The cytoprotective role of gemcitabine-induced autophagy associated with apoptosis inhibition in triple-negative MDA-MB-231 breast cancer cells

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Abstract. Triple-negative breast cancer (TNBC), which is estrogen receptor (ER)-negative, progesterone receptor-negative and is also negative for HER2 expression, remains a great clinical challenge due to its strong resistance to chemotherapy at the late stage of treatment and relatively unfavorable prognosis. Gemcitabine has been approved by the FDA/SFDA for use as a first-line therapeutic drug against advanced or metastatic breast cancer. Therefore, the clarification of the mechanisms underlying gemcitabine-acquired resistance is of particular importance for the optimal management of TNBC. A number of studies have revealed that autophagy, which has been found to protect cancer cells from anti-cancer drug-induced death, may contribute to the development of drug resistance. However, the association between autophagy and gemcitabine treatment in TNBC cells has yet to be defined. Our study clearly demonstrates that gemcitabine is able to induce mTOR-independent autophagy in human triple-negative MDA-MB-231 breast cancer cells. In addition, we demonstrate that autophagy protects MDA-MB-231 cells from gemcitabine-induced cell growth inhibition and apoptosis, indicating that gemcitabine can activate autophagy to impair the sensitivity of MDA-MB-231 cells. Furthermore, as shown by our results, the inhibition of gemcitabine-induced autophagy by chloroquine shifts the expression of the p53 protein, Bcl-2 family proteins and the relative Bax/Bcl-xL ratio in favor of promoting apoptosis. These results reveal that the inhibition of apoptosis may be one of the mechanisms of

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autophagy-induced cytoprotection in gemcitabine-treated MDA-MB-231 cells. The apoptotic and autophagic processes constitute a mutual inhibition system and jointly seal the fate of TNBC cells that are exposed to gemcitabine. Thus, our study suggests that the combination of an autophagic inhibitor and gemcitabine as a therapeutic strategy may represent a promising approach with greater clinical efficacy for patients with TNBC. However, extended preclinical trials are required to further determine the positive effects of the inhibition of autophagy on the efficacy of gemcitabine.

Introduction

Breast cancer is the most prevalent malignancy and was expected to be the second leading cause of cancer-related mortality among women in the United States in 2013 (1). Triplenegative breast cancer (TNBC), accounting for approximately 15% of breast cancer cases, is an immunohistochemicallydefined phenotype and is estrogen receptor (ER)-negative, progesterone receptor (PR)-negative and is also negative for HER2 expression (2). Currently, TNBC remains a tremendous clinical challenge due to its aggressive behavior and relatively unfavorable prognosis (3). It is not only associated with an early relapse and a high risk of visceral and central nervous system metastases, but also with an early mortality during the first 3-5 years of follow up (4-7). Since TNBC is refractory to endocrine therapies, such as tamoxifen and aromatase inhibitors, as well as current HER2-targeted therapies, such as trastuzumab, chemotherapy remains the mainstay of treatment for patients with TNBC (2). Therefore, the improvement of chemotherapeutic efficacy is of particular importance to the optimal management of TNBC.

Gemcitabine is a deoxycytidine analogue, which competes with deoxycytidine triphosphate for incorporation into DNA, and thus inhibits DNA synthesis mainly in its triphosphate form (8). It has been used in a wide spectrum of malignancies, both as a single agent and in combination with other cytotoxic drugs. As a single agent, the response rates of gemcitabine range from 14 to 37% as a first-line therapy for advanced breast cancer and 12-30% in patients previously treated with anthracyclines and/or taxanes (9). In combination with taxanes and/

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or anthracyclines as doublets or triplets, gemcitabine has been determined as a first-line therapy in clinical practice for the treatment of patients with metastatic breast cancer, achieving overall response rates ranging from 39 to 75% (10-12). However, intrinsic and acquired resistance to gemcitabine is common, whereas the molecular mechanisms underlying chemoresistance to gemcitabine are not yet fully understood (13).

Autophagy is an evolutionarily well-conserved lysosomal degradation pathway that maintains cytoplasmic homeostasis by eliminating protein aggregates and damaged organelles (14). It is characterized by the formation of double-membraned autophagosomes that engulf cytoplasmic components and eventually fuse with lysosomes to form autolysosomes for the degradation of the contents (15). Considering the role of autophagy in helping cells to adapt to starvation or unfavorable stress conditions (e.g., hypoxia, radiation therapy and cytotoxic drugs), autophagy is usually identified as a cellular pro-survival mechanism (16). By contrast, under certain circumstances, autophagy may also lead to cell death directly, causing autophagic cell death or type II programmed cell death (PCD; type II PCD), which is distinct from apoptosis (type I PCD) or necrosis (type III PCD).

As regards the connections between the apoptotic and autophagic processes, a complex interplay between them has come to light, taking into account that numerous genes, such as p53, the death-associated protein kinase (DAPK) family and Bcl-2 family members, are shared between these two response machineries. This suggests that apoptosis and autophagy jointly seal the fate of cells (21).

A number of studies have demonstrated that gemcitabine induces autophagy in pancreatic cancer cells and the modulation of autophagy may improve the efficacy of gemcitabine (22-25). However, whether autophagy is associated with gemcitabine treatment for TNBC remains unknown. In the current study, we aimed to explore whether gemcitabine treatment induces autophagy in triple-negative MDA-MB-231 breast cancer cells, and establish the function of such inducible autophagy in the therapeutic efficacy of gemcitabine. Moreover, the possible interplay between autophagy and apoptosis in triple-negative MDA-MB-231 cells is also explored.

Materials and methods

Reagents and antibodies. The chemicals used in the present study were the following: gemcitabine (G6423), chloroquine diphosphate salt (C6628) and monodansylcadaverine (MDC; 30432) (all from Sigma-Aldrich, St. Louis, MO, USA). The Annexin V FITC Apoptosis Detection kit (88-8005) and the propidium iodide (PI) staining solution (00-6990) were from eBioscience, Inc. (San Diego, CA, USA). The primary antibodies were the following: anti-LC3B antibody produced in rabbit (L7543; Sigma-Aldrich), beclin 1 antibody (NB500-249; Novus Biologicals, Littleton, USA), anti-Bax rabbit polyclonal antibody (AB20073a), anti-Bcl-2L1 rabbit polyclonal antibody (AB20306a) (both from Life Science Products & Services), anti-p53 (WBL, K0181-3) and mTOR rabbit antibody (#2972; Cell Signaling Technology, Danvers, MA, USA). The secondary antibodies, HRP-conjugated goat anti-rabbit (sc-2357) and goat anti-mouse IgG (sc-2371), were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Cell culture. The monolayer culture of MDA-MB-231 cells [obtained from the American Type Culture Collection (ATCC), Manassas, VA, USA] was maintained in L-15 medium supplemented with 10% fetal bovine serum, 100 μ g/ml streptomycin and 100 U/ml penicillin in a humid incubator without CO₂ at 37°C.

MTT assay. Cells seeded in 96-well plates were allowed to attach overnight, and treated with various concentrations of the reagents for the indicated periods of time. Subsequently, $20 \ \mu$ l MTT (5 mg/ml) were added to each well for 4 h of incubation. The supernatant was then removed and 150 μ l DMSO were added to each well to dissolve the blue-purple formazan crystals. The plates were agitated for 15 min. Absorbance values at 490 nm were detected with a model 680 MicroPlate Reader (Bio-Rad, Hercules, CA, USA).

Flow cytometry with Annexin V-FITC (AV-FITC)/PI staining. The cells were seeded at 4x10⁵ cells/well in 6-well plates and allowed to attach overnight. The cells were then treated for the indicated periods of time and were then collected and dyed with AV-FITC and PI according to the instructions of the manufacturer. Cell viability was analyzed by flow cytometry (BD FACSCalibur; BD Biosciences, San Jose, CA, USA).

Western blot analysis. The cells were treated for the indicated periods of time. Both the detached and attached cells were collected, and whole cell lysates were prepared. Approximately 30 μ g total proteins were separated by SDS-PAGE with a proper concentration and transferred onto PVDF membranes (Bio-Rad). The PVDF membranes were blocked by 5% non-fat dry milk for 90 min at room temperature, and were then incubated with primary antibodies overnight with light agitation at 4°C and secondary antibodies for 2 h at room temperature. Visualization was carried out with electrochemiluminescence (ECL; Bio-Rad).

Visualization of MDC-labeled vacuoles. The cells were cultured on coverslips in 24-well plates and treated for the indicated periods of time. Subsequently, fresh medium-containing MDC (50 μ mol/l) was added to the plates for 15 min. Subsequently, the cells were fixed using a freshly-prepared 4% formaldehyde solution for 10 min at room temperature. The specimens were analyzed under a BX61W1-FV1000 confocal microscope (Olympus, Tokyo, Japan).

EGFP-LC3 plasmid transfection. The EGFP-LC3 plasmids were transiently transfected into the cells using Lipo-fectamine 2000^{TM} (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the instructions of the manufacturer. After 24 h, the cells were treated with the chemicals for the indicated periods of time, and then fixed with 4% formaldehyde solution for 10 min at room temperature. The fluorescence of EGFP was analyzed using a BX61W1-FV1000 confocal microscope (Olympus). The number of LC3-II⁺ puncta was counted manually in confocal images from random fields to quantify the EGFP-LC3-tagged autophagosomes.

Transmission electron microscopy (TEM). The treated cells were fixed for 1 h in ice-cold 2.5 glutaraldehyde and 1%



Figure 1. Gemcitabine decreases cell viability and induces apoptosis in MDA-MB-231 breast cancer cells. (A) Chemical structure of 2'-deoxy-2',2'-difluorocytidine (gemcitabine hydrochloride). (B) Cell viability following treatment with gemcitabine ($0.04-2.5 \mu g/m$) for 48 h was determined by MTT assay in MDA-MB-231 cells. Results are expressed as the relative percentage of each group compared with the control. The results [means ± standard error (SE)] were from 3 independent experiments. (C) Apoptosis was measured by Annexin V-FITC (AV-FITC)/propidium iodide (PI) staining and flow cytometry following treatment with gemcitabine ($1 \mu g/m$) for 0 h (untreated control) or 12, 24 and 48 h. The scatter plots and statistical analysis of Annexin V-FITC and PI uptake are shown. The results (means ± SE) were from 3 independent experiments.

osmium tetroxide for a further 1 h. The samples were stained with 4% uranyl acetate for 30 min, dehydrated through a graded series of ethanol solutions (ranging from 50 to 100%) and embedded in Epon resin. Ultrathin sections (120 nm) were post-stained with uranyl acetate and lead citrate, and were then examined under a TECNAI 10 transmission electron microscope (Philips, Amsterdam, The Netherlands) at 60 kV.

RNA interference. The cells were transfected with either scrambled siRNA or beclin 1-targeting siRNA using interfering siRNA transfection reagent (PolyPlus Transfection SA, Illkirch, France) according to the recommendations of manufacturer. The si-BECN1 targeting sequence was UUC AAC ACU CUU CAG CUC AUC AUC C and the scrambled siRNA sequence was UUC UCC GAA CGU GUC ACG UTT. All siRNAs were obtained from GenePharma, Ltd. (Shanghai, China).

Statistical analyses. To determine whether the difference between 2 groups was statistically significant, Student's t-tests were performed. The significance level was set at $P \le 0.05$.

Results

Gemcitabine decreases cell viability and induces apoptosis in MDA-MB-231 cells. The MDA-MB-231 cells were treated with gemcitabine at various concentrations, and cell viability was assessed with by MTT assay. The results revealed that cell viability (% of the control) gradually decreased with the increasing concentrations of gemcitabine (Fig. 1B). The half maximal inhibitory concentration (IC₅₀) of 48 h of treatment with gemcitabine in the MDA-MB-231 cells was 1.2 μ g/ml. In this study, 1 μ g/ml was selected as the treatment concentration in the subsequent experiments. Flow cytometry with AV-FITC/ PI staining was used to determine the apoptotic rate of the MDA-MB-231 cells following treatment with gemcitabine for different periods of time. The AV-FITC-positive cells were defined as apoptotic and double-negative cells were considered viable. The percentage of AV-FITC-positive cells increased with the increasing duration of gemcitabine treatment (Fig. 1C).

Gemcitabine induces mTOR-independent autophagy in triple-negative MDA-MB-231 cells. To evaluate gemcitabine-induced autophagy, we first tested the conversion of LC3-I into LC3-II in the MDA-MB-231 cells. As shown in Fig. 2A, gemcitabine induced the conversion of LC3-I into LC3-II in a time-dependent manner. Compared with that at 24 h, the ratio of LC3-II/β-actin at 48 h was decreased, possibly due to the fact that autophagy is a highly dynamic, multi-step process. We further examined the autophagic flux by monitoring the protein level of LC3-II relative to β -actin after 24 h of treatment with gemcitabine with or without chloroquine (CQ) (Fig. 2B). LC3-II protein expression was markedly increased following treatment with CQ, and the relative level of LC3-II vs. actin was significantly higher than that observed with CQ treatment alone following treatment with gemcitabine plus CQ. No apparent change in the mTOR protein levels was observed following treatment with



Figure 2. Gemcitabine induces mTOR-independent autophagy in triple-negative MDA-MB-231 cells. (A) The conversion of Lc3-I (18 KDa) into LC3-II (16 KDa) was assayed in cells treated with gemcitabine (1 μ g/ml) for 0 h (untreated control) or 12, 24 and 48 h. (B) Cells were treated with gemcitabine, chloroquine (CQ; 5 μ M; added prior to treatment with gemcitabine for 1 h), or gemcitabine plus CQ for 0 h (control) or 24 h, then the conversion of Lc3-I into LC3-II was assessed. (C) The levels of mTOR protein were assessed following treatment with gemcitabine (0, 0.5 and 1, 2 μ g/ml) for 24 h. (D) Cells exposed to gemcitabine, CQ, or gemcitabine plus CQ for 0 h (control) or 24 h, then the samples were examined under a confocal microscope. (E) Cells transiently transfected with EGFP-LC3 plasmids were exposed to gemcitabine, CQ, or gemcitabine plus CQ for 0 h (control) or 24 h. A confocal microscope was used to measure EGFP fluorescence distribution. The punctate EGFP-LC3 was indicative of autophagosomes. The number of LC3-II* puncta was counted in at least 75 cells from random fields and the fold change was calculated by normalizing to the amount of the control group. (F) Cells were treated with gemcitabine for 0 (control) or 24 h. The ultrastructural characteristics of the cells were analyzed by transmission electron microscopy (TEM). Autophagical vacuoles with typical double-layer membrane containing remnants of organelles are highlighted by white arrows. *P<0.05;**P<0.01. Gem, gemcitabine; Ctrl, control.

various concentrations of gemcitabine, indicating that the gemcitabine-induced autophagy in MDA-MB-231 cells was possibly mTOR-independent (Fig. 2C).

MDC is a selective fluorescent dye used to observe for autophagic vacuoles. As shown in Fig. 2D, gemcitabine treatment increased the number of bright blue puncta. Moreover, the number of MDC-labeled puncta was markedly increased and the average puncta volume was significantly larger following treatment with gemcitabine plus CQ. The transient transfection of EGFP-LC3 plasmids was performed to further determine gemcitabine-induced autophagy in the MDA-MB-231 cells. The number of EGFP-LC3 puncta per cell in the group treated with gemcitabine plus CQ was markedly higher than that observed in the cells treated with gemcitabine alone or CQ alone (Fig. 2E), thus indicating the increased autophagic flux level in triple-negative MDA-MB-231 cells treated with gemcitabine. Finally, TEM confirmed that numerous autophagic vacuoles with a typical double-layer membrane containing organelle remnants were present in the gemcitabine-treated MDA-MB-231 cells as opposed to the untreated (control) cells (Fig. 2F). Our results indicated that gemcitabine treatment induce autophagy in TNBC cells.

Gemcitabine-induced autophagy plays a cytoprotective role in triple-negative MDA-MB-231 cells. As shown by the results of MTT assay, CQ treatment alone (5 μ M) had little toxic effect on the MDA-MB-231 cells (Fig. 3A). However, the inhibition of autophagy by CQ significantly enhanced the inhibitory effects of gemcitabine on cell viability at 12 and 48 h (Fig. 3B). Flow cytometry revealed that after the cells



Figure 3. Inhibition of autophagy by chloroquine (CQ) enhances the cytotoxicity of gemcitabine in triple-negative MDA-MB-231 cells. (A) The effect of chloroquine (1.25-160 μ mol/l) for 48 h on MDA-MB-231 cell growth was determined by MTT assay. The results are expressed as the relative percentage of each group compared with the control. (B) The viability of MDA-MB-231 cells exposed to gemcitabine alone (0.16-2.5 μ g/ml) or gemcitabine plus CQ (5 μ M; added prior to treatment with gemcitabine for 1 h) for 12 or 48 h was determined by MTT assay. The results are expressed as the relative percentage of each group compared with the control. The data [means ± standard error (SE)] were from 3 independent experiments. (C) Apoptosis was measured by Annexin V-FITC (AV-FITC)/ propidium iodide (PI) staining and flow cytometry following treatment with gemcitabine (1 μ g/ml) or gemcitabine plus CQ for 12 or 48 h. The scatter plots and the statistical analysis of Annexin V-FITC and PI uptake are shown. *P<0.05; **P<0.01; ***P<0.001. ns, not significant; Gem, gemcitabine.

were treated with gemcitabine plus CQ, the number of apoptotic markedly increased compared with the cells treated with gemcitabine alone (Fig. 3C).

We further inhibited autophagy by silencing the beclin 1 gene, as beclin 1 is one of the essential autophagy-related genes (Atgs) (26). Transfection with beclin 1-targeting siRNA resulted in a marked reduction in beclin 1 protein expression, while the scrambled siRNA-transfected group showed no change (Fig. 4A). As shown in Fig. 4B, beclin 1 knockdown markedly reduced the viability of cells treated with gemcitabine at 12 and 48 h. Of note, si-BECN1 alone also inhibited cell viability, thus indicating that beclin 1 plays a positive role in the growth of TNBC cells. As shown by flow cytometry, treatment with scrambled siRNA plus gemcitabine produced results that were comparable to those that were obtained by treatment with gemcitabine alone, while treatment with gemcitabine plus si-BECN1 led to a more significant increase in the percentage of apoptotic cells (Fig. 4C). These results demonstrate that beclin 1-mediated autophagy induced by gemcitabine contributes to TNBC cell growth and survival.

Autophagy inhibition by CQ shifts the expression levels of p53 and Bcl-2 family proteins in favor of promoting apoptosis. The p53 and Bcl-2 family member proteins play a critical role in the intrinsic apoptotic pathway, which regulates apoptosis by modulating mitochondrial outer membrane integrity (27).

We investigated the protein levels of p53, the anti-apoptotic Bcl-xL gene and the pro-apoptotic Bax gene. As shown by our results, the Bcl-xL protein levels increased, while the p53 and

Bax protein levels decreased in a concentration-dependent manner following treatment with gemcitabine (Fig. 5A, left panel). Following treatment with gemcitabine plus the autophagic inhibitor, CQ, the Bcl-xL protein levels markedly decreased with a concomitant increase in the p53 and proapoptotic Bax levels in a concentration-dependent manner, which was in contrast to what was observed following treatment with gemcitabine alone (Fig. 5A, right panel). We further assessed the relative Bax/Bcl-xL ratio following treatment with gemcitabine with or without CQ. With the increasing gemcitabine concentration, the relative Bax/Bcl-xL ratio slightly decreased, while it markedly increased following treatment with CQ plus gemcitabine (Fig. 5B).

Discussion

A number of studies have demonstrated that anti-cancer therapy induces autophagy in cancer cells, while there is an ongoing debate on the association between autophagy and therapeutic efficacy (28). A series of studies have reported that chemotherapeutic drugs induce autophagy, promoting the survival of cancer cells (29-31), thus suggesting that autophagy is involved in the development of resistance to chemotherapy.

CQ has been extensively used as an autophagic inhibitor that prevents the acidification of the lysosome, thus blocking the late stage of the autophagy process (32). It is noteworthy to mention that some clinical trials testing CQ-mediated autophagy inhibition, using hydroxychloroquine (HCQ) combined with ixabepilone for metastatic breast cancer and CQ for ductal



Figure 4. Inhibition of autophagy by si-BECN1 enhances the efficacy of gemcitabine in triple-negative MDA-MB-231 cells. (A) The levels of beclin 1 protein were detected following treatment with scrambled siRNA or si-BECN1 for 48 h. (B) Cells were treated with scrambled siRNA or si-BECN1 for 48 h. (Cell viability was measured by MTT assay and the results are expressed as the relative percentage of each group compared with the control. The results [means ± standard error (SE]] were from 3 independent experiments. (C) Cells were treated with or without scramble siRNA or si-BECN1 for 48 h, followed by treatment with gemcitabine for 12 or 48 h, followed by treatment with gemcitabine for 12 or 48 h, and apoptosis was determined by Annexin V-FITC (AV-FITC)/propidium iodide (PI) staining and flow cytometry. The results (means ± SE) were from 3 independent experiments. *P<0.05; **P<0.01. Gem, gemcitabine.

carcinoma *in situ*, have been carried out (http://www.clinical trials.gov/ct2/show/results/NCT00765765) or are ongoing (http://www.clinicaltrials.gov/ct2/show/NCT01023477); this indicates that chloroquine is clinically applicable. Our results demonstrated that the addition of CQ to gemcitabine treatment greatly enhanced the inhibitory effect on cell viability and increased cell apoptosis in the MDA-MB-231 cells. Our data support the therapeutic potential of CQ in combination with gemcitabine for enhancing the efficacy of gemcitabine in TNBC.

Beclin 1 is an essential Atg that signals the onset of the autophagy process (33). In our study, the silencing of beclin 1



Figure 5. Autophagy inhibition by chloroquine (CQ) shifts the expression of the p53 and Bcl-2 family proteins in favor of promoting apoptosis. (A) p53, Bax and Bcl-xL protein levels were detected following treatment with gemcitabine (0, 0.5, 1 and 2 μ g/ml) or gemcitabine plus CQ (5 μ M, added prior to treatment with gemcitabine for 1 h). (B) The fold change of the Bax/Bcl-xL ratio relative to the control group with the increasing gemcitabine concentration was calculated. Gem, gemcitabine.

significantly enhanced the inhibitory effect of gemcitabine on the growth and viability of MDA-MB-231 cells, indicating that beclin 1-mediated autophagy plays a cytoprotective role in TNBC cells. Of note, treatment with si-BECN1 alone markedly inhibited the viability of MDA-MB-231 cells, indicating that the beclin 1 gene plays a positive role in the growth of TNBC cells. By contrast, the study by Liang et al (26) produced different results. They evaluated the effects of beclin 1 on the growth of ER⁺ MCF-7 cells before and after stably transfecting MCF-7 cells with a vector containing flag-beclin 1. They also evaluated cell proliferation and tumor formation in nude mice with MCF-7 tumors. The number of MCF-7 beclin 1 clones was significantly lower compared with the control group. Therefore, the authors concluded that beclin 1 acts as a negative regulator of mammary cell growth and tumorigenesis (26). Thus, it can be hypothesized that the effects of beclin 1 on the growth of breast cancer cells may depend on the status of steroid hormone receptors.

There are multiple connections between the apoptotic and autophagic processes (21). In this study, we demonstrated that pre-treatment with CQ (to inhibit autophagy) prior to gemcitabine treatment shifted the p53 protein level and the Bax/Bcl-xL ratio in favor of promoting apoptosis. This indicates that gemcitabine-induced autophagy inhibits the apoptotic process to a certain extent, and that the inhibition of the induced autophagy may promote apoptosis, resulting in an improved efficacy of gemcitabine in TNBC cells. Our results also suggest that the inhibitory effect of autophagy on apoptosis is another mechanism behind autophagy-induced cytoprotection other than the basic cellular functions of autophagy in triple-negative MDA-MB-231 cells.

In conclusion, the present study clearly demonstrates that gemcitabine induces mTOR-independent autophagy in triple-negative MDA-MB-231 cells and suggests that gemcitabine-induced autophagy plays an active role in the refractory response of TNBC cells to gemcitabine. Our results also indicate that the downregulation of cellular apoptotic levels by autophagy constitutes another mechanism responsible for autophagy-induced cytoprotection in gemcitabine-exposed TNBC cells. Thus, the combination of an autophagic inhibitor with gemcitabine may be a promising approach to promote greater therapeutic efficacy in patients with TNBC. However, more preclinical trials are required to further determine the positive effects of autophagic inhibitors on gemcitabine treatment in patients with TNBC.

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