Abstract. The autophagy involved in the occurrence, development and prognosis of human epidermal growth factor receptor 2 (HER2) gene-amplified cancer also controls the resistance of this type of cancer to the monoclonal antibody, trastuzumab (Tzb). In the present study, Tzb resistance was established in HER2-positive NCI-N87 cell lines (Tzb-refractory cells). The cell viability, clonogenic assay, ratios of light chain 3 II/I, sequestosome 1 expression, and the phosphorylation of protein kinase B (Akt) and mechanistic target of rapamycin (mTOR) were investigated in the parental and Tzb-refractory cells. The viability of parental NCI-N87 and Tzb-refractory cells with an autophagy inhibitor or inducer was also examined. The results of the present study indicated that autophagic flux may have an important function in the resistance of HER2-positive human gastric cancer NCI-N87 cells to Tzb. Tzb resistance in NCI-N87 cells prevents cell apoptosis via autophagic flux inhibition. Tzb may activate the Akt/mTOR pathway to inhibit autophagic flux in gastric cancer cell lines. Everolimus, an mTOR inhibitor, may inhibit cell viability, indicating that the mTOR pathway may serve a function in HER2-positive gastric cancer and that the resistance of HER2-positive gastric cancer to Tzb may, at least partially, be due to activation of the mTOR pathway.

Introduction

Gastric cancer is one of the most common causes of cancer-associated mortality globally and clinical approaches for treatment of gastric cancer are limited, with the median survival time for advanced gastric cancer remaining at 8-10 months (1). Dysplasia and Barrett's esophagus have been reported to be associated with the development of esophageal adenocarcinoma, while Helicobacter pylori infection, atrophic gastritis, intestinal metaplasia and dysplasia are associated with gastric adenocarcinoma (2). In 20-30% of gastric and gastro-esophageal junction cancer cases, gastric cells over-express human epidermal growth factor receptor 2 (HER2), which is indicative of a poor prognosis (3).

Trastuzumab (Tzb) is a humanized monoclonal antibody that targets the HER2 gene. Tzb is one of the first molecular-targeting drugs to be developed and was originally introduced for the treatment of HER2-positive advanced breast cancer (4). Tzb has also been widely used to treat HER2-positive gastric cancer (1). Tzb, induces antibody-dependent cellular cytotoxicity and confers an overall survival benefit in HER2-positive advanced gastric cancer (3). However, Tzb treatment remains under investigation in order to further elucidate its potential utilization and underlying mechanisms (5). Tzb in combination with chemotherapy may be considered as a novel standard option for patients with HER2-positive advanced gastric or gastro-esophageal junction cancer (6). However, with increased durations of Tzb treatment, the risk of developing resistance to the drug is also increased. In addition, details of the mechanisms underpinning Tzb resistance remain unclear. Therefore, it is important to explore the mechanisms underlying drug resistance in order to combat this problem.

Autophagy is the cellular degradation process in which cellular proteins and organelles are engulfed by double-membrane autophagosomes and are degraded in lysosomes (7). Perturbations in autophagy have been observed in gastric cancer (8,9). In cancer cells, autophagy has both pro-survival and pro-death functions and, thus, the action of autophagy in cancer cells remains controversial. Autophagy
may act as a survival mechanism that provides energy and protects cancer cells from the cell death induced by multiple antitumor treatments; however, autophagy is also a cell death mechanism in response to anticancer therapies (10). Furthermore, autophagy modulates the development of gastric cancer by affecting a range of pathological events, including tumor angiogenesis and changes to the tumor microenvironment (11).

Wu et al (10) revealed that loss of the autophagy regulator beclin 1 is significantly correlated with HER2 amplification in patients with breast cancer. Notably, HER2 signaling and responsiveness to Tzb appear to dynamically interact with the tumor-suppressive and tumorigenic functions of autophagy (12). Previously, autophagy has been reported to protect against Tzb-induced cytotoxicity in HER2-overexpressing breast tumor spheroids (13). A study has revealed that the autophagy inhibitor, chloroquine, overcomes Tzb resistance in HER2-positive breast cancer SK-BR3 cells and have confirmed that HER2-overexpressing breast cancer cells may require autophagy in order to maintain the Tzb-resistant phenotype (14). However, these studies are focused on breast cancer, with only limited data regarding the association between autophagy and HER2 expression in gastric adenocarcinoma being reported. The present study investigated the function of autophagic flux in a Tzb-resistant gastric cancer cell line in order to study its mechanism of action.

Materials and methods

Materials. Tzb was provided by Ningbo No. 2 Hospital (Zhejiang, China), solubilized in water (stock solution at 21 mg/ml), stored at 4°C and used within 1 month. Dimethylsulfoxide (DMSO), 3-methyladenine (3MA), MTT, crystal violet, hydroxychloroquine (HCQ) and bafilomycin A1 (BafA1) were purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). Everolimus was provided by the China State Institute of Pharmaceutical Industry (Shanghai, China). RPMI-1640 medium, 10 U/ml penicillin-streptomycin (P/S) and RPMI-1640 medium, supplemented with 10% FBS (Waltham, MA, USA). Cell lysis buffer, polyvinylidene difluoride (PVDF) membranes, and Tween-20 were purchased from Fisher Scientific, Inc. (Waltham, MA, USA). Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan). Cell lysis buffer, polyvinylidene difluoride (PVDF) membranes, and Tween-20 were purchased from Weiao Inc. (Shanghai, China). Glutaraldehyde, Epon 812, DDSA, NMA and DMP-30 were purchased from Sinopharm Inc. (Beijing, China).

Cell culture. Human gastric cancer NCI-N87 and SGC 7901 cell lines, and the human breast cancer SK-BR3 cell line, which was used as the positive control, were purchased from the Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). The cells were cultured in full medium (P/S and RPMI-1640 medium, supplemented with 10% FBS) at 37°C in a humidified atmosphere with 5% CO₂.

Establishment of Tzb-acquired auto-resistance in HER2-positive NCI-N87 cells. Establishment of NCI-N87/Tzb-resistant pools was performed as previously described (15), with Tzb-naive NCI-N87 cells being treated with increasing concentrations of Tzb for 10 months. The NCI-N87 cells were initially treated with 50 µg/ml Tzb for 2 months (twice weekly) followed by 100 µg/ml Tzb for 3 months (twice weekly). Two pools (Tzb POOL1 and Tzb POOL2), which resisted continuous growth in 250 µg/ml Tzb for 5 months (twice weekly), were selected for further study. The resistant cells were maintained in RPMI-1640 medium without Tzb for ≥2 days prior to further experiments.

MTT assay. The NCI-N87, POOL1 or POOL2 cells were seeded at 4,000 cells/100 µl into 96-well plates, and allowed to attach overnight. The medium was removed, and 95 µl fresh full medium with 5 µl Tzb (final concentration 1, 3.2, 10, 32, 100 µg/ml), BafA1 (50 nM), 3MA (1 µM), HCQ (50 µM) or everolimus (0.1, 1, 10 µM). The control cells treated with 5 µl PBS was used as vehicle. For testing the combination effect of Tzb (10 µg/ml) and autophagy inhibitors, the concentration of BafA1, 3MA, and HCQ is 25 nM, 500 and 25 µM respectively. After 72 h culture (37°C, 5% CO₂), 10 µl MTT solution (0.5 mg/ml) was added into each well at 37°C for 4 h and 100 µl DMSO was used to dissolve the purple formazan. Following agitation, the optical densities were measured at 595 nm using a microplate reader (BioTek PowerWave XS2; BioTek Instruments, Inc., Winooski, VT, USA).

CCK-8 assay. The NCI-N87 or POOL2 cells were seeded at 1,000 cells/100 µl into 96 well plates, and allowed to attach overnight. The medium was removed, and 95 µl fresh full medium with 5 µl Tzb (final concentration 1, 3.2, 10, 32 or 100 µg/ml), or everolimus (0.1, 1 or 10 µM). The control cells treated with 5 µl PBS was used as vehicle. When assessing the combination effect of Tzb (10 µg/ml) and an autophagy inhibitor, the concentration of BafA1, 3MA, and HCQ is 25 nM, 500 and 25 µM respectively. After a 72 h culture (37°C, 5% CO₂), 10 µl CCK-8 solution was added into each well at 37°C for 2 h and the optical densities were measured at 450 nm using a microplate reader (PowerWave XS2; BioTek Instruments, Inc.).

Clonogenic assay. Cells were cultured in 6-well plates at a density of 100 cells/well and were incubated (37°C, 5% CO₂) for 24 h with full medium to allow for attachment. Following incubation, cells were treated with 100 µg/ml Tzb for 72 h. The control cells treated with 5 µl PBS was used as vehicle. Allowed to grow in a Tzb-free full medium for 14 days (37°C, 5% CO₂), fixed with cold ethanol for 15 min and stained with 0.1% crystal violet in PBS for 30 min at 25°C. Images from light microscopy (magnification, x4; Olympus IX73) were captured using CellSens Dimension software (version 1.15) (both from Olympus Corporation, Tokyo, Japan).

Western blot analysis. Protein lysates were obtained with cell lysis buffer for 30 min at 4°C. The protein (20 µg/lane) was fractionated by 10% SDS-PAGE and transferred onto PVDF membranes. After 1 h blocking with 5% BSA at 25°C, the membranes were incubated at 4°C overnight with different primary antibodies diluted in 3% BSA in 1% Tween-20 in TBS. The membranes were probed with the following antibodies: HER2 (cat. no. 4290; Cell Signaling Technology,
Inc., Danvers, MA, USA; dilution 1:1,000), light chain 3B (LC3B) (cat. no. L7543; dilution 1:1,000; Sigma-Aldrich; Merck KGaA), sequestosome 1 (SQSTM1; cat. no. PM045; dilution 1:2,000; MBL International Corporation, Woburn, MA, USA), phosphorylated (p)-protein kinase B (Akt; cat. no. 2965; dilution 1:1,000), Akt (cat. no. 2966; dilution 1:1,000), p-mechanistic target of rapamycin (mTOR; cat. no. 2983; dilution 1:1,000) and mTOR (cat. no. 5536; dilution 1:1,000) (all from Cell Signaling Technology, Inc.). GAPDH (cat. no. 5014; dilution 1:2,000; Cell Signaling Technology, Inc.) was used as the reference protein. Subsequently, the membranes were incubated with horseradish peroxidase-conjugated secondary anti-rabbit IgG antibody (cat. no. 7074; dilution 1:2,000; Cell Signaling Technology, Inc.) for 1 h at room temperature. Protein bands were visualized using an enhanced chemiluminescence (ECL) kit using ECL blotting detection reagents (cat. no. 54-61-00; KPL, Inc., Gaithersburg, MD, USA).

Transmission electron microscopy (TEM). A total of 1x10⁷ cells were digested with 0.25% trypsin at 37°C and washed twice with PBS. The cell precipitation was fixed with 2.5% glutaraldehyde in PBS for 3 h at 4°C, followed by 1% OsO₄ for 2 h at 4°C. After washing with PBS, the samples were progressively dehydrated in a graded series of ethanol solutions (50, 70, 80, 90, 95 and 100%) at 4°C and embedded in the mixture of Epon 812, DDA, NMA and DMP-30 at 37°C for 12 h, and at 60°C for 48 h. Following dehydration, the cells were cut in to 70 nm sections, and stained with 3% uranyl acetate and lead citrate for 15 min at 25°C, the ultrastructure of cells were analyzed with a TEM (magnification, x16,000; cat. no. JEM-1230; JEOL, Ltd., Tokyo, Japan).

Statistical analysis. All of the data are presented as the mean ± standard deviation of three independent experiments. Statistical comparisons were performed using Student's t-test and repeated-measures one-way analysis of variance followed by Dunnett's post hoc test using SPSS software (version 19; IBM Corp., Armonk, NY, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Tzb induces gastric cancer NCI-N87 cell death. HER2 was expressed in human gastric cancer NCI-N87 cells, but not in SGC 7901 cells (Fig. 1A). Breast cancer SKBR 3 cells were used as a positive control. The viability of NCI-N87 and SGC 7901 cells treated with Tzb was examined using MTT and CCK-8 assays. Tzb (1-100 µg/ml) was revealed to significantly
induce NCI-N87 cell death (Fig. 1B and C), but did not induce the death of SGC 7901 cells (Fig. 1D and E), suggesting that NCI-N87 cells are more sensitive to Tzb.

HER2-positive Tzb-resistant gastric cancer cells were established by treating NCI-N87 parental cells with increasing concentrations of Tzb. After 10 months of being maintained with Tzb, two pools of Tzb-refractory cells (Tzb POOL1 and Tzb POOL2) were obtained. Resistance to Tzb was examined using an MTT assay. A concentration of 100 µg/ml Tzb did not significantly decrease cell viability in POOL1 or POOL2 cells compared with the parental cells (Fig. 2A), and as the resistance of POOL2 was stronger than that of POOL1. The data obtained from the CCK-8 assay demonstrated that the viability of POOL2 cells did not significantly alter with Tzb treatment (Fig. 2B). The NCI-N87 parental cells and the Tzb-refractory cells (Tzb-resistant POOL2) were incubated with 100 µg/ml Tzb for 72 h and were observed under a light microscope. The number of parental cells was visibly decreased following Tzb treatment (Fig. 2C), while the effect of Tzb was less notable in the POOL2 cells. Additionally, as demonstrated in Fig. 2D, NCI-N87 cells treated with 100 µg/ml Tzb for 72 h did not form colonies, indicating that Tzb may inhibit the proliferation of parental cells. However, 100 µg/ml Tzb did not affect the colony-forming ability of POOL2, suggesting that POOL2 is a Tzb-refractory cell line.

Tzb-refractory cells exhibit autophagic flux inhibition. Western blot analysis revealed that the top band (18 kDa) expressed LC3-I and the bottom band (16 kDa) expressed LC3-II, which is a typical autophagosome marker (Fig. 3A). Furthermore, Tzb reduced the expression of LC3 I, and the Tzb-resistant POOL1 and POOL2 cells exhibited an increased expression of LC3-II, suggesting that HER2 may be associated with autophagy. POOL2 demonstrated increased resistance compared with POOL1, therefore POOL2 was selected for further study. SQSTM1 is a link between LC3 and ubiquitinated substrates. The inhibition of autophagic flux is correlated with increased expression of SQSTM1 (16). In the present study, SQSTM1 degradation was observed and Tzb increased the expression of SQSTM1, suggesting that Tzb may inhibit autophagic flux. POOL1 and POOL2 cells exhibited an elevated SQSTM1 expression compared with the parental cells, suggesting that the Tzb-refractory cells exhibited autophagic flux inhibition. The TEM data (Fig. 3B) demonstrated that the autolysosome in the POOL2 cells was smaller than that in the parental cells. As demonstrated in Fig. 3C, 100 µg/ml
Tzb did not alter the autophagic flux in POOL2. These results indicated that Tzb-refractory cells exhibited less autophagic flux and improved viability compared with parental cells, suggesting that autophagy may induce the cell death of gastric cancer cells.

BafA1 is a vacuolar H^+ -ATPase inhibitor known to block autophagic flux to prevent fusion between autophagosomes and lysosomes, 3MA is an inhibitor of the formation of the pre-autophagosomal structures and HCQ is an autophagic inhibitor that prevents endosomal acidification and blocks autophagosome-lysosome fusion (13,17,18). In the present study, cell viability was studied in both parental NCI-87 and Tzb-refractory cells following blocking of autophagy with BafA1 (50 nM), 3MA (1 mM) or HCQ (50 µM). The three
Autophagy inhibitors, BafA1, 3MA and HCQ, were revealed to induce parental NCI-N87 cell death (Fig. 4A-C). However, no change in the viability of POOL1 or POOL2 cells treated with BafA1 or 3MA was observed, suggesting that these autophagy inhibitors did not alter the viability of Tzb-refractory cells. HCQ overcame Trz resistance, but its effect was less notable than that in the parental NCI-N87 cells. In addition, the results of MTT and CCK-8 assays revealed that the combination of Tzb (10 µg/ml) with an autophagy inhibitor (BafA1, 3MA or HCQ) may inhibit the growth of parental cells, but neither BafA1 nor 3MA induced Tzb-refractory cell death (Fig. 4D and E). Although HCQ may induce Tzb-refractory cell death, this effect was significantly lower than in the parental cells. These results indicated that cell Tzb resistance may be due to autophagic flux inhibition.

**Tzb induces resistance via the Akt/mTOR pathway.** The Akt/mTOR signaling pathway is a key negative regulator of autophagy in gastric cancer cells (8). Therefore, the present study investigated whether the Akt/mTOR pathway is involved in the Tzb resistance of HER2-positive cancer cells. As demonstrated in Fig. 5A, the phosphorylation of Akt and mTOR was increased in Tzb-resistant HER2 positive cancer cells compared with the parental cells, suggesting that Tzb resistance is partially due to alterations in the Akt/mTOR pathway. Notably, everolimus, a derivative of rapamycin that is functionally similar to rapamycin and is an allosteric inhibitor of mTOR, is also able to induce autophagy (19,20), and significantly induced cell death in parental and Tzb-refractory gastric cancer cells in a dose- and time-dependent manner (Fig. 5B and C). As demonstrated in Fig. 5D, Tzb had little effect on the phosphorylation of Akt and mTOR in POOL2, however, combining everolimus with Tzb reduced the activation of Akt and mTOR. Taken together, these results suggest that Tzb-induction in cells may alter the balance of cellular autophagic flux, via autophagic flux inhibition or the use of an autophagy inducer, such as everolimus.

**Discussion**

Although a number of studies have reported that autophagy induction is associated with the viability of HER2-amplified human breast cancer cells in response to the anti-HER2 monoclonal antibody Tzb, few studies have investigated the association between autophagy and HER2 in gastric cancer. To the best of our knowledge, the present study is the first to investigate the function of autophagy in HER2-refractory gastric cancer.

The present study revealed that Tzb reduced the expression of LC3 I and increased the expression of SQSTM1 in NCI-N87 cells, suggesting that Tzb may inhibit autophagic flux. Tzb-refractory cells exhibited less autophagic flux and increased viability compared with the parental cancer cells, suggesting that autophagy may induce cell death in gastric cancer cells. The involvement of autophagy in tumorigenesis and cancer therapy is complex. In the present study, the...
autophagy inhibitors, BafA1 and 3MA, induced NCI-N87 cell death, but did not induce the death of Tzb-refractory cells. HCQ was able to overcome Tzb resistance, but had a weaker effect than that observed in the parental NCI-N87 cells. These results suggest that, in Tzb-refractory NCI-N87 cells, cell apoptosis is prevented via autophagy inhibition and not autophagy activation. Certain studies have also reported that the autophagy inhibitor, chloroquine, is an effective treatment for Tzb-refractory HER2 gene-amplified metastatic breast cancer (14), which is in line with the results of the present study in NCI-N87 cells. As HCQ is not only an autophagy inhibitor, having other functions that include Toll-like receptor regulation (21) and AMP-activated protein kinase induction (22), it may induce cancer cell death via various pathways not associated with autophagic flux.

However, certain aspects of the results obtained in NCI-N87 cells in the present study are not consistent with the previous results associated with breast cancer (13,15). One reason for this is that the cell lines used were different. It has been reported that HER2 protein overexpression and gene amplification are more heterogeneous in gastric cancer than in breast cancer (5). In gastric and gastroesophageal cancer, HER2 overexpression varies and prognostic relevance is inconsistent (5), which may explain, to a certain extent, the inconsistency in the mechanisms of autophagy in different Tzb-refractory cells. Additionally, Tzb resistance time and the method used to achieve Tzb resistance differ to those used previously.

The Akt/mTOR signaling pathway is an important negative regulator of autophagic flux (23-25). In the present study, the phosphorylation of Akt and mTOR in Tzb-refractory cells and parental NCI 87 cells was examined. The data demonstrated that Tzb may activate the Akt/mTOR pathway to inhibit autophagic flux.

Everolimus has been approved by the US Food and Drug Administration for the treatment of renal and breast cancer (26). The present study demonstrated that everolimus induces the cell death of parental gastric cancer cells and Tzb-refractory cells, suggesting that the mTOR inhibitor inhibits Tzb-refractory cell growth. Fuereder et al (27) reported that another mTOR inhibitor, BEZ235, inhibited the growth of NCI-N87, but not that of MK45 or MKN28 (HER2-negative gastric cancer) xenografts. Furthermore, Zhu et al (28) reported that BEZ235 exerts extensive antitumor activity in HER2-positive gastric cancer (NCI-N87 and SNU216) in vitro and in vivo. These results implied that mTOR may serve an important function in HER2-positive gastric cancer and that the resistance of HER2 to Tzb may, at least partially, be due to mTOR activation.

In conclusion, the present study suggests that autophagy may be involved in the resistance of human gastric cancer cells to Tzb. Furthermore, it was also revealed that the Akt/mTOR pathway is involved in the Tzb resistance of gastric cancer cell lines and that the autophagy inducer, everolimus, is able to inhibit cell growth in these cell lines. These results suggest that Tzb-refractory cells prevent cell apoptosis via autophagic flux inhibition, and that everolimus may overcome resistance to Tzb in HER2-positive gastric cancer. Although these effects are not fully understood and the details of such mechanisms require further investigation, autophagy inhibited by Tzb appears to provide cells with the capacity to evolve and develop Tzb resistance, thereby preventing gastric cancer cell death.

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