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Pirarubicin induces an autophagic cytoprotective response through suppression of the mammalian target of rapamycin signaling pathway in human bladder cancer cells

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ABSTRACT

Pirarubicin is widely used in intravesical chemotherapy for bladder cancer, but its efficacy is limited due to drug resistance; the mechanism has not been well studied. Emerging evidence shows that autophagy can be a novel target for cancer therapy. This study aimed to investigate the role of autophagy in pirarubicin-treated bladder cancer cells. Bladder cancer cells EJ and J82 were treated with pirarubicin, siRNA, 3-methyladenine or hydroxychloroquine. Cell proliferation and apoptosis were tested by cell survival assay and flow cytometric analysis, respectively. Autophagy was evaluated by immunoblotting before and after the treatments. The phosphorylated mammalian target of rapamycin, serine/threonine kinase p70 S6 kinase, and eukaryotic translation initiation factor 4E binding protein 1 were also investigated by immunoblotting. We found that pirarubicin could induce autophagy in bladder cancer cells. Inhibition of autophagy by 3-methyladenine, hydroxychloroquine or knockdown of autophagy related gene 3 significantly increased apoptosis in pirarubicin-treated bladder cancer cells. Pirarubicin-induced autophagy was mediated via the mTOR/p70S6K/4E-BP1 signaling pathway. In conclusion, autophagy induced by pirarubicin plays a cytoprotective role in bladder cancer cells, suggesting that inhibition of autophagy may improve efficacy over traditional pirarubicin chemotherapy in bladder cancer patients.

1. Introduction

Bladder cancer is the most common malignancy of the urinary tract in China. 75–85% of bladder cancers are grouped as non-muscle invasive bladder tumors. Intravesical chemotherapy is the standard adjuvant treatment of non-muscle invasive bladder cancer [1,2]. Pirarubicin (also known as THP) has been widely used in intravesical chemotherapy and decreased the tumor recurrence rate significantly, but 10–30% of patients still relapse and metastasize within 5 years [3–5]. Previous studies have demonstrated that pirarubicin can interfere with transcription and prevent DNA synthesis [1]. However, detailed effects and mechanisms of pirarubicin on bladder cancer cells have not been fully explored.

Autophagy is a dynamic catabolic process in which cytoplasmic proteins and damaged organelles are delivered to lysosomes for degradation [6]. Emerging evidence has shown that autophagy can be a novel target for cancer therapy, but whether autophagy causes survival or death in cancer cells is controversial [7–9]. Although several anticancer drugs have been proved to mediate crosstalk of autophagy and apoptosis, the role of pirarubicin has been little studied.

In this work, we determined the effect of autophagy induced by pirarubicin in bladder cancer cells. We demonstrate that pirarubicin can induce autophagy in bladder cancer cells, a cytoprotective response against pirarubicin-induced cytotoxic effects.

Abbreviations: ATG, autophagy-related gene; LC3B, microtubule-associated protein light chain 3B; 3-MA, 3-methyladenine; HCQ, hydroxychloroquine; mTOR, mammalian target of rapamycin; p70S6K, serine/threonine kinase p70 S6 kinase; 4E-BP1, eukaryotic translation initiation factor 4E binding protein 1; MAPK, mitogen-activated protein kinase; JNK, Jun N-terminal kinases; STAT, signal transducer and activator of transcription.

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Furthermore, we found that the mTOR/p70S6K/4E-BP1 signaling pathway played a major role in the regulation of autophagy in pirarubicin-treated bladder cancer cells.

2. Materials and methods

2.1. Cell lines and Cell culture

Human bladder cancer cell lines T24, J82, UM-UC-3, 5637 and EJ were obtained from the American Type Culture Collection (ATCC) and cultured in RPMI-1640 (Gibco), which contained 10% fetal bovine serum (Gibco), under a 5% CO_2 atmosphere. Cells were treated with pirarubicin (Sigma, P8624), 3-methyladenine (Sigma, M9281) or hydroxychloroquine (Sigma, H0915). The incubation times and concentrations of reagents are described in the figure legends.

2.2. siRNA transfection

siRNA or negative control oligonucleotides (RiboBio) were transfected into cells with Lipofectamine RNAi MAX Transfection Reagent (Invitrogen, 13378-075) according to the manufacturer's instructions. After 6 h of incubation with siRNA (50 nM), the medium containing siRNA-Lipofectamine RNAi MAX complex was replaced with fresh growth medium and the cells were cultured for further experiments.

2.3. Western blot assay

Protein extracts from EJ and J82 cells were separated by 6%–15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and were electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore Corporation). Then the membrane was incubated with primary antibodies against LC3B (2775, Cell Signaling Technology), ATG3 (3415), ATG5 (8540), Phospho-mTOR (Ser2448) (5536), Phospho-p70S6 Kinase (Ser371) (9280) or Phospho-4E-BP1 (Thr37/46) (2855), followed by horseradish peroxidase (HRP)-labeled secondary antibodies. Detection used chemiluminescence (Millipore Corporation). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a protein loading control. The intensity of protein fragments was quantified with Quantity-One software (Bio-Rad Laboratories).

2.4. Cell survival assay

[3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] (MTS) (Promega, G3580) was used to analyze cell survival according to the manufacturer's instructions. Briefly, cells were plated in 96-well plates in triplicate at 2×10^3 cells per well and cultured in growth medium. Then cells were treated with pirarubicin at different concentrations (2.5 μg/ml, 5 μg/ml, 10 μg/ml) for 24 h. MTS reagent (5 mg/ml) was added and incubated at 37 °C for 4 h. The absorbance was monitored at 490 nm using a microplate reader (Multiscan MK3, Thermo Fisher Scientific).

2.5. Apoptosis assays

Cells were treated with pirarubicin, siRNA, 3-methyladenine or hydroxychloroquine as indicated. Then the cells were harvested and resuspended in 200 μl of binding buffer. After the addition of 5 μl Annexin V conjugate (BD Bioscience) for 10 min of incubation, the samples were resuspended in 200 μl binding buffer and 5 μl propidium iodide (PI). Finally, samples were analyzed using a MACS Quant® Analyzer (Miltenyi Biotec Inc). Annexin V-positive cells were designated as apoptotic cells. The data were analyzed using FlowJo software (Tree Star Inc).

2.6. Statistical analysis

Statistical analysis was performed using SPSS software 17.0. Differences were assessed by Student's t-test. A p value of <0.05 was regarded as statistically significant.

3. Results

3.1. Pirarubicin induced autophagy in bladder cancer cells

To determine the cytotoxicity of pirarubicin in bladder cancer cells, we treated five bladder cancer cell lines, T24, EJ, 5637, J82 and UM-UC-3, with pirarubicin at different concentrations (2.5 μg/ml, 5 μg/ml, 10 μg/ml). Pirarubicin significantly increased the death of all five cell lines in a dose-dependent manner (Fig. 1A).

To investigate whether pirarubicin could induce autophagy, we detected microtubule-associated protein light chain 3B (LC3B) conversion levels (LCB3-I to LC3B-II) by Western blot analysis. LC3B is widely used to investigate autophagy, and the ratio of LC3B-II/LCB3-I positively correlates with the number of autophagosomes [10]. After treatment with 5 μg/ml pirarubicin for different times (12 h, 24 h, 48 h), or with pirarubicin at different concentrations (2.5 μg/ml, 5 μg/ml, 10 μg/ml) for 24 h, LC3B-II levels increased in a time-dependent (Fig. 1B) and dose-dependent manner (Fig. 1C). Autophagy-related genes (ATGs) are critical regulators of autophagy and we found that ATG3, but not ATG5, increased after treatment of EJ and J82 cells with pirarubicin (Fig. 1B and C). These data suggested that pirarubicin could induce autophagy and selectively induce ATG expression in bladder cancer cells.

To examine autophagic flux induced by pirarubicin, both EJ and J82 cells were transfected with si*ATG3* for 48 h. Then the cells were treated with pirarubicin (5 μg/ml) for 24 h. Treatment with siRNA decreased pirarubicin-induced levels of LC3B-II in EJ and J82 cells (Fig. 2A and B). Pharmacological inhibition of autophagy has been widely used in autophagy research. We found that autophagy inhibitor 3-methyladenine (3-MA), which blocks the initiation stage of autophagy, decreased pirarubicin-induced expression of LC3B-II in EJ and J82 cells (Fig. 2C). After co-incubation of cells with pirarubicin (5 μg/ml) and hydroxychloroquine (HCQ, 3 μg/ml) whch block the late stage of autophagy, we found a remarkable accumulation of LC3B-II in both cell lines (Fig. 2D). In addition to LC3B, p62/SQSTM1, as a degraded substrate, has been used as an autophagy marker. Evidence confirms that inhibition of autophagy correlates with increased levels of p62 [11,12]. In this study, we found that p62 levels increased on inhibition of autophagy by 3-methyladenine or HCQ, or on knockdown of *ATG3* (Fig. 2). Taken together, these findings demonstrate that pirarubicin can induce autophagic flux, which confirmed that pirarubicin induced autophagy in bladder cancer cells.

3.2. Inhibition of autophagy enhanced pirarubicin-induced apoptosis in bladder cancer cells

There role of autophagy in cell survival or death is highly controversial. In certain circumstances, inhibition of autophagy can increase apoptosis, and upregulation of autophagy protects against the onset of apoptosis. By contrast, autophagy has been found to promote cell death through autophagic cell death after treatment with specific anticancer agents [13–15]. To investigate the role of autophagy in pirarubicin-induced apoptosis, we suppressed

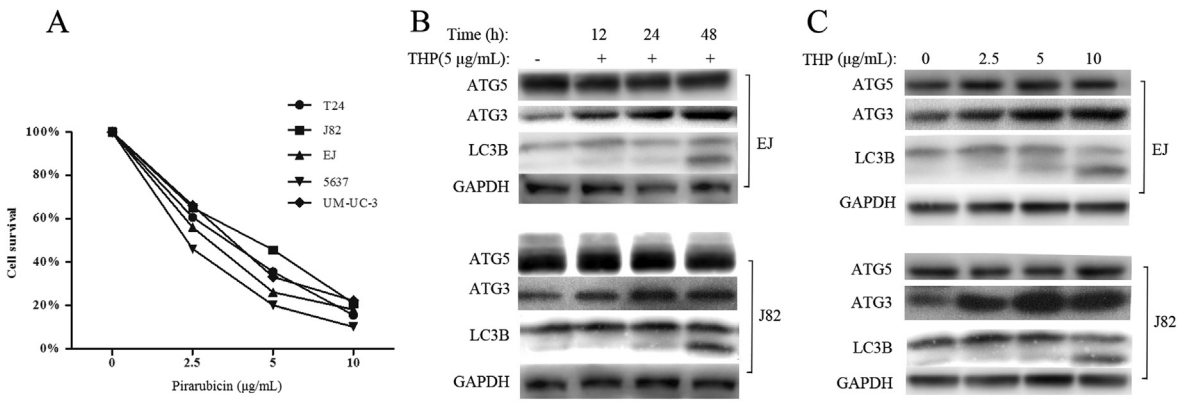

Fig. 1. Pirarubicin induces autophagy in human bladder cancer cells. (A) T24, J82, UM-UC-3, 5637 and EJ cells were treated with pirarubicin (also known as THP, 2.5 μg/ml, 5 μg/ml or 10 μg/ml) for 24 h and analyzed by MTS assay. (B) EJ and J82 cells were treated with 5 μg/ml pirarubicin for 12 h, 24 h or 48 h and analyzed by Western blot using antibodies against ATG3, ATG5, GAPDH and LC3B. (C) EJ and J82 cells were treated with pirarubicin (2.5 μg/ml, 5 μg/ml or 10 μg/ml) for 24 h and analyzed by Western blot using antibodies against ATG3, ATG5, LC3B and GAPDH. Results shown are representative of at least three independent experiments.

autophagy by *ATG3* knockdown in EJ and J82 cells. Pirarubicin-induced apoptosis was significantly increased by si*ATG3* (Fig. 3A–D) compared to the scramble siRNA-treated control group. We further examined the apoptotic effect of pirarubicin co-incubated with 3-MA or HCQ. Fig. 3C and D shows that 3-MA (0.75 mg/ml) or HCQ (3 μg/ml) effectively enhanced pirarubicin-induced apoptosis in EJ and J82 cells. Additionally, compared with the pirarubicin-treated group, co-incubation with pirarubicin and si*ATG3*, 3-MA or HCQ significantly decreased the survival of EJ and J82 cells (Fig. 3E). Collectively, these results show that autophagy

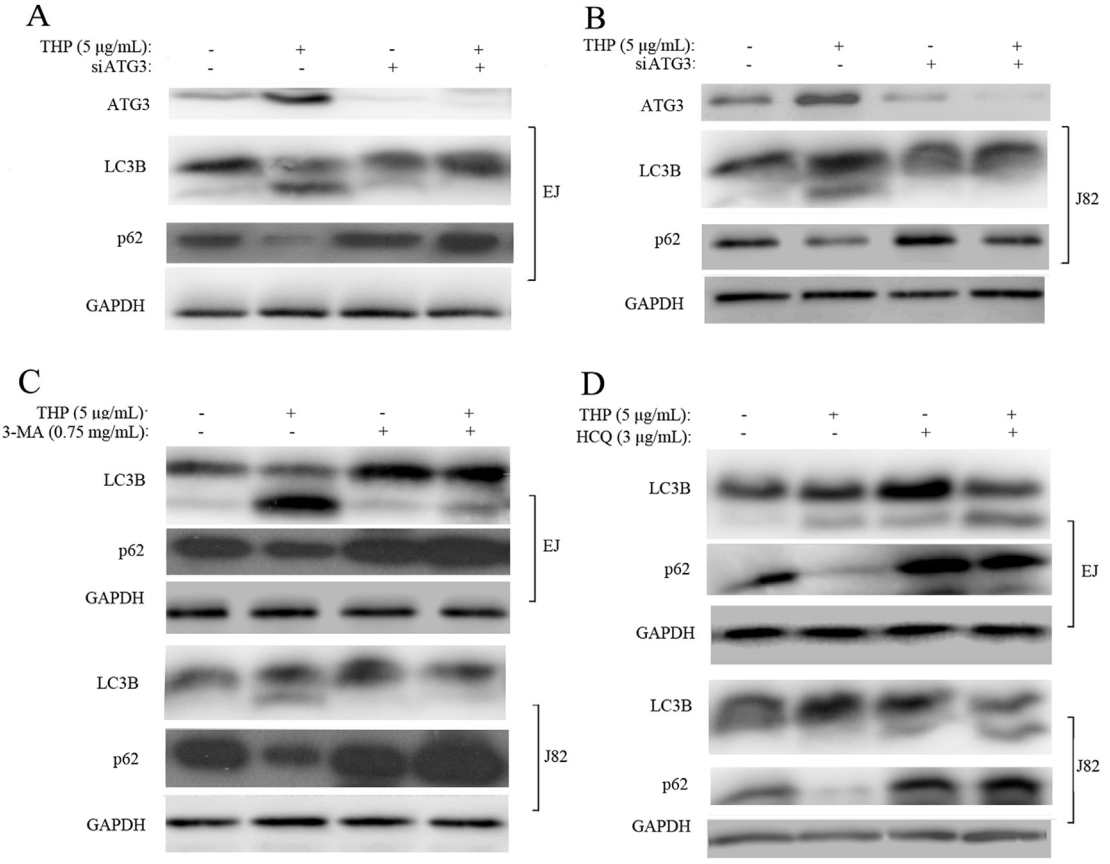

Fig. 2. Pirarubicin induces autophagic flux in human bladder cancer cells. (A) EJ cells were transfected with si*ATG3* or negative siRNA for 48 h. Then cells were treated with 5 μg/ml pirarubicin or DMSO for 24 h, and the cells were harvested for Western blot using antibodies against ATG3, LC3B, p62 and GAPDH. (B) J82 cells were transfected with si*ATG3* or negative siRNA for 48 h. Then cells were treated with 5 μg/ml pirarubicin or DMSO for 24 h, and the cells were harvested for Western blot using antibodies against ATG3, LC3B, p62 and GAPDH. (C) EJ and J82 cells were treated with 3-MA (0.75 mg/ml) for 1 h. Then cells were co-incubated with pirarubicin (5 μg/ml) for another 24 h, and the cells were harvested for Western blot using antibodies against LC3B, p62 and GAPDH. (D) EJ and J82 cells were treated with HCQ (3 μg/ml) for 1 h. Then cells were co-incubated with pirarubicin (5 μg/ml) for another 24 h, and the cells were harvested for Western blot using antibodies against LC3B, p62 and GAPDH. Results shown are representative of at least three independent experiments.

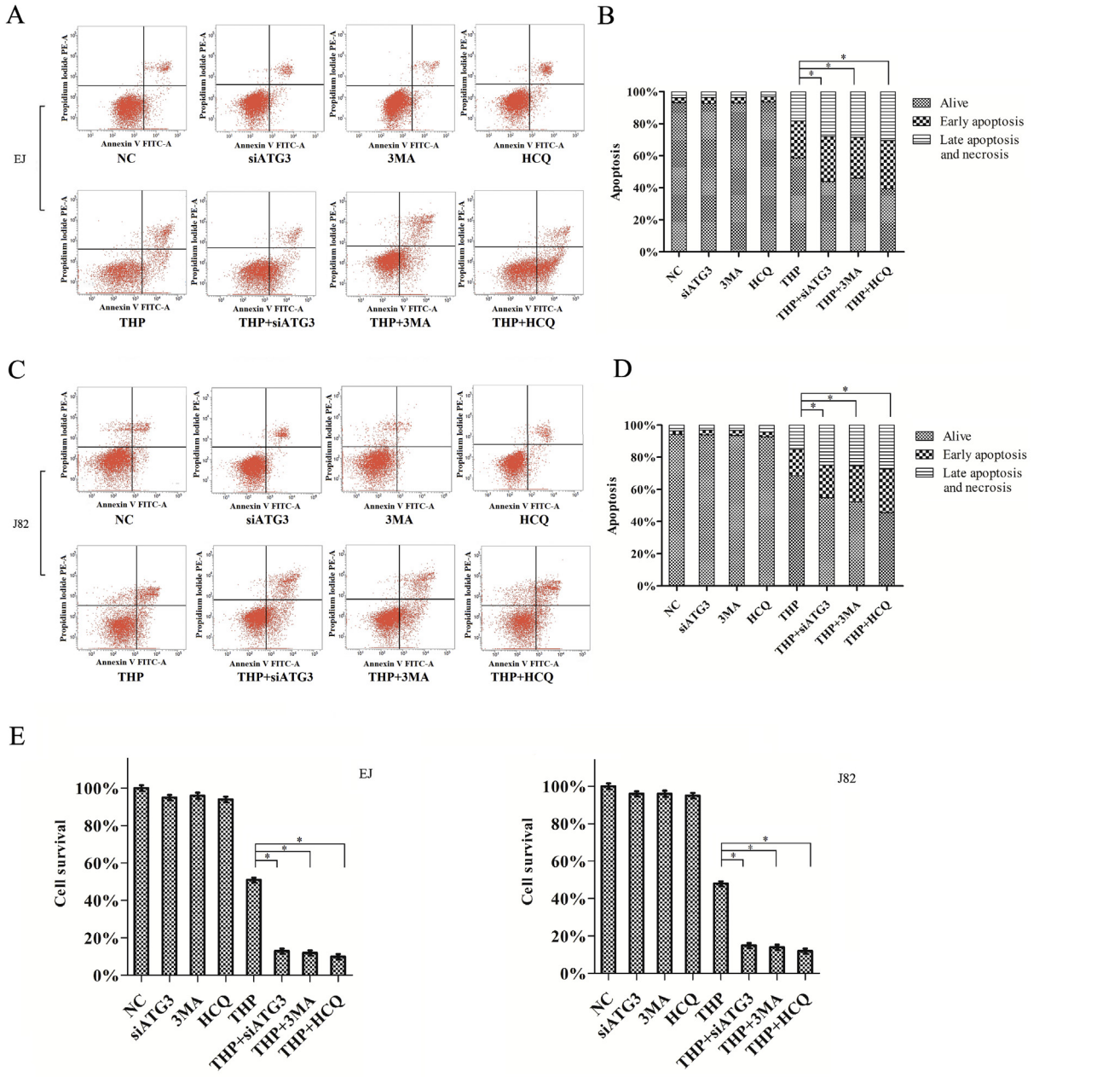

Fig. 3. Inhibition of autophagy enhanced pirarubicin-induced apoptosis in bladder cancer cells. (A) EJ cells were transfected with si*ATG3* for 48 h, then treated with 3-MA (0.75 mg/ml) for 1 h or HCQ (3 μg/ml) for 1 h. Then cells were treated with pirarubicin (5 μg/ml) or DMSO for 24 h, and the cells were stained with Annexin V and detected by flow cytometry analysis. (B) Histogram of apoptosis of EJ cells. The data show the percentages of cells that were alive, early apoptotic, and late apoptotic/necrotic. The data shown are representative of three independent experiments (*$p < 0.05$). (C) J82 cells were transfected with si*ATG3* for 48 h, then treated with 3-MA (0.75 mg/ml) for 1 h or HCQ (3 μg/ml) for 1 h. Then cells were treated with pirarubicin (5 μg/ml) or DMSO for 24 h, and the cells were stained with Annexin V and detected by flow cytometry analysis. (D) Histogram of apoptosis of J82 cells. The data show the percentages of cells that were alive, early apoptotic, and late apoptotic/necrotic. The data shown are representative of three independent experiments (*$p < 0.05$). (E) EJ and J82 cells were treated with siRNA or autophagy inhibitors and analyzed by MTS assay. The data is displayed in bar graphs showing cell viability; the data shown are representative of three independent experiments (*$p < 0.05$).

was a cytoprotective response against pirarubicin-induced apoptosis in bladder cancer cells.

3.3. Pirarubicin induced autophagy through inhibition of mTOR/p70S6K/4E-BP1 in bladder cancer cells

Since mTOR/p70S6K/4E-BP1 is an important pathway in regulating autophagy, we hypothesized that mTOR/p70S6K/4E-BP1 might be involved in pirarubicin-induced autophagy. To determine whether pirarubicin regulates the mTOR/p70S6K/4E-BP1 pathway, we measured phosphorylation of mTOR, p70S6K and 4E-BP1, key points of the mTOR pathway. After treatment of EJ and J82 cells with pirarubicin at the indicated concentrations for 24 h, or with 5 μg/ml for the indicated times, we found that the phosphorylation of the three proteins significantly decreased in both dose-dependent (Fig. 4A) and time-dependent manners (Fig. 4B). This result was negatively related to the observed increase in LC3B-II (Fig. 1B and C), demonstrating that pirarubicin-induced autophagy was regulated via the mTOR/p70S6K/4E-BP1 signaling pathway in bladder cancer cells.

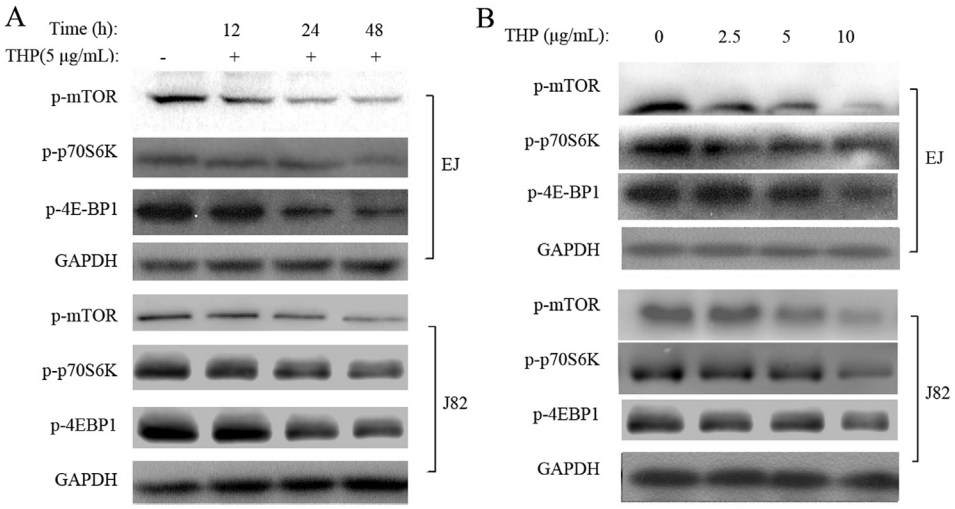

Fig. 4. Pirarubicin induced autophagy through inhibition of mTOR/p70S6K/4E-BP1 in bladder cancer cells. (A) EJ and J82 cells were treated with 5 μg/ml pirarubicin for 12 h, 24 h or 48 h and analyzed by Western blot using specific antibodies against Phospho-mTOR, Phospho-p70S6 kinase, Phospho-4E-BP1, and GAPDH. (B) EJ and J82 cells were treated with pirarubicin (2.5 μg/ml, 5 μg/ml, 10 μg/ml) for 24 h and analyzed by Western blot using specific antibodies against Phospho-mTOR, Phospho-p70S6 kinase, Phospho-4E-BP1, and GAPDH.

4. Discussion

Pirarubicin is a widely used intravesical instillation agent against bladder cancer. Although instillation of pirarubicin significantly decreases the risk of recurrence in patients with bladder cancer, 10–30% of patients will relapse and suffer metastasis within 5 years. The mechanism of drug resistance remains unclear. We, for the first time, determined that pirarubicin induces an autophagic cytoprotective response against its cytotoxicity in human bladder cancer cells.

Autophagy is a conserved homeostatic process that degrades damaged organelles and proteins. It is a principal mechanism in regulating cell metabolism and energy balance in response to metabolic stress, cellular damage or chemotherapy [16,17]. In this study, our results showed that pirarubicin could prompt the conversion of LC3B-I to LC3B-II in bladder cancer cells. However, although the conversion of LC3B-I to LC3B-II indicates the quantity of autophagosomes, the accumulation of autophagosomes represents either the activation of autophagosome formation and/or the inhibition of autophagosomal maturation and degradation in the autophagy process. Therefore, it is necessary to distinguish whether autophagosome accumulation is due to induction of autophagy, or rather to an inhibition in autophagosomal maturation and degradation. In this study, we used autophagic flux assays to distinguish between these two possibilities. Cotreatment of cells with pirarubicin and 3-MA or RNA interference of *ATG3*, which block the upstream steps of autophagy, obviously decreased the LC3B conversion level. Conversely, cotreatment with pirarubicin and chloroquine, which blocks the downstream steps of autophagy, increased the LC3B conversion level. These results confirmed that pirarubicin could induce autophagy in bladder cancer cells.

Increasing evidence has shown that autophagy, paradoxically, acts both in protective and lethal roles depending on the context. In pro-survival function, autophagy is employed to degrade damaged proteins or organelles and to provide the cells with energy supporting survival. However, many studies indicate that overstimulation of autophagy results in cell death by triggering apoptosis. Several anti-cancer drugs have been found to induce autophagy in bladder cancer. Temsirolimus, pazopanib and sunitinib are reported to trigger autophagic death of bladder cancer cells. By contrast, the AKT inhibitor AZ7328 and mTORc1/TORc2 inhibitor OSI-027 are found to support cell survival by increasing the autophagy level [18–21]. These reports have proved that the apparent conundrum of the balance between autophagy and apoptosis exists in bladder cancer. As we have described above, pirarubicin can induce autophagy in bladder cancer cells. Thus, the question emerges, as a widely used intravesical instillation agent, is pirarubicin-induced autophagy involved in autophagic cell death or autophgaic survival? In our study, we investigated the relationship between autophagy and apoptosis in pirarubicin treated bladder cancer cells and found that when combined with pirarubicin, apoptosis was increased by the autophagy inhibitors 3-MA, HCQ and by *ATG3* knockdown in bladder cancer cells, which reveals that autophagy plays a pro-survival role in pirarubicin treated bladder cancer cells.

Mammalian target of rapamycin (mTOR), a central checkpoint regulating autophagy, positively regulates the serine/threonine kinase p70 S6 kinase (p70S6K) and eukaryotic translation initiation factor 4E binding protein 1 (4E-BP1) [22,23]. Mechanistic studies reveal that mTOR/p70S6K/4E-BP1 is a key signaling pathway in regulating cell proliferation and differentiation [24]. In our study, we demonstrated that pirarubicin could decrease the phosphorylation of mTOR, p70S6K and 4E-BP1 in both a time-dependent and dose-dependent manner in bladder cancer cells. We believe that the mTOR/p70S6K/4E-BP1 pathway was the key regulator of pirarubicin-induced autophagy. However, it is of note that MAPK/JNK, Wnt and STAT signaling pathways are also related to autophagy in different cancers [25–27]. Whether these pathways are involved in pirarubicin-induced autophagy remains unclear. Our findings need to be verified by further research.

We showed that ATG3 had effects on pirarubicin-induced cell cytotoxicity. ATG3 is an E2-like enzyme involved in autophagy and mitochondrial homeostasis. It catalyzes the conjugation of ATG8-like proteins to phosphatidylethanolamine (PE), which is critical in autophagy [28]. We found pirarubicin could increase expression of ATG3 in a time and dose-dependent manner. Moreover, si*ATG3* inhibited pirarubicin-induced autophagy, which confirms that ATG3 is a downstream effector of pirarubicin-induced autophagy. We also found that knockdown of *ATG3* increased pirarubicin-induced apoptosis, providing a novel experimental strategy to decrease drug resistance in pirarubicin-treated bladder cancer cells.

HCQ prevents the degradation of autophagosomes relying on autophagosome-lysosome fusion and cargo degradation by lysosomal hydrolases [29]. Interestingly, we found that HCQ, as a single agent, had little anticancer activity in bladder cancer cells. However, HCQ can enhance pirarubicin-induced apoptosis and significantly potentiated bladder cancer cell growth. This result was similar to those in other studies in which chloroquine was particularly effective in potentiating tumor regression without escalating toxicity when used in combination with other conventional or targeted therapies [30–32]. We think the explanation is that autophagy occurs at a low level in the absence of chemotherapy pressure and therefore inhibition of autophagy has little impact on cell growth. However, in the presence of chemotherapy pressure, autophagy is employed in negative feedback inhibition of apoptosis. In these conditions, inhibition of autophagy can prompt significant cell death. As commonly used in clinical medicine, chloroquine or its derivative (HCQ) has proven to be safe and therefore could be a powerful adjuvant drug in combination with conventional anticancer therapies.

In summary, pirarubicin can induce autophagy, which protects against pirarubicin-induced cell death of bladder cancer cells. Inhibition of the mTOR/p70S6K/4E-BP1 signaling pathway participated in this process. These findings enhance the understanding of drug resistance mechanisms during treatment of bladder cancer with pirarubicin. Additional work is required to explore whether inhibition of autophagy clinically contributes to enhancing the efficacy of anti-bladder cancer chemotherapy.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgments

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