Trastuzumab induces PUMA-dependent apoptosis and inhibits tumor growth in gastric cancer
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Keywords
apoptosis; gastric cancer; GC; GSK3β; PUMA; trastuzumab

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(Received 13 June 2018, revised 15 August 2018, accepted 24 August 2018)
doi:10.1002/2211-5463.12522

Gastric cancer (GC) is one of the most prevalent cancers worldwide [1]. Unfortunately, most GC patients are diagnosed at the terminal stage of the disease [1,2]. Many patients develop metastasis even if radical excision is performed [1,3]. Remarkable progress has been made in the surgical treatment for GC, yet the 5-year overall survival rate is limited to 24.5% in Europe and merely 40-60% in Asia. This latter finding may be attributable to an elevated recurrence rate [2]. Previous studies have proved that the median survival of patients with terminal GC lacking chemotherapy is no longer than six months [4,5]. Thus far, the understanding of how chemotherapy drugs counteract malignancies is insufficient.

As a humanized antibody acting against human epidermal growth factor receptor 2 (HER2), trastuzumab has been approved by the FDA to treat metastatic GC, gastroesophageal junction cancer, and breast cancer. However, the mechanisms involved in trastuzumab-induced GC cell apoptosis remain largely unknown. In this study, we investigated the underlying mechanisms of trastuzumab-mediated suppression of GC cell growth both in vitro and in vivo. We found that trastuzumab treatment induces p53 upregulated modulator of apoptosis (PUMA) expression in GC cells, through the NF-κB pathway following AKT inhibition and glycogen synthase kinase 3β (GSK3β) activation. We also observed that PUMA was necessary for trastuzumab-induced apoptosis in GC cells. Moreover, PUMA deficiency suppressed apoptosis and the antitumor effect of trastuzumab in xenograft models. Finally, computerized tomography (CT) and immunohistochemistry results showed that patients with increased activation of PUMA were more sensitive to trastuzumab treatment than those with low PUMA expression. These results indicate that trastuzumab induces PUMA-dependent apoptosis and inhibits tumor growth in GC, suggesting that PUMA plays a critical role in mediating the antitumor effects of trastuzumab in GC. PUMA induction may be used as a marker of trastuzumab sensitivity.

Abbreviations
ATCC, American Type Culture Collection; CT, computerized tomography; DSCT, dual-source computed tomography; GC, gastric cancer; GSK3β, glycogen synthase kinase 3β; HER2, human epidermal growth factor receptor 2; PUMA, p53 upregulated modulator of apoptosis; RT-PCR, reverse transcriptase–real-time PCR; TFs, transcriptional factors.
PUMA mediates trastuzumab-induced apoptosis

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Pertuzumab is another type of humanized antibody acting against HER2. The supplementation of pertuzumab with trastuzumab remarkably postponed trastuzumab response and promoted its ability to counteract the development of subsequent malignancy [11,12]. Nevertheless, the objective response rate to this therapy is merely 24.2%, while a complete response is limited to 8% [12,13]. P53 upregulated modulator of apoptosis (PUMA), which is transcriptionally targeted by p73 and p53, can stimulate cell apoptosis in diverse malignancies [14]. PUMA, which is a member of the Bcl-2 family, possesses one ‘BH3-only’ domain. [15]. It is located in the mitochondria where it stimulates cell apoptosis when modulated by mitochondrial malfunction [16]. It acts against the activities of Mcl-1 as well as Bel-X<sub>L</sub> via Bax/Bak [17]. It has been indicated by various studies that cancer growth can be suppressed by PUMA [18]. Additionally, PUMA independent of or accompanied by chemotherapy or radiotherapeutic agents displays a remarkable capacity to counteract the development of various types of malignancies [19,20]. Although trastuzumab triggers diverse reactions in HER2-positive GC patients, the mechanisms underlying its GC inhibitory effect remain unclear.

In this study, we aimed to elucidate the mechanisms of trastuzumab involved in suppressing GC cell growth both in vitro and in vivo.

Materials and methods

Cell culture

Human GC line NCI-N87 was acquired from American Type Culture Collection (ATCC, Manassas, VA, USA). DMEM was supplemented with 100 µg/mL streptomycin, 100 units/mL penicillin, and 10% v/v of heat-inactivated newly born calf serum (Invitrogen, Carlsbad, CA, USA). Drugs applied to counteract malignancy such as BAY 11-7082 (Merck, Kenilworth, NJ, USA) as well as trastuzumab (Genentech, South San Francisco, CA, USA) were dissolved in DMSO. AKT, which was constitutively activated, was obtained from Addgene (Cambridge, MA, USA).

MTS

Cells (1×10<sup>4</sup> cells/well) were cultured in 96-well microtiter plates. Subsequent to overnight incubation, the wells were supplemented with the indicated dilution range of trastuzumab. Subsequently, plates were incubated for a further 72 h. Following this, cellular proliferation was determined using the MTS assay kit (Promega, Madison, WI, USA). Wallac Victor 1420 Multilabel Counter, provided by PerkinElmer, was used to detect luminescence. All measurements were made in triplicate, and assays were repeated three times.

Reverse transcriptase–real-time PCR

TRIzol RNA Kit (Invitrogen) was used to purify total RNA in accordance with the manufacturer’s guidelines. In order to obtain cDNA, we utilized 1 µg total RNA as template in a reaction catalyzed by SuperScript II reverse transcriptase (provided by Invitrogen and used as recommended by the manufacturer). PCRs to detect differences in expression between the indicated genes were carried out in triplicate (final volume 20 µL) using SsoFast<sup>TM</sup> Probes Supermix (Bio-Rad Laboratories, Hercules, CA, USA). Detection was carried out using the CFX96<sup>TM</sup> Real-time PCR System (Bio-Rad Laboratories) using standard thermal cycling conditions over thirty-five amplification cycles. The relative expression of related genes was assessed using standard TaqMan Gene Expression Real-Time PCR assays. The results were measured as threshold cycles (Ct). Comparative Ct approaches (ΔΔCt) were applied to measure the expression of the targeted transcripts, and differential expression was evaluated using 2−ΔΔCt approaches.

Western blotting

Western blotting was carried out as outlined previously [21]. Antibodies applied included anti-AKT, GSK3β, FoxO3a, and p65 with or without phosphorylation, caspase 3 and 9 subsequent to cleavage, PUMA (Abcam, Cambridge, MA, USA), cytochrome c, E2F1, β-actin, Bid, Bak, Bim, lamin A/C (Santa Cruz Biotechnology, Santa Cruz), STAT1, p-STAT1, Cox IV (Cell Signaling Technology, Beverly, MA, USA), Mcl-1, and Bel-X<sub>L</sub> (BD, San Jose, CA, USA).

Apoptosis assay

Cell apoptosis was assessed using nuclear staining approaches (Hoechst 33258, Invitrogen) [22]. PI (Annexin V/propidium iodide) staining was performed using both Annexin-Alexa 488 (Invitrogen) and PI. To evaluate the release of cytochrome C, cytosolic fractions were separated with the help of differential centrifugation, and indicated fractions were subsequently probed by western blotting.

Transfecting and siRNA/shRNA knockdown

Lipofectamine 2000 (Invitrogen) was used to stably transfect cells. Knockdown assays were carried out using siRNA (200 pm) prior to trastuzumab treatment. SiRNA for scrambled control, for p65 (sc-29410), as well as for GSK3β (sc-35527) were obtained from Santa Cruz Biotechnology. Plasmid DNA permitting the expression of shRNA and including the nucleotide sequence targeted by PUMA (CCT
GGA GGG TCA TGT ACA ATC TCTT) or the same plasmid vector including scrambled sequence as a negative control was used to stably transfect NCI-N87 cells. Subsequently, 96-well plates were seeded with cells, with the growth media supplemented with 5 µg·mL\(^{-1}\) puromycin. Western blotting was used to evaluate clones that were resistant to puromycin.

**Chromatin immunoprecipitation**

A Chromatin Immunoprecipitation Assay Kit provided by Milipore was used for the Chromatin immunoprecipitation (ChIP) assay using anti-p65 antibody (Cell Signaling Technology). PCR was applied to evaluate the sediment using primers (5’-CCC GCG TGA CGC TAC GGC CC-3’ and 5’-GTC GGT CTG TGT ACG CAT CG-3’) for the detection and amplification of the PUMA promoter, including the potential κB site.

**Animal models of malignancies**

Experimental procedures using animals were carried out according to the ethical principles of Tongde Hospital in Zhejiang Province. Nude mice were purchased from the Experimental Animal Center of Chinese Academy of Sciences in Shanghai. PUMA-KD cells and parental cells were obtained. Media (0.1 mL) containing 4 × 10⁶ cells were subcutaneously injected into the back of female nude mice whose thymus had been excised. Tumors began to grow after 7 days. Mice received a supplement of trastuzumab (daily, 3 mg·kg\(^{-1}\), twice a week). Calipers were applied to measure the volume of the tumors based on the formula of \(\frac{1}{2} \times \text{length} \times \text{width}^2\). The tumors were excised as soon as the volume reached 1.0 cm\(^3\). Informed consent of each subject. The study methodologies conformed to the standards set by the Declaration of Helsinki.

**Immunohistochemistry examination**

Cancer slices were placed on slides in order to carry out immunohistochemical evaluation. Deparaffinized tumor slices were incubated in citrate buffer (pH 6.0) for 40 min at 95°C in order to recover antigens. Anti-PUMA antibodies resuspended at a dilution of 1:100 were consequently added to these slices and incubated at room temperature for 2 h. The avidin–biotin–peroxidase complex approach was utilized to terminate immunoreactive staining.

**Computerized tomography and tissue specimens**

Each computerized tomography (CT) scan was acquired using a dual-source computed tomography (DSCT) scanner. Twenty GC patients who received trastuzumab treatment were recruited from Tongde Hospital of Zhejiang Province, China. CT was conducted prior and subsequent to treatment, in order to measure the size of the tumors. Informed consent was acquired from patients, and the Ethics Committee of Tongde Hospital of Zhejiang Province approved our study. GC specimens were obtained from patients during the course of their surgeries. Personal information of patients was maintained confidential. Every sample was pathologically analyzed and categorized in agreement with WHO criteria. The experiments were undertaken with the understanding and written consent of each subject. The study methodologies conformed to the standards set by the Declaration of Helsinki.

**Statistical analysis**

GraphPad Prism IV (La Jolla, CA, USA) software was applied to conduct statistical analysis. Student’s t-test was used to analyze data. \(P < 0.05\) indicated statistical significance. Results are presented as means ± SD.

**Fig. 1.** Trastuzumab induces p53-independent PUMA induction in gastric cancer cells. (A) NCI-N87 cells were treated with 10 µmol·L\(^{-1}\) trastuzumab at indicated time point. PUMA mRNA induction by trastuzumab was analyzed by reverse transcriptase–real-time (RT-PCR), with β-actin as a control. The results were expressed as the means ± SD of three independent experiments. ***\(P<0.001\); **\(P<0.01\) (Student’s t-test). (B) NCI-N87 cells were treated with 10 µmol·L\(^{-1}\) trastuzumab at indicated time point. PUMA expression was analyzed by western blotting. (C) NCI-N87 cells were treated with trastuzumab at indicated concentration for 24 h. PUMA expression was analyzed by western blotting. (D) NCI-N87 cells transfected with p53 siRNA or control siRNA were treated with trastuzumab at indicated concentration for 24 h. PUMA expression was analyzed by western blotting. (E) NCI-N87 cells treated with 10 µmol·L\(^{-1}\) trastuzumab at indicated time point. The expression of indicated Bcl-2 family members was analyzed by western blotting.
Results

Trastuzumab triggered PUMA stimulation independent of p53 in GC cells

In order to explore how cell apoptosis was triggered by trastuzumab in GC cells, expression of PUMA was examined in NCI-N87 cells supplemented with trastuzumab. Addition of trastuzumab to NCI-N87 cells remarkably promoted transcription as well as translation of PUMA depending on dose and time (Fig. 1A–C). Transcription and translation of PUMA were also triggered by trastuzumab in p53-KD NCI-N87 cells (Fig. 1D). Trastuzumab supplementation did not promote the protein level of Bim, but reduced the protein level of antiapoptotic, such as Mcl-1 and Bcl-XL (Fig. 1E). These results indicate that trastuzumab can trigger the expression of PUMA based on p53 and that the malignancy counteraction effect of trastuzumab could be attributable to PUMA.

PUMA participated in cell apoptosis triggered by trastuzumab

Subsequently, the role of PUMA in cell apoptosis triggered by trastuzumab was explored using PUMA-KD NCI-N87 cells. When supplemented with trastuzumab, cell apoptosis was noticeably stimulated in NCI-N87 cells, whereas it was considerably suppressed in PUMA-KD cells (Fig. 2A). The limited expression of PUMA in NCI-N87 cells suppressed cell apoptosis triggered by trastuzumab, which was verified using Annexin V/PI staining (Fig. 2B). Trastuzumab supplementation triggered the stimulation of caspase-9 as well as caspase-3, and the release of cytochrome c, which were inhibited in PUMA-KD cells (Fig. 2C,D). These results show that PUMA is essential for cell apoptosis triggered by trastuzumab in GC cells.

NF-κB modulated expression of PUMA triggered by trastuzumab

The mechanisms by which PUMA was stimulated by trastuzumab were then explored. Some transcriptional factors (TFs) that modulate the stimulation of PUMA in p53-KD NCI-N87 cells were evaluated in order to reveal the mechanisms of how PUMA was stimulated. Previous works reported that Her2 inactivation promotes PUMA upregulation in FoxO3a-dependent increase of PUMA expression, which in turn triggers apoptosis [23,24]. Our results showed that FoxO3a was not included as a result of its unaltered suppression of phosphorylation following trastuzumab supplement (Fig. 3A). STAT1 and p73 were eliminated
because of a lack of stimulation in phosphorylation/activation assays (Fig. 3A).

It was previously reported that NF-κB participated in PUMA transcription after the addition of Aurora kinase inhibitors [17], TNF-α, or regorafenib [25]. NCI-N87 cells receiving trastuzumab triggered p65 (S536) phosphorylation in a time-dependent manner (Fig. 3B). PUMA stimulation was inhibited by p65 knockdown with the help of siRNA via supplementation with trastuzumab (Fig. 3C). Subsequently, p65 was translocated to the nucleus, which could be inhibited by the specific suppressor of NF-κB, BAY 11-7082 (Fig. 3D). NF-κB suppression eliminated stimulation of PUMA as well as phosphorylation of p65 triggered by trastuzumab, indicating that p65 stimulation/nuclear translocation modulated PUMA promotion via trastuzumab (Fig. 3E). Consequently, the ability of NF-κB to bind to the promoter of PUMA was explored. It was discovered by ChIP that p65 was recruited to the PUMA promoter after the addition of trastuzumab (Fig. 3F). Thus, our research proved that the expression of PUMA was modulated by p65 via direct binding to various NF-κB sites as a result of trastuzumab supplementation.

**GSK3β is necessary for PUMA activation by trastuzumab**

Subsequently, we determined whether GSK3β participated in the stimulation of p65 triggered by trastuzumab. First, we found that GSK3β siRNA but not the control siRNA suppressed trastuzumab-induced nuclear translocation of p65 (Fig. 4A). GSK3β exhaustion...
eliminated PUMA stimulation triggered by trastuzumab in NCI-N87 cells (Fig. 4B). Supplementation with trastuzumab remarkably inhibited AKT (Ser473) stimulation in a time-dependent manner (Fig. 4C). Furthermore, treatment with trastuzumab inhibited GSK3β Ser9 phosphorylation, which inhibited its kinase activity (Fig. 4C). Excessive expression of AKT that was constitutively activated in turn inhibited stimulation of PUMA as well as p65 triggered by trastuzumab (Fig. 4D). Our research indicates that AKT suppression modulated stimulation of GSK3β, bringing about translocation of p65 as well as stimulation of PUMA via trastuzumab.

**PUMA participated in malignancy counteraction of trastuzumab in vivo**

Whether cell apoptosis was modulated by PUMA participating in trastuzumab-mediated counteraction of malignancy was then explored in xenografts, which were generated with the help of PUMA-KD as well as parental NCI-N87 cells in nude mice. Mice developing cancer received trastuzumab (3 mg-kg⁻¹) or vehicle twice a week. The size of the tumors was examined every 48 h. PUMA-KD, as well as parental tumors, displayed nearly identical growth patterns in groups without trastuzumab supplementation. Parental cancer growth was inhibited by 70–80% trastuzumab supplementation (Fig. 5A). Contrarily, PUMA-KD tumors displayed weaker growth suppression subsequent to trastuzumab supplementation in comparison with parental cancers (Fig. 5A), suggesting that PUMA shortage eliminated trastuzumab-mediated counteraction of malignancy. Trastuzumab promoted the expression of PUMA as well as phosphorylation of p65 in xenografts (Fig. 5B). It was suggested by TUNEL staining that cell apoptosis was noticeably promoted in parental cancers when supplemented with trastuzumab.
However, TUNEL staining of PUMA-KD cancers supplemented with trastuzumab (Fig. 5C) was not positive. Consequently, our results indicate that PUMA was essential for trastuzumab-mediated counteraction of malignancy in vivo.

**PUMA determines trastuzumab sensitivity in GC patients**

In order to assess the role of PUMA in trastuzumab sensitivity in GC patients, twenty GC patients who had received trastuzumab therapy were studied. The size of each tumor was examined by using CT prior and subsequent to trastuzumab supplement. Immunohistochemical and RT-PCR analyses were used to detect PUMA expression. Additionally, PUMA expression and alterations in cancer sizes in twenty GC tissues were examined using RT-PCR and CT. Our study showed that the expression of PUMA was linked with cancer size suppression (Fig. 6A,B), indicating that the expression of PUMA served as a reliable predictor of trastuzumab sensitivity in treatment of GC.

**Discussion**

Developing drug resistance remains the first challenge of antitumor treatment [26,27]. Furthermore, when the occurrence of breakdowns in the normal functioning of shared molecular pathways triggers carcinogenesis, the choice of antitumor treatments can be greatly complicated [28,29]. Our research aimed at revealing the link between expression of PUMA and trastuzumab-mediated mechanisms of counteracting malignancy in GC. In our study, the role of trastuzumab in GC was explored. Our findings are the first to demonstrate that trastuzumab-mediated mechanisms of counteracting malignancy are modulated by autonomic cell apoptosis, which results from AKT suppression, nuclear translocation of p65, as well as GSK3β stimulation, thereby bringing about PUMA stimulation and mitochondrial-dependent cell apoptosis. Our results indicate that trastuzumab induces PUMA-dependent apoptosis and inhibits tumor growth in GC, suggesting that PUMA plays a critical role in mediating the antitumor effects of trastuzumab in GC and PUMA induction may be used as an indicator of trastuzumab sensitivity.

Illuminating the response to trastuzumab remains an essential challenge in order to treat GC patients not responding to trastuzumab-dependent chemotherapy, because HER2 serves as a promising target of GC cells that are HER2 positive. Until now, the roles played by individual contributors to the trastuzumab response are not sufficiently well understood. However, it has been discovered that trastuzumab sensitivity is linked to excessive expression of HER2 in diverse malignancies. Previous study demonstrated that HER2 directly phosphorylates PUMA and this leads to PUMA degradation and suppression of apoptosis [30]. In our research, we demonstrated that the expression of PUMA is triggered by trastuzumab via the GSK-3β/NF-κB pathway subsequent to AKT suppression and triggers cell apoptosis depending on the number of mitochondria in GC cells. PUMA stimulation is involved in cell apoptosis brought about by chemotherapeutic drugs and serves as a promising predictor of sensitivity. It has been demonstrated in a previous study that PUMA stimulation is linked with diverse EGFR TKIs sensitivity and that insufficient PUMA stimulation is linked with EGFR TKI resistance [31]. Additionally, it has been recently reported that the reaction of separated mitochondria from mutations to BH3 domains of PUMA is related to chemotherapy response [31–33]. The findings of our
study indicate that the expression of PUMA can serve as a reliable predictor of malignancy counteraction of trastuzumab in GC cells. Despite the fact that acquiring biopsies from GC patients receiving chemotherapy following surgery is difficult, it is nonetheless a promising line of inquiry to examine PUMA stimulation in noninvasive ways, including evaluation of cancer cells in circulation.

Conclusions
In conclusion, our study elucidated an innovative way in which malignancy can be counteracted using trastuzumab, via cell apoptosis modulated by PUMA, independent of p53. Furthermore, trastuzumab-mediated alterations in PUMA expression may serve as a reliable predictor in clinical experiments and can assist in determining essential implications for therapeutic progression as well as for applications in the future.

Acknowledgements
This work was supported by the National Natural Science Foundation of China (No. 81301200) and Pudong New Area Health and Family Planning Commission, Shanghai (No. PWRd2016-07).

Author contributions
HL and QX conceived and designed the project. LL, HL, and QX performed the experiments. LL and QX wrote the manuscript. HL and QX revised the final manuscript.

Conflict of interest
The authors declare no conflict of interest.

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