Original Article

Inhibition of autophagy results in a reversal of taxol resistance in nasopharyngeal carcinoma by enhancing taxol-induced caspase-dependent apoptosis

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Abstract: Drug resistance in nasopharyngeal carcinoma remains a major obstacle of clinical therapy. We found that the taxol-resistant cells demonstrated higher basal levels of autophagy than parental cells, which could be inhibited by 3-MA and Beclin-1-siRNA. We further revealed that inhibition of autophagy enhanced taxol-induced caspase-dependent apoptosis, resulted in partial reversal of the acquired taxol resistance in taxol-resistant cells. Our results suggest that the combination of an autophagy inhibitor with taxol may be a promising approach to promote therapeutic efficacy in patients with nasopharyngeal carcinoma.

Keywords: Nasopharyngeal carcinoma, taxol, autophagy, apoptosis, chemoresistance

Introduction

Nasopharyngeal carcinoma (NPC) is one of the most common head and neck cancers in Southern China and Southeast Asia [1]. Most patients with early-stage NPC have been cured successfully due to the improvement of diagnostic and therapeutic approaches. Even chemotherapy is an effective treatment against middle-late stage NPC combined with radiotherapy; the survival rate is still not satisfactory because of the development of acquired drug resistance, which leads to relapse or metastasis and patient death [2]. As taxol is one of the widely used chemotherapeutic agents against NPC, it is important to understand the underlying molecular mechanisms responsible for the development of taxol resistance in NPC cells.

Autophagy is an evolutionarily conserved cellular process that basically consists of degradation and recycling of defective organelles and proteins to maintain cellular homeostasis [3]. The function of autophagy in anticancer treatments is extremely complicated. There are evidences supporting that autophagy is activated as a protective mechanism to mediated the acquired resistance phenotype in some cancers during chemotherapy [4-6]. On the other hand, there are other evidences to prove that autophagy acts as a killing mechanism under chemotherapeutic agents mediate autophagic cell death [7, 8]. Therefore, whether inducers of autophagy will be bad or good for cancer therapies may depend on the context of the drug, the cancer type, or both. Till now, the role of autophagy in taxol treatment for NPC cells is still unclear.

In this work, it was investigated whether or not autophagy plays a role in the treatment with taxol against NPC cells, if this participation could be cytotoxic or cytoprotective and we tried to link such inducible autophagy to the taxol-induced cell apoptosis.

Materials and methods

Cell culture and reagents

The human NPC parental cell lines CNE-1 and HNE-2 were gifts from the Cancer Research
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Institute of Central South University (China). The taxol-resistant cell lines CNE-1/Taxol and HNE-2/Taxol were established previously in our laboratory by exposing parental cells to gradually increasing concentrations of taxol [9]. The cells were cultured in RPMI-1640 medium (Hyclone, Logan, UT, USA) containing 10% fetal bovine serum (Gibco BRL, Gaithersburg, MD, USA) in a humidified atmosphere containing 5% CO₂ at 37°C. Taxol and 3-Methyladenine (3-MA) were obtained from Sigma (Sigma-Aldrich, St. Louis, MO, USA). Rabbit polyclonal antibodies against Beclin-1, LC3, cleaved Caspase 3, GAPDH were obtained from Santa Cruz Biotechnology (Santa Cruz, California, USA).

Transmission electron microscopy

Cells were collected and fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer for 2 h at 4°C, followed by post-fixation in 1% osmium tetroxide for 1 h at room temperature. Samples were dehydrated in graded ethanol solutions, and infiltrated and embedded in Spurr’s low-viscosity medium (TED PELLA, Redding, CA). Ultrathin sections of 50-100 nm were cut in a Leica microtome, double-stained with uranyl acetate and lead acetate, and examined in a Hitachi 7500 transmission electron microscope at an accelerating voltage of 80 kV.

Lysosomal function: Lyso-ID green detection kit

Acidic vesicles (endosomes, lysosomes, late autophagosomes) were detected with Lyso-ID Green detection kit (EnzoLifesciences, Farmingdale, NY, USA). Cells were seeded in 96-well plates (90% confluent). After treatment, 1 μl Lyso-ID green dye and 1 μl Hoechst 33324 from the kit were added to 1 ml cell medium. Subsequently, the cells were incubated in dark for 30 min. For imaging, the cells were washed twice with cold PBS and changed with a live cell imaging solution (Gibco). Images were obtained using a Leica confocal microscope.

Western blot analysis

After treatment, the cells were collected, washed and lysed with ice-cold RIPA lysis buffer (Beyotime Inst. Biotech) with 1 mmol/L of PMSF. Protein concentrations were calculated using BCA assay kits (Beyotime Inst. Biotech). Total cellular protein (20 μg) was subjected to 12% SDS-PAGE and transferred to PVDF mem-

branes (Millipore). The membranes were blocked with 5% defatted milk powder at room temperature for 2 h, followed by immunoblotting with primary antibodies at 4°C overnight and immunoblotting with HRP-conjugated secondary antibody at room temperature for 1 h. Following each step, the membranes were washed three times with PBST for 5 min. Finally, the blots were developed using an enhanced chemiluminescence system (Pierce). GAPDH was used as a loading control.

RNA interference for inhibiting Beclin-1 expression in taxol-resistant cells

The taxol-resistant cell lines CNE-1/Taxol and HNE-2/Taxol were plated at 2×10⁵ cells/well in 6-well plates. The cells were incubated in medium without serum for 1 hour. A mixture of 200 nM siRNA (the human Beclin-1-specific siRNAs were 5’-UGGAAUGGAAUGGAAUUAATT-3’ and 5’-AAGAUUGAAGACACAGGAGGC-3’) and Oligofectamine (Invitrogen, Gaithersburg, Maryland) was added to each well for 6 hours, after which PRMI media containing 30% serum were added. Cells were harvested 48 hours later. Western blot was used to confirm the Beclin-1 protein level. Cells with Beclin-1-siRNA also were assayed for cell growth inhibition by taxol.

Drug treatments and cell growth inhibition assay

Six hundred exponentially growing cells were plated in six-well cell culture plates in a total volume of 2 ml of medium and incubated for 3 h. Then 2 mM of 3-MA or Beclin-1-siRNA were added to the medium, and incubation continued for 6 h, followed by different concentrations of taxol for 24 h. At the end of the incubation, the drug-containing medium was exchanged for fresh medium, and colony formation was monitored. Ten days later, the colonies were fixed with 75% ethanol and stained with methylene blue. After washing, the colonies were counted. The IC₅₀ values of taxol for both NPC cell lines with or without 3-MA/Beclin-1-siRNA were calculated.

Flow cytometry for quantitative analysis of apoptosis

After treatment, 3×10⁵ of cells were collected used for each sample. For apoptosis detection, cells were stained using the Annexin V-FITC
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Apoptosis Detection Kit (BD Biosciences, San Diego, CA) according to the manufacturer’s recommendation. The stained cells were determined by flow cytometry (BD FACS Canto) and analyzed by the FCS Express v2.0 software.

Microarray hybridization and analysis
cDNA microarray was hybridized using HG-U133 Plus 2.0 array (Affymetrix, Santa Clara, CA), and the array slides scanned with a GeneChip Scanner 3000 (Affymetrix, Santa Clara, CA, US); the raw data were normalized by the MAS 5.0 algorithm using the GeneSpring Software version 11.0 (Agilent technologies, Santa Clara, CA, US).

Statistical analysis
Statistical evaluations are expressed as the mean ± S.E.M. Independent-Samples T Test of SPSS was used to make statistical comparisons. P<0.05 was considered as statistically significant.

Results
The chemosensitivity in human NPC parental and taxol-resistant cell lines
The effect of taxol on 2 NPC parental cell lines and 2 taxol-resistant cell lines was examined by exposing them to different concentrations of taxol. The IC_{50} value of taxol was 1.32 ± 0.06 nM for CNE-1, 0.95 ± 0.14 nM for HNE-2, 10.78 ± 0.43 nM for CNE-1/Taxol and 5.59 ± 0.30 nM for HNE-2/Taxol, as determined by the colony formation assays and the growth inhibition curves (Figure 1A, 1B).

The basal levels of autophagy in human NPC parental and taxol-resistant cell lines
Transmission electron microscopy (TEM), one of the most reliable methods for detecting autophagy [10], demonstrated that autophagic vacuoles were observed in both NPC parental cells (CNE-1, HNE-2) and taxol-resistant cells (CNE-1/Taxol, HNE-2/Taxol). But the basal levels of autophagy in taxol-resistant cells are higher than that in parental cells (Figure 1C). These results indicated that autophagy was involved in taxol resistance of NPC cells.

Taxol induces autophagy in human NPC parental and taxol-resistant cell lines
To determine if taxol induced an autophagic response in NPC cells, we used the Lyso-ID green detection kit, which labels acidic compartments within the cytoplasm including autophagosomes. Taxol treatment (5 nM, 12 hours) of CNE-1, HNE-2, CNE-1/Taxol and HNE-2/Taxol cells increased both the number and size of acidic vesicles within the cytoplasm (Figure 2A).

Inhibition of autophagy sensitizes human NPC taxol-resistant cell lines to taxol treatment
To further confirm the involvement of autophagy in taxol resistance, western blot was used to analyze the expression of Beclin-1, LC3-II. Protein levels of Beclin-1, LC3-II were markedly increased by taxol in CNE-1, HNE-2, CNE-1/Taxol and HNE-2/Taxol cells (Figure 2B, 2C).
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Figure 2. Taxol induces autophagy in NPC parental and taxol-resistant cell lines. The 2 NPC parental cell lines and 2 taxol-resistant cell lines were treated with taxol (5 nM) for 12 h. A. LysoID fluorescence measurements detected an increase in acidic functional lysosomes in cells treated with taxol. Lysosomes were stained with the Lyso-ID green dye. The nuclei were stained with Hoechst 33342 (blue). B, C. Western blot was performed to analyze the expression of autophagic protein Beclin-1, LC3-II. B1: CNE-1, B2: CNE-1+Taxol, B3: CNE-1/Taxol, B4: CNE-1/Taxol+Taxol; C1: HNE-2, C2: HNE-2+Taxol, C3: HNE-2/Taxol, C4: HNE-2/Taxol+Taxol. Bar graphs indicated relative expression of Beclin-1, LC3-II/LC3-I normalized to GAPDH. All quantitative data shown represent the means ± SEM of at least 3 independent experiments. *P<0.05, **P<0.01.
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To further assess the role of autophagy in taxol resistance, Autophagy inhibitor 3-MA (2 mM, 6 hours) was pretreated in CNE-1/Taxol and HNE-2/Taxol cells, followed by different concentrations of taxol, and colony formation was employed to determine the cell growth inhibition rate. The IC\textsubscript{50} value of taxol was 6.25 ± 1.0 nM for CNE-1/Taxol with 3-MA, 2.19 ± 0.19 nM for HNE-2/Taxol with 3-MA. These results indicated that the growth inhibition was significantly higher in 3-MA pretreated NPC taxol-resistant cells (P < 0.01) and that NPC taxol-resistant cells were more sensitive to taxol treatment while the cells were pretreated with 3-MA (Figure 3A).

To further confirm the role of autophagy described above, siRNA experiments were performed in taxol-resistant NPC cells (CNE-1/Taxol, HNE-2/Taxol). As shown in Figure 3C, 3D, the siRNA targeting Beclin-1 decreased its expression to 29.35% in CNE-1/Taxol and 20.03% in HNE-2/Taxol compared with the control siRNA, demonstrating the efficacy of this siRNA in the knockdown of Beclin-1 expression. The sensitivity of taxol on CNE-1/Taxol and HNE-2/Taxol with Beclin-1-siRNA were significantly higher than CNE-1/Taxol and HNE-2/Taxol with a scrambled siRNA. The IC\textsubscript{50} value of taxol was 5.69 ± 1.23 nM for CNE-1/Taxol and 3.08 ± 0.67 nM after transfection with Beclin-1-siRNA (Figure 3B).

**Autophagy inhibition enhances Taxol-induced apoptosis in human NPC parental and taxol-resistant cells**

To determine whether the inhibition of autophagy influenced Taxol-induced apoptosis in CNE-1 and CNE-1/Taxol cells, apoptosis were detected by Flow cytometry with or without 3-MA before taxol treatment. The results indicated that 3-MA could significantly increase taxol-induced apoptosis in both cells (Figure 4A-C).

**Different expression of Caspase family genes in cDNA microarray between human NPC parental and taxol-resistant cells**

Caspase is an important protein family for the procedure of apoptosis. To explore the different
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Figure 4. Autophagy inhibition enhances Taxol-induced apoptosis and increases cleaved Caspase 3 protein level in CNE-1 and CNE-1/Taxol cells. Cells were treated with taxol (5 nM) for 24 h with or without 3-MA (2 mM pretreated for 6 h). A. Flow cytometry was used to quantify the apoptotic rate. Cells were stained with PI and Annexin V-FITC. The positive-stained cells were counted using FACSscan. B, C. Quantitative analysis of apoptosis rate. D-F. Western blot was performed to analyze the expression of cleaved Caspase 3. D1, E1: Blank, D2, E2: 3-MA, D3, E3: Taxol, D4, E4: 3-MA+Taxol. Bar graphs indicated relative expression of cleaved Caspase 3 normalized to GAPDH. All quantitative data shown represent the means ± SEM of at least 3 independent experiments. *P<0.05, **P<0.01.
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Table 1. Different expression of Caspase family genes between human NPC parental and taxol-resistant cells (Fold change)

<table>
<thead>
<tr>
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<tr>
<td>Caspase 10</td>
<td>0.68</td>
<td>0.38</td>
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<tr>
<td>Caspase 9</td>
<td>0.46</td>
<td>1.37</td>
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<td>Caspase 8</td>
<td>0.18</td>
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<td>Caspase 7</td>
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<td>1.73</td>
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<td>Caspase 6</td>
<td>2.19</td>
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<td>Caspase 4</td>
<td>0.34</td>
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<tr>
<td>Caspase 3</td>
<td>0.17</td>
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<tr>
<td>Caspase 2</td>
<td>0.78</td>
<td>0.32</td>
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<td>Caspase 1</td>
<td>0.66</td>
<td>0.86</td>
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expression of Caspase protein between NPC parental and taxol-resistant cells, global gene expression was analyzed by comparing the transcriptome profiles of the NPC parental (CNE-1, HNE-2) and taxol-resistant (CNE-1/Taxol, HNE-2/Taxol) cells. The microarray analysis showed that the Caspase 10, Caspase 8, Caspase 4, Caspase 3, Caspase 2 and Caspase 1 were downregulated in both taxol-resistant cells compared with that in parental cells, in which the expression levels of Caspase 3 and Caspase 8 decreased most obviously; Caspase 7 and Caspase 6 were slightly upregulated; Caspase 9 was downregulated in the CNE-1/Taxol, but upregulated in HNE-2/Taxol (Table 1).

Autophagy inhibition increases cleaved Caspase-3 protein level

To further determine the contributions of apoptosis and autophagy to chemosensitivity of human NPC cells, cleaved Caspase3, as the specific hallmark of apoptosis, was detected in CNE-1 and CNE-1/Taxol cells with or without 3-MA before taxol treatment. We found that 3-MA together with taxol induced Caspase 3 cleavage in both CNE-1 and CNE-1/Taxol cells. These results suggested that inhibition of autophagy could contribute to taxol-induced caspase-dependent apoptosis in human NPC cells (Figure 4D-F).

Discussion

As we all known, nasopharyngeal carcinoma has a particularly high incidence in Southern China and South-East Asia, with early neck lymph nodes metastasis and a high occurrence rate [11, 12]. For patients with advanced NPC, taxol treatment is offered as one of the most efficacious auxiliary treatment, but the overall efficacy is still far from satisfactory, due to the acquired drug resistance, even great progress has been made in the treatment strategy of NPC in the past decade [13, 14]. Considering the widespread application of taxol in the treatment of NPC, it is urgent to find therapeutic targets associated with taxol resistance, so as to increase its clinical efficiency.

Previous studies have focused on discovering the mechanisms underlying drug resistance, including reduced drug absorption, increased efflux pump and so on [15, 16]. To date, these mechanisms are not entirely understood. Autophagy mechanisms have captured increasing attention, and autophagy-related genes have become viable prospects for potential targets in cancer treatment [17, 18]. Nevertheless, the role of autophagy in drug resistance remains controversial. As in taxol treatment, autophagy has been demonstrated to protect cancer cells from death [19-22]; conversely, autophagy was reported to enhance the sensitivity of taxol [23, 24]. The present study, to our best knowledge, is the first to explore the role of autophagy in taxol treatment for NPC cell lines. Our results showed that the NPC taxol-resistant cell lines expressed greater basal levels of autophagy than parental cell lines and inhibition of autophagy induced by taxol could partially reverse the taxol-resistant phenotype. The results described above indicated that autophagy may play a pro-survival role in NPC cell lines against taxol treatment.

Maiuri MC et al. [25] demonstrated that apoptosis and autophagy coexist in the same cells, and mutually influence the execution of the other. To investigate the relationship between autophagy and apoptosis in NPC cells under taxol treatment, apoptosis was detected by flow cytometry, it was found that taxol together with 3-MA significantly induced apoptosis. To further explore the potential mechanisms of autophagy inhibition on taxol-induced apoptosis. The cDNA microarray analysis revealed that most of the caspase family genes were downregulated in taxol-resistant cell lines, and western blot showed that 3-MA together with taxol significantly increased the expression levels of...
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cleaved Caspase 3, indicating that autophagy inhibitors may be used to augment taxol-induced caspase-dependent apoptosis in NPC cells.

In conclusion, taxol-induced autophagy may protect NPC cells from caspase-dependent apoptotic death, and inhibition of autophagy can increase the taxol sensitivity. The combination of an autophagy inhibitor with taxol may be a promising approach to promote therapeutic efficacy in patients with nasopharyngeal carcinoma.

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Disclosure of conflict of interest

None.

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