Original Article

3-MA sensitize oral squamous carcinoma cells to cisplatin by antagonizing cisplatin-initiated autophagy

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Abstract: Cisplatin (DDP) is one of the most effective chemotherapeutic agents commonly used for several malignancies including oral squamous cell carcinoma cells (OSCC). DDP-based combinatorial treatments can eventually result in tumor resistance response. Autophagy represents a survival mechanism and has been demonstrated to promote drug resistance in many cancers. In this study, cell death and apoptosis induced by DDP in OSCC cell line (CAL-27) was revealed using MTT assay, flow cytometry, and active-caspase-3 immunoblotting. The autophagy activation induced by DDP treatment was measured by green fluorescent protein-light chain 3 plasmid transfection (GFP-LC3), LC3 and p62 immunoblotting. 3-methyladenine (3-MA) inhibited autophagy and significantly enhanced DDP-induced cell death and apoptosis. In conclusion, our results indicate inhibition of autophagy by 3-MA sensitizes OSCC to DDP treatment. Correspondingly, autophagy inhibition could have promising therapeutic potential as DDP-based adjuvant chemotherapy against OSCC.

Keywords: Oral squamous cell carcinoma, DDP, autophagy, apoptosis, 3-MA

Introduction

Oral squamous cell carcinoma (OSCC) is a growing global public health problem for which standard therapeutic strategies have failed to contribute significantly to improve the 5-year survival rates that have remained around 50% over the past three decades [1, 2]. Although chemotherapy is still widely used as a non-surgical treatment for OSCC, drug-resistant has becoming a huge impediment to chemotherapy [3].

As a first line anti-tumor drug, cisplatin (DDP) is one of the most effective chemotherapeutic agents commonly used for several malignancies including OSCC [4], however long term of chemotherapy with DDP can eventually result in resistance response and reduced sensitivity of cancer cells to apoptosis, which is a major cause of uncontrolled progression or recurrence of cancers [5, 6]. Therefore, new therapeutic strategies and/or new adjuvant drugs still need to be explored to antagonize DDP-induced drug resistance. Bcl-2 up-regulation has been demonstrated to be implicated in the resistance to cisplatin, and it is suggested that Bcl-2 silencing could be a promising strategy for reversing DDP drug-resistance [7, 8]. Autophagy has also been proved to result in drug resistance [9-12].

Autophagy is an evolutionarily conserved process involving lysosomal degradation of intracellular components, damaged organelles, misfolded proteins, and toxic aggregates, reducing oxidative stress and protecting cells from damage and maintaining intracellular stability [13]. This ability makes autophagy a good candidate for a survival mechanism in response to several stresses, including the tumor cell transformation [14-16]. In particular, recent studies suggested that autophagy functions as a pro-death mechanism within different tumor contexts [17]. It is widely reported that autophagy represents a survival mechanism and has been demonstrated to promote antitumor drug resistance in many types of cancers [11, 12]. The autophagy is consist of four phases: initiation, nucleation, maturation and merging with lysosomes.
About 30 autophagy-associated genes/proteins in conjunctions with several signal pathways are involved in regulating autophagy [18]. Adenosine monophosphate kinase (AMPK) can regulate the rate of autophagy vesSEL formation in the initiation phase [19]. Vacuolar protein sorting 34 (Vps34), a class 3 phosphatidylinositol-3-kinase (PIK3) controls the nucleation phase through binding to a complex consisting of Atg6/Beclin-1, p150, and Atg14L [20]. Atg8/microtubule-associated protein 1 light chain 3 (LC3) binding to the membrane is involved in the transport and matura-tion of the autophagosome. LC3-II is also involved in trafficking proteins into the proteosome and facilitates the autophagic degrada-tion of ubiquitinated protein aggregates by binding to adaptor protein p62/sequestosome-1 (SQSTM1). Inhibition of Vps34 with 3-methyl adenine (3-MA) is commonly used to inhibit autophagy by inhibiting Vpa34.

Cellular stress triggers a fascinating decision-making process in cells; they can either attempt to survive until the stress is resolved through the activation of cytoprotective pathways, such as autophagy, or can commit suicide by apoptosis in order to prevent further damage to surrounding healthy cells [21, 22]. Although autophagy and apoptosis constitute distinct cellular processes with often opposing outcomes, their signalling pathways are extensively inter-connected through various mechanisms of crosstalk. The physiological relevance of the autophagy-apoptosis crosstalk is not well understood, but it is presumed to facilitate a controlled and well-balanced cellular response to a given stress signal [21, 23].

Autophagy, as a kind of resistance to DDP-induced apoptosis, may be a major obstacle to successful treatment of OSCC. In our study, we investigated the role of autophagy inhibitor 3-methyl adenine (an inhibitor of class III phosphatidylinositol-3 kinase; 3-MA) in the apoptosis induced by DDP on CAL-27 cells and detected the molecular changes of autophagy and apoptosis.

Materials and methods

Reagents and antibodies

Anti-LC3B antibody, EBSS and 3-MA were purchased from Sigma Aldrich (St. Louis, MO, USA). Anti p62, β-actin, Beclin1, AMPK, p-AMPK, active-caspase-3 antibodies were purchased from Abcam.

Cell culture and treatments

Human OSCC cell line CAL-27 was bought from American Tissue Culture Collection (ATCC). Cells were grown in DMEM (Gibco) with 10% fetal bovine serum (FBS; Gibco), 100 μg/ml penicillin, and 100 μg/ml streptomycin in a humidified 37°C incubator with 5% CO₂. Cells in a mid-log phase were used in the experiments. The cells were divided into four groups: control, 3-MA, DDP, and DDP plus 3-MA groups. After 24 h, the culture medium were replaced with fresh medium containing none (control group), 3-MA (3-MA group), DDP (DDP group), and the combination (DDP plus 3-MA group). CAL-27 cells were treated with 12 μg/ml DDP for 24 h and/or 10 mm/l 3-MA for 1 h and then deprived of nutrition for 24 h.

MTT assay

Cells were seeded in 96-well plates at a density of 1 × 10⁴ cells, 100 μl per well. MTT assays (Beyotime, China) were performed by adding 20 μl MTT bromide (5 mg/ml in PBS) to the cells for 4 h. Then, 150 μl of dimethyl sulfoxide (Beijing Chemical Industry Limited Company, China) was added to each well. After shaking for 10 min, Optical density was measured at 490 nm with background subtraction at 630 nm using a plate microreader (TECAN SPETRA).

Western blot analysis

To extract the total protein of cells, treated cells were rinsed with ice-cold PBS, and lysed in lysis buffer (10 mM RAPA and 0.1 mM PMSF). The extracts were centrifuged at 20,000 × g at 4°C for 30 min, and the clear supernatants containing total protein were collected. After preparation of protein, the protein concentration was measured using the BCA protein assay, an equal amount of protein was subjected to SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes. After blocking with 5% nonfat milk, the membranes were probed with designated first and second antibodies, developed with the enhanced chemiluminescence method and visualized with LAS-4000. The value of band density was quantified by the quantity one image processing program.

Analysis of apoptosis by FCM of AV/PI dual staining

In this study, apoptosis was detected using AnnexinV-FITC (fluorescein isothiocyanate)/prop...
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According to the manufacturer's recommendations (Keygene), then, the samples were analyzed by FACScan flow cytometer (Becton Dickinson) to quantify the apoptotic rate. Apoptotic rate was determined as the percentage of D1+D2. All experiments reported in this study were performed in triplicate.

Green fluorescent protein-light chain 3 plasmid transfection

CAL-27 cells were grown in 6-well plate at a density of 106 cells/dish with 2 ml medium per well, and after 24 hours the cells were transfected with green fluorescent protein-light chain 3 (GFP-LC3) plasmid (Shanghai Genechem Co.Ltd, China) by use of a mixture of Lipofectamine 2000 (invitrogen) and GFP-LC3 plasmid in Opti-MEM medium (Life Technologies) at a ratio of 4 μg plasmid/10 μl Lipofectamine 2000 per well. After 5 hours of incubation, cells were placed in regular complete media and cultured for one day. The culture medium were replaced with fresh medium containing none (control group), 3-MA (3-MA group), DDP (DDP group), and the combination (DDP plus 3-MA group). CAL-27 cells were treated with 12 μg/ml DDP for 24 h and/or 10 mM/1 3-MA for 1 h and then deprived of nutrition for 24 h. The wells were examined on a Nikon fluorescent microscope and color print pictures were taken. The numbers of LC3 fluorescent punctate structures per cell was counted by an observer blinded to experimental condition. LC3 is a microtubule-associated protein that is a critical component of the autophagosome. The use of the GFP-tagged LC3 plasmid has become an effective marker for the autophagosome.

Statistical analysis

Statistical analysis was calculated using One-way ANOVA by SPSS program version 16.0. Differences were considered significant when the P value was less than 0.05.

Results

3-MA facilitated DDP induced cell death and apoptosis in CAL-27 cells

The IC50 of DDP against CAL-27 cells was 13.45±0.87 μg/ml for 24 h treatment, which was examined via MTT assay (data not shown?).
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Therefore, we used 12 μg/ml DDP in CAL-27 cells for 24 h in the subsequent experiment.

To examine whether 3-MA could facilitated cell death and apoptosis induced by DDP. We measured the cell death rate or apoptosis rate after CAL-27 cells treated with DDP or 3-MA or a combination of DDP and 3-MA. 3-MA could significantly increase DDP-induced cell death rate although 3-MA itself could not induce cell death (Figure 1A). We also detected the apoptosis rate and the expression of active-caspase-3. The apoptosis rate of the CAL-27 cells significantly increased by combinational treatment with DDP and 3-MA compared with the DDP alone (Figure 1B). Furthermore, the expression of active-caspase-3 induced by DDP was also significantly increased by 3-MA (Figure 1C).

3-MA inhibited DDP-induced autophagy in CAL-27 cells

Since DDP could induce chemoresistance through autophagy and 3-MA is an autophagy inhibitor, we hypothesized that autophagy may be responsible for 3-MA facilitating DDP-induced cell apoptosis. DDP increased the expression of p62 and decreased the ratio of LC3-II/LC3-I; while the increased p62 and decreased ratio of LC3-II/LC3-I was antagonized by 3-MA (Figure 2A). In order to detected whether 3-MA antagonized DDP-induced autophagy in CAL-27 cells, we overexpressed GFP-LC3 into CAL-27 cells. The number of punctate GFP-LC3 structures per cell is usually an accurate measure of autophagosome number. After the CAL-27 cells treated with DDP, the number of punctate GFP-LC3 structures per cell significantly increased (Figure 2B), indicating that DDP could initiate the autophagy of CAL-27 cells; while the DDP-induced punctuate GFP-LC3 was...
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reversed by 3-MA treatment (Figure 2B), suggesting that 3-MA could antagonize DDP-induced autophagy.

3-MA antagonized DDP induced Bcl-2 upregulation and AMPK activation

Since it is strongly suggested that there is crosstalk between DDP-induced apoptosis and autophagy, we checked expression of the bcl-2 which is reportedly implicated in both apoptosis and autophagy. By immunoblot analysis, we found that DDP increased the expression of bcl-2 in CAL-27 cells (Figure 3). By immunoblotting, we also found that with DDP treatment, expression and phosphorylation of AMP-activated protein kinase (AMPK) increased significantly in CAL-27 cells (Figure 3). It is strongly suggested that 3-MA antagonized DDP-induced autophagy and correspondingly enhanced DDP-induced cell apoptosis through inhibition of Bcl-2 and activation of AMPK.

Discussions

In this study we proved that inhibition of autophagy could sensitize OSCC cells to DDP. First 3-MA could facilitate DDP-induced cell apoptosis and cell death in CAL-27 cells. Second, 3-MA could inhibited DDP-induced autophagy in CAL-27 cells. Third, 3-MA could antagonize DDP induced Bcl-2 upregulation and AMPK activation.

Autophagy is a cellular homeostatic mechanism that involves protein and organelle degradation, and has a number of connections to human physiology and diseases [24, 25]. In different type of cancers autophagy in tumor parenchyma may acts as either a tumor-promoting role or a tumor-inhibiting role [24]. Stimulation of autophagy promotes the survival of tumor cells under stressful metabolic and environmental conditions [26, 27]. Autophagy is also implicated in promoting chemoresistance of cancer cells to attenuate efficacy of chemotherapy [14, 28]. In our study, DDP-induced OSCC cell autophagy was examined using the makers of autophagy, LC3, p62, and GFP-LC3. We observed that DDP significantly increased the level of autophagy compared with control groups. 3-MA is a well known autophagy inhibitor that inhibits the activity of type III PI3K (a kinase that is essential for vesi-

![Figure 3. Bcl-2 and AMPK may participate in DDP-induced autophagy response. Immunoblotting for Bcl-2, AMPK, p-AMPK were performed. Data are representative of three independent experiments (**P<0.01).](image-url)
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cle nucleation, the first phase of autophago-

some formation) [29, 30]. We also found DDP-
 induced OSCC cell autophagy was inhibited by
3-MA. 3-MA has also been verified to enhance
the effect of cancer therapy in breast, glioma,
and prostate cancer [31-33]. In our study, DDP-
induced OSCC cell death by enhancing apopto-
sis was augmented by 3-MA. Therefore, we pre-
sume inhibition of autophagy inhibition could
sensitize OSCC to DDP. This is a new proof
about inhibition of autophagy inducing chemother-
apy, as already proved in other cancers,
such as breast cancer, multiple myeloma, and
prostate cancer [31, 33, 34].

Autophagy and apoptosis triggered by several
common upstream signals play important roles
in the development, cellular homeostasis and,
especially, oncogenesis of mammals [35, 36].
Our study showed DDP-induced OSCC cell
apoptosis was augmented by 3-MA, suggesting
there may be crosstalk between autophagy and
apoptosis in OSCC cells. Bcl-2 and Bcl-xL, the
well-characterized apoptosis guards, also ap-
pear to be important factors in autophagy, as
they could inhibit Beclin 1-mediated autophagy
by binding to Beclin 1 [35]. We observed DDP
increased the expression of Bcl-2 in CAL-27
cells compared with DDP plus 3-MA groups.
Therefore, Bcl-2 may represent a molecular link
between autophagy and apoptosis in OSCC
cells.

The AMPK is a cellular energy sensor and signal
transducer that is regulated by a wide ar-
ray of metabolic stresses, and serves as a sig-
aling for regulating cellular metabolism, ener-
gy homeostasis, and cell growth [19, 37]. The
dysregulation of AMPK pathway may contribute
to the development of metabolic disorders
such as obesity, type 2 diabetes, and cancer
[19, 38]. AMPK has recently been connected to
cellular processes such as autophagy [39, 40].
We found that with DDP treatment, expression
and phosphorylation of AMPK increased
significantly compared with control groups or
DDP plus 3-MA groups in CAL-27 cells. The
underlying mechanism of AMPK participating in
DDP-induced autophagy response needs fur-
ter detection. Our results suggest that AMPK
may be a possible therapeutic target in OSCC
cells.

In conclusion, our results indicate DDP-induc-
ed autophagy may be a possible mechanism
for chemoresistant in human OSCC. Correspond-
ingly, autophagy inhibition could have promising therapeutic potential as DDP-based
adjuvant chemotherapy against OSCC. Next, it
is necessary to assess combination treatment
of DDP and 3-MA as a useful approach to anti-
cancer treatment in vivo experiments.

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Disclosure of conflict of interest

None.

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