	274.68±17.45*	114.35±6.79
RAPA	55.76±5.11*	179.11±10.51	195.47±9.88*
3-MA	127.89±15.77	42.16±7.64*	51.23±5.64*
GFT + LY294002	19.46±6.14**	209.86±12.89*	314.57±15.82**
GFT + RAPA	27.31±7.03**	119.67±5.08	354.61±16.83**
GFT + 3-MA	50.48±4.88*	281.73±19.78*	51.04±6.11*

Compared with the control group, *P<0.05, **P<0.01.

Table 6. GFP-LC3 positive cells were detected by flow cytometry

Groups	GFP-LC3 + proportion (%)
Control	29.46±2.98
GFT	51.36±1.67*
LY294002	45.40±5.45*
RAPA	86.09±5.25**
3-MA	19.20±1.57
GFT + LY294002	73.75±2.94**
GFT + RAPA	93.54±3.02**
GFT + 3-MA	86.54±2.79**

Compared with the control group, *P<0.05, **P<0.01.

degree of cells autophagy [24]. PI3K molecular signaling pathway participates in cell proliferation, apoptosis, recurrence and metastasis. In addition, PI3K/Akt plays an important role in cell proliferation, apoptosis, recurrence and metastasis, as well as extracellular matrix degradation and so on [25-27]. Interfere of PI3K/Akt abnormal activation could promote cell death, which can inhibit tumor cell growth [28]. Therefore, drugs targeted PI3K/Akt have become more and more important in cancer research [29]. Autophagy can remove abnormal organelles, nucleus, and proteins, and protect cells [30]. In the treatment of tumor, autophagy can protect cells, remove the toxic substances from the cells, reduce the toxicity of chemotherapy drugs, as well as reduce the sensitivity to chemotherapy from mental stress [31-33]. At the same time, the activation of autophagy can accelerate the cells-apoptosis [34]. Many studies focus on the regulatory mechanism in cancer development of autophagy, how to improve cell autophagic activity, and the role of autophagy in sensitivity to chemotherapeutic drugs, which are the important direction in the clinical treatment of anti-tumor in the future [35-37].

In this study, results showed that Gefitinib, a chemotherapy drug commonly used in clinical, can induce the autophagy-activity of MG63 cells effectively, inhibit the cell proliferation-activity, and affect the cell cycle and so on. Autophagy plays an important role in the inhibition of cell proliferation with Gefitinib, and the cell proliferation may be inhibited by enhancing autophagy. However, it may reduce the sensitivity to Gefitinib of MG63 cells via promoting autophagy. At the same time, PI3K plays an important role in the process of autophagy. PI3K inhibitors can significantly increase the sensitivity of MG63 cells to Gefitinib, which provide a new method for the clinical treatment. The regulatory function of autophagy will play an important role in tumor therapy, which provides a crucial theoretical basis for the clinical treatment of osteosarcoma by using autophagy regulator combined with chemotherapy drugs.

Disclosure of conflict of interest

None.

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Table 7. Effect of drugs on MG63 cells cycle

Groups	G1	G2	S
Control	54.67±3.45%	22.07±2.33%	29.92±1.87%
GFT	70.24±4.32%**	8.52±1.01%*	13.77±3.64%*
LY294002	72.06±5.02%**	12.07±1.23%	15.33±2.99%
RAPA	85.39±4.95%**	6.53±0.98*	7.92±3.21%**
3-MA	57.66±3.11%	11.90±1.78	35.03±4.18%
GFT + LY294002	88.07±4.56%**	7.06±0.34*	6.26±0.75%**
GFT + RAPA	91.81±5.62%**	6.94±0.42*	3.68±0.43%**
GFT + 3-MA	81.03±3.87%**	4.69±0.33*	16.91±2.44%

Compared with the control group, *P<0.05, **P<0.01.

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