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PTEN loss is associated with a poor response to trastuzumab in HER2-overexpressing gastroesophageal adenocarcinoma

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Abstract

Background Although trastuzumab improves outcome of patients with HER2 overexpressing gastric or gastro-esophageal junction adenocarcinoma (GEA), no clinical response is observed in a substantial population of patients. A predictive biomarker of trastuzumab response is required. The aim of this study was to evaluate whether the hyper-activation of downstream PI3K pathway, due to PTEN loss or *PIK3CA* mutations, could provide trastuzumab resistance in GEA.

Methods Expression of HER2 and PTEN, and *PIK3CA* gene mutations were screened in surgically resected 264 GEA specimens. The effects of PTEN knock-down on the response to trastuzumab on cell viability, HER2 downstream signaling, apoptosis, and cell cycle were evaluated in HER2 overexpressing NCI-N87 gastric and OE19 esophageal adenocarcinoma cell lines. Inhibition of xenograft tumor growth by trastuzumab was investigated using OE19 cells with or without PTEN knock-down. The PTEN expression and objective response were analyzed in 23 GEA patients who received trastuzumab-based therapy.

Results PTEN loss was identified in 34.5 % of HER2 overexpressing GEA patients, whereas *PIK3CA* mutations were rare (5.6 %). Trastuzumab-mediated growth suppression, apoptosis, and G1 cell cycle arrest were inhibited by PTEN knock-down through Akt activation in NCI-N87 and OE19 cells. PTEN knock-down impaired the anti-proliferative effect of trastuzumab in OE19 xenograft models. A clinical response was observed in 50 % of PTEN positive tumors (9/18), while in 0 % of tumors with PTEN loss (0/5).

Conclusions PTEN loss was frequently found in HER2 overexpressing tumors, and was associated with a poor response to trastuzumab-based therapy in patients with GEA.

Mini-abstract

PTEN loss is frequently found in HER2 overexpressing tumors and is closely associated with a poor response to trastuzumab-based therapy in patients with gastric or esophageal adenocarcinoma.

Keywords

Gastric adenocarcinoma, Esophageal adenocarcinoma, PTEN, HER2, Trastuzumab

Introduction

Gastric cancer is the fifth most common malignancy and the third leading cause of cancer death in the world [1]. Most gastric cancer patients have highly advanced disease at presentation, despite improvements in the diagnosis, and thus the treatment option is mainly limited to systemic chemotherapy. Patients with advanced gastric cancer rarely achieve long-term survival and the median survival remains 8 to 14 months [2, 3]. In addition, the incidence of gastro-esophageal junction adenocarcinoma has increased considerably in the past 40 years, especially in industrialized countries [4].

The ToGA (Trastuzumab for Gastric Cancer) trial, a recent international randomized controlled trial, demonstrated a significant survival benefit of trastuzumab combined with chemotherapy for human epidermal growth factor receptor 2 (HER2) overexpressing gastric or gastro-esophageal junction adenocarcinoma (collectively referred to as gastro-esophageal adenocarcinoma; GEA) [5]. However, more than half of the patients in the ToGA trial did not exhibit an objective response by trastuzumab, despite HER2 overexpression. Moreover, trastuzumab has several unique adverse effects, such as cardiotoxicity, which causes severe cardiac dysfunction in 0-3.9 % of the patients [6]. Therefore, a predictive biomarker is required to select patients who are likely to benefit from trastuzumab-based therapy.

The phosphatidylinositol 3-kinase (PI3K) pathway is a major downstream signal transduction pathway of HER2. In breast cancer, constitutive activation of the PI3K pathway due to phosphatase and tensin homolog (PTEN) deficiency or phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA*) gene mutations was identified to be one of the important mechanisms of trastuzumab resistance [7-9].

However, the mechanisms of trastuzumab resistance in GEA have yet to be clarified. We hypothesized that the hyper-activation of the PI3K pathway might contribute to trastuzumab resistance also in GEA. The first aim of this study was to evaluate the frequency of PTEN

loss and *PIK3CA* mutations, and to evaluate the relationship between alterations in the PI3K pathway and HER2 overexpression in GEA. The second aim was to explore whether PTEN loss or *PIK3CA* mutation is associated with trastuzumab resistance and influences the response to trastuzumab therapy in patients with GEA.

Materials and Methods

Patients

Two separate cohorts of patients were investigated in the study. First, 326 patients who underwent R0 curative resection for primary GEA at the Kyoto University Hospital between January 2001 and December 2010 were diagnosed with pathologic T2-T4 and pathologic TNM stage IB-IIIC (Seventh Edition of the Union for International Cancer Control TNM classification). Sixty-two patients received preoperative chemotherapy and were excluded from the study. Therefore, surgical specimens from a total of 264 patients were screened for HER2 expression, PTEN expression and *PIK3CA* mutation status (screening cohort).

The second cohort included 24 patients with HER2 overexpressing advanced GEA who received trastuzumab-based therapy from November 2010 to December 2014 at Kyoto University Hospital. Specimens for HER2 and PTEN evaluation were obtained by biopsy or surgical resection, but a specimen was not available in one patient. Therefore, a total of 23 patients were included in the study to investigate the influence of PTEN loss on trastuzumab sensitivity (investigation cohort). Trastuzumab was administered to these patients with oral fluoropyrimidine drugs (capecitabine or S-1) with or without cisplatin. The objective response was assessed using RECIST v1.1 [10].

All patients gave informed consent for additional molecular analyses at the time of biopsy or surgery, and formalin-fixed paraffin-embedded tissue specimens were obtained under the

protocol approved by the Institutional Review Board of Kyoto University (E1752). Clinicopathological information was retrieved from medical charts and pathological reports.

Evaluation of HER2 expression and amplification

The HER2 expression was analyzed by immunohistochemistry (IHC), and *HER2* amplification was evaluated by dual-color *in situ* hybridization (DISH) as previously described [11]. Briefly, IHC staining of HER2 with the PATHWAY® HER2/neu (4B5) antibody (Ventana Medical Systems) was performed using an automated slide staining system (Bench-Mark XT; Ventana Medical Systems). The scoring system defined in the ToGA study was employed for IHC scoring (evaluated by membranous reactivity in ≥10 % of tumor cells.) [5]. DISH was performed using the INFORM Dual ISH HER2 kit (Ventana Medical Systems). The post hoc analysis of the ToGA study showed that IHC 0/1+ patients with *HER2* gene amplification (ISH +) did not benefit from trastuzumab-based chemotherapy [5]. Therefore, HER2 overexpression was defined as either IHC 3+ or IHC 2+ with DISH positive in this study.

Evaluation of PTEN expression

The evaluation of PTEN expression was performed by IHC using an anti-PTEN monoclonal antibody (clone 138G6, #9559, Cell Signaling Technology, Danvers, MA; diluted 1:200) according to the manufacturer's protocol. PTEN IHC was subjectively scored as absent (0) if no immunostaining was detectable in cancer cells; as weak (1+) if cytoplasmic staining was low; as moderate (2+) if cytoplasmic staining was intermediate between weak and strong; and as strong (3+) if the cytoplasmic staining was intense (**Fig. 1**). PTEN loss was determined when there was negative staining (score 0) of cells in more than 75 % of the tumor as previously reported [12]. A PTEN positive status was defined as weak,

moderate, or strong staining (score 1+ to 3+) of cells in >25 % of the tumor. Interpretation was performed by 2 independent observers (Y.D. and S.M.), and discrepancies were discussed to gain a final result.

PIK3CA mutation analysis

Samples from 97 of the 264 patients that underwent surgery before 2004 were not in the appropriate status for mutational analysis. DNA was successfully extracted from macro-dissected tumor tissue (>75 % tumor content) from the remaining 167 patients, using the NucleoSpin DNA FFPE XS kit (Macherey-Nagel, Germany) according to the manufacturer's instructions. Mutation analysis of the hot spot regions of *PIK3CA* (exon 9 and 20) was performed by PCR amplification followed by direct sequencing using a 3130xl Genetic Analyzer (Applied Biosystems, USA).

Cell lines, cell culture and reagents

The human gastric adenocarcinoma cell lines, NCI-N87, AGS, KATO-III, and SNU-1 were purchased from American Type Culture Collection (ATCC, Manassas, VA), and MKN1, MKN45, MKN7, and MKN74 were purchased from RIKEN BRC Cell Bank (Ibaraki, Japan). The human esophageal adenocarcinoma cell lines, OE19 and OE33, and the gastric adenocarcinoma cell line, HGC-27, were purchased from European Collection of Cell Culture (ECACC). All cell lines were cultured in RPMI 1640 medium supplemented with 10 % fetal bovine serum (FBS; Life technologies, Carlsbad CA, USA), penicillin (100 units/ml) and streptomycin (100 μg/ml; Life Technologies), and were incubated at 37 ˚C in a humidified chamber containing 5 % CO_2.

Trastuzumab was kindly provided by Chugai Pharmaceutical Co., Ltd. (Tokyo, Japan) for non-clinical investigations. The anti-HER2 Affibody® Molecule (FITC) (ab31891) was

purchased from AbCam (AbCam, Cambridge, MA) for flow cytometric analysis of membranous HER2 expression.

Small interfering RNA (siRNA) and short hairpin RNA (shRNA)

Two distinct siRNA species targeting PTEN (siPTEN #1, Hs_PTEN_6 FlexiTube siRNA, SI00301504; siPTEN #2, Hs_PTEN_8 FlexiTube siRNA, SI03048178) and non-silencing control siRNA (AllStars Negative Control siRNA, SI03650318) were purchased from Qiagen, and were transfected using Lipofectamine RNAiMAX (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's reverse-transfection protocol. Briefly, siRNA at final concentration of 3 mM with Lipofectamine RNAiMAX in OPTI-MEM was first added to wells or dishes and incubated at room temperature for 30 minutes, and then cells were seeded. Cells were incubated overnight and the culture media was changed to fresh media or 10 μg/ml of trastuzumab containing media.

The control shRNA vector (pLKO.1 scrambled shRNA) and packaging vector (psPAX2, plasmid 12260) and envelop vector (pMD2.G, plasmid 12259) were purchased from Addgene Inc. (Cambridge, MA). The shRNA sequences targeting PTEN was made as the same sequences as the purchased siRNA (shPTEN #1 as siPTEN #1 and shPTEN #2 as siPTEN #2), and cloned into pLKO.1 vectors. Lentivirus containing shRNA constructs was made according to the manufacturer's protocol, and infected to cell lines. Stable knock-down clones were selected by culturing cells with puromycin (2 μg/ml) for at least 4 weeks.

Western blotting

Cells were washed with ice-cold PBS and lysed in SDS lysis buffer supplemented with Protease Inhibitor Cocktail (Nacalai Tesque Inc., Kyoto, Japan) and Phosphatase Inhibitor Cocktail (Nacalai Tesque Inc., Kyoto, Japan). The protein concentration was determined with

the BCA Protein Assay kit (Thermo Scientific, Rockford IL, USA). A total of 20 μg of whole cell lysate were subjected to SDS-PAGE and transferred to PVDF membrane (Merck Millipore Co.). Membranes were probed with specific primary antibodies against HER2 (polyclonal, #2242), PTEN (clone 138G6, #9559), pan-Akt (clone C67E7, #4691), phospho-Akt (Ser473, clone D9E, #4060), p44/p42 MAPK (ERK1/2) (clone 137F5, #4695), and phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204, clone D13.14.4E, #4370) (Cell Signaling Technology, Danvers, MA), and horseradish peroxidase (HRP)-conjugated secondary antibody (Dako). HRP-conjugated anti-ACTB antibody (Sigma) was used as a loading control. Bands were visualized using Pierce Western Blotting Substrate kit (Thermo Scientific).

Cell viability and cell growth inhibition assay

The cell viability was measured by the WST-8 colorimetric assay (Cell Counting Kit-8(CCK-8), Dojindo, Kumamoto, Japan). Exponentially growing cells (2-10 × 10^3/100 μl/well) were seeded in triplicate into 96-well plates. The cells were treated with or without trastuzumab for 72 hours, and 10 μl of the CCK-8 was added into each well and the plates were further incubated for 3 hours. The absorbance at 450 nm and 600 nm was measured to calculate the numbers of viable cells in each wells using GloMax-Multi Detection System (Promega). Cell growth inhibition (%) was calculated using the formula: (1-experimental absorbance (treated well)/control absorbance (untreated well)) × 100.

Apoptosis assay

Exponentially growing cells (5-10 × 10^3/100 μl/well) were plated in triplicate in 96-well plates. The cells were treated with or without trastuzumab for 72 hours, and caspase3/7 activity was measured using the Caspase-Glo3/7 Assay (Promega) according to the

manufacturer's protocol. Luminescence was measured using GloMax-Multi Detection System (Promega). Caspase activity was normalized to the cell number using the CCK-8 assay performed simultaneously under the same conditions [13].

Cell cycle analysis

The cells were dissociated with trypsin/EDTA, PBS washed and fixed with ice cold 70 % ethanol at –20 ˚C overnight. The cells were treated with ribonuclease I (Nacalai Tesque Inc.) for 10 min. at 37 ˚C to digest RNA and stained with 100 μg/ml of propidium iodide (Sigma). The cells were analyzed by flow cytometry (BD Accuri^{TM} C6 Flow Cytometer).

Xenograft experiments

Six-week-old female KSN/slc athymic nude mice (Japan SLC, Shizuoka, Japan) were purchased and maintained under specific pathogen-free conditions. A total of 3×10^6 scrambled or stable PTEN knock-down OE19 cells in 100 μl of PBS were injected subcutaneously into 3 to 4 points of the back. Treatment with intraperitoneal injection of trastuzumab (30 mg/kg as a loading dose, then 15 mg/kg weekly) or normal saline was started 2 weeks after inoculation, when tumors became palpable and measurable. The tumor volume was estimated by the formula; 0.5 × L×W×W (L: length, W: width). The experiments were approved by the institutional Animal Ethics and Research Committee (MedKyo15513).

Statistical analysis

All values were expressed as mean ± standard deviation (SD). All *in vitro* experiments were repeated at least three times. Statistical analyses were performed using JMP pro 11 software (SAS). Student *t*-test was used to compare continuous variables. Dunnett's test was

used for multiple comparisons. Categorical variables were analyzed by the Fisher's exact test. Differences were considered statistically significant at a p value < 0.05.

Results

PTEN loss is frequent whereas *PIK3CA* mutation is rare in HER2 overexpressing GEA

The expression/amplification of HER2, expression of PTEN, and mutations of *PIK3CA* gene were screened in a total of 264 patients with GEA. The characteristics of the screening cohort are shown in **Table 1**. IHC scores of HER2 were 3+ in 22 patients and 2+ in 13 patients. A heterogeneous expression pattern of HER2 was often observed (**Fig. 2a**). DISH was positive in 7 of the 13 IHC 2+ patients. Therefore, 29 tumors (11.0 %) were defined as positive for HER2 overexpression (IHC 3+, or IHC 2+ and DISH positive; **Table 1**).

Expression of PTEN was evaluated by IHC using the scoring system described in the Materials and Methods (**Fig. 1**). PTEN loss was found in 62 patients (23.5 %, **Table 1**). Homogeneous expression of PTEN (≥90 % of tumor cells) was observed in most PTEN positive cases (**Fig. 2b**).

The mutation status of *PIK3CA* gene was successfully evaluated in 167 patients and a total of 17 *PIK3CA* mutations were identified in 16 patients (9.6 %, **Table 1**): 6 mutations in exon 9 (E545K in 5 cases and E545A in 1 case), and 11 mutations in exon 20 (H1047R). One patient had mutations both in exon 9 and exon 20. A comparison of the frequency of these alterations in relation to HER2 status revealed that the incidence of PTEN loss was higher in HER2 overexpressing tumors in comparison to HER2-negative tumors, although the difference was not significant (34.5 % *vs.* 22.1 %, $p = 0.163$, Fisher's exact test; **Table 1**). On the other hand, *PIK3CA* mutation was less frequent both in HER2 overexpressing and HER2-negative tumors (5.6 % *vs*. 10.1 %, $p = 1.000$, Fisher's exact test; **Table 1**).

PTEN knock-down induces trastuzumab resistance in trastuzumab-sensitive cell lines

The screening data suggested that PTEN loss is clinically more relevant in comparison to *PIK3CA* mutation. Therefore, we focused on PTEN deficiency, since this might have a major impact on trastuzumab resistance in GEA. Nine gastric and 2 esophageal adenocarcinoma cell lines were screened to evaluate HER2 and PTEN expression and trastuzumab sensitivity. Western blotting revealed that the NCI-N87 gastric adenocarcinoma cell line and OE19 esophageal adenocarcinoma cell line overexpressed HER2 protein, and that both cell lines expressed PTEN protein **(Fig. 3a**). Flow cytometry also revealed that HER2 protein was strongly expressed on the cell surface both in NCI-N87 cell line and OE19 cell line (data not shown). The cell viability was examined with different concentrations of trastuzumab in a total of 11 cell lines to determine the trastuzumab sensitivity. Two HER2 overexpressing cell lines, NCI-N87 and OE19, were sensitive to trastuzumab treatment at a concentration of 1 μg/ml (**Fig. 3b**).

PTEN knock-down was performed using non-silencing control siRNA (Ns) and 2 different sequences of siRNA targeting PTEN (referred to as siPTEN #1 and siPTEN #2) to investigate the influence of PTEN deficiency on the trastuzumab sensitivity. PTEN expression was sufficiently knocked-down in both NCI-N87 and OE19 cell lines at 72 hours and 96 hours from transfection of siRNA (**Fig. 3c, 3d**). Further analyses were performed between 72 hours and 96 hours after transfection.

The cell growth inhibition rate was calculated as a percentage reduction of cell viability when treated with 10 μg/ml of trastuzumab for 72 hours in comparison to untreated controls. Both the NCI-N87 and OE19 cell lines showed a significantly lower cell growth inhibition rate in PTEN knock-down cells, in comparison to cells with control siRNA (19.3 and 19.5 % *vs.* 39.9 % in NCI-N87, $p < 0.01$; 19.5 and 22.1 % *vs*. 34.1 % in OE19, $p < 0.01$; **Fig. 3e, 3f**).

PTEN knock-down reverses trastuzumab-induced Akt inactivation, inhibits apoptosis, and partially suppresses G1 cell cycle arrest

The phosphorylation status of Akt and ERK, two key proteins in the PI3K and the mitogen-activated protein kinase (MAPK) downstream signaling pathways of HER2, were evaluated to investigate the mechanisms by which PTEN knock-down induces trastuzumab resistance. Phosphorylation of Akt and ERK were both considerably reduced in the non-transfected NCI-N87 and OE19 cells by treatment with 10 μg/ml of trastuzumab for 24 hours (**Fig. 4a**, Nt). However, phosphorylation of Akt was mostly maintained in spite of trastuzumab treatment following PTEN knock-down with siPTEN #1 and #2, while ERK was dephosphorylated to a similar extent to cells transfected with non-silencing control siRNA (**Fig. 4a**, Ns).

The relative caspase 3/7 activity, which represents apoptosis activity, following treatment with 10 μg/ml of trastuzumab for 72 hours was measured in comparison to the untreated controls in NCI-N87 and OE19 cells transfected with non-silencing control or PTEN targeting siRNAs. While trastuzumab treatment increased the relative caspase 3/7 activity in both cell lines with non-silencing control siRNA, increments of the caspase activity with trastuzumab treatment were significantly lower in PTEN knock-down cells (1.56 *vs.* 1.13 and 1.14 in NCI-N87, $p < 0.01$; 1.66 *vs.* 1.20 and 1.43 in OE19, $p < 0.01$; **Fig. 4b**).

The fraction of G1 phase was significantly increased following treatment with 10 μg/ml of trastuzumab for 72 hours in both NCI-N87 and OE19 cells with non-silencing control siRNA (69.8 % to 74.4 % in NCI-N87, $p < 0.01$; 72.4 % to 75.3 % in OE19, $p < 0.01$), indicating that trastuzumab induced G1 cell cycle arrest. On the other hand, the proportion of G1 phase was not increased by trastuzumab treatment in the PTEN knock-down NCI-N87 cells. Similar

results were also obtained in OE19 cells, although a moderate increase of G1 phase was observed in cells with siPTEN #1 (**Fig. 4c**).

PTEN knock-down induces trastuzumab resistance in xenograft models

This study further explored the influence of PTEN knock-down on trastuzumab sensitivity using xenograft models. Stable PTEN knock-down clones and scrambled control clones were established using lentiviral infection of shRNAs and puromycin selection in NCI-N87 and OE19 cells. The PTEN expression was sufficiently knocked-down in OE19 cells (**Fig. 5a**), whereas the PTEN knock-down was not sufficient in NCI-N87 cells (data not shown). The scrambled control OE19 cells (scramble) or stable PTEN knock-down OE19 cells (referred to as shPTEN #1 and shPTEN #2) were inoculated subcutaneously, and treatment with intraperitoneal injection of normal saline or trastuzumab (30 mg/kg loading dose followed by 15 mg/kg weekly) was started 2 weeks after inoculation, when tumors became palpable and measurable. The representative images of the xenograft tumors with normal saline treatment or trastuzumab treatment are shown in **Fig. 5b** and **Fig. 5c**, respectively. The tumor volume in the scrambled control tumors was significantly decreased in response to trastuzumab treatment in comparison to normal saline treatment (**Fig. 5d**, $p = 0.035$). In contrast, the proliferation of the tumors derived from PTEN knock-down cells was not suppressed by the trastuzumab treatment (**Fig. 5e** and **5f**, $p = 0.200$ and $p = 0.886$). HER2 expression and PTEN expression in xenograft tumor tissues after trastuzumab treatment were evaluated by IHC (**Fig. 5g**). Expression of both HER2 and PTEN was homogeneous in these tissues. HER2 expression was similar among scrambled tumors and PTEN knock-down tumors, whereas PTEN expression was strong in scrambled tumors and negative in PTEN knock-down tumors.

Patients with PTEN loss show a poor response to trastuzumab-based therapy

We further investigated the clinical significance of PTEN loss in trastuzumab therapy for GEA in the investigation cohort. Twenty three patients that received trastuzumab-based therapy were examined. Nineteen of those patients were treated by capecitabine and cisplatin with trastuzumab, 3 patients were treated by S-1 and cisplatin with trastuzumab, and 1 patient was treated by capecitabine with trastuzumab. **Table 2** shows that PTEN loss was found in 5 patients. None of these 5 patients with PTEN loss showed an objective clinical response to trastuzumab-based therapy. On the other hand, 9 out of 18 PTEN positive patients (50 %) responded to the trastuzumab-based therapy including 1 complete response and 8 partial responses, although the difference in the response was not statistically significant between PTEN loss and PTEN positive patients (**Table 2**, $p = 0.142$).

Discussion

Trastuzumab has significantly improved survival in patients with HER2 overexpressing GEA [5]. However, a limited response and resistance to trastuzumab-based therapy remain as obstacles. Only a few reports have examined the relationship between PTEN loss and trastuzumab resistance in gastric cancer [14-16], although PI3K pathway alteration, including PTEN loss, is well known as an inducer of trastuzumab resistance in breast cancer [7-9]. The current study is the first to comprehensively investigate the impact of PTEN deficiency on trastuzumab response in HER2 overexpressing esophageal adenocarcinoma.

The current screening for PTEN loss and *PIK3CA* gene mutations, which involve two major aberrations of the PI3K pathway, revealed that 23.5 % of patients showed PTEN loss and 9.6 % showed *PIK3CA* mutations in a cohort of 264 curatively resected GEA patients. These findings are consistent with those of previous reports in gastric cancer (11-21.5 % with

PTEN loss [17-19], and 2.5-15.9 % with *PIK3CA* mutations [18, 20-22]). More importantly, as many as 34.5 % of patients with HER2 overexpression who are thought to be candidates for trastuzumab therapy showed PTEN loss, whereas *PIK3CA* mutations were found in only 5.6 % of these patients. These data suggest the potential importance of PTEN loss in affecting the response to trastuzumab-based therapy in GEA.

Zaitsu *et al*. showed that loss of heterozygosity of PTEN, which confers PTEN deficiency, was associated with HER2 overexpression [19]. However, some reports have shown that loss of PTEN expression or deletion of the *PTEN* gene is rarely found (3.7-5.3 %) in HER2 overexpressing gastric cancer [23, 24]. The discrepancy in these results might be due to variations in the definition of PTEN loss. Thus, a validated standard definition of PTEN loss may be required to evaluate the influence of PTEN expression on the response to trastuzumab.

HER2 expression in GEA is often heterogeneous, as we have previously reported [11]. While HER2 expression was also highly heterogeneous in the current screening cohort, PTEN expression was relatively homogeneous (**Fig. 2**). A previous study also found that PTEN deletion was often homogeneous in gastric cancer [24]. Given the homogeneous distribution of PTEN-negative cells in GEA, evaluation of PTEN expression in a biopsy specimen is likely to be a reliable method for evaluating PTEN loss.

Trastuzumab suppresses the HER2 signaling of both the PI3K pathway and MAPK pathway, and thus induces apoptosis and G1 cell cycle arrest, which contribute to the anti-proliferation effect [25]. The current study showed that the cell growth inhibition by trastuzumab was significantly suppressed in PTEN knock-down cells, in which Akt remained phosphorylated despite trastuzumab treatment, and trastuzumab-induced apoptosis and G1 cell cycle arrest were both prevented. These findings suggest that constitutively activated Akt by PTEN knock-down leads to cell survival and cell proliferation by inhibiting apoptosis and

G1 cell cycle arrest, resulting in trastuzumab resistance. The importance of the inhibition of the PI3K pathway on the anti-tumor effect of trastuzumab should be different among different cell types, because trastuzumab can act via multiple mechanisms. These could explain why the degree of cell cycle inhibition by trastuzumab and its recovery by PTEN knock-down was slightly different between NCI-N87 and OE19 cells. Although G1 cell cycle arrest by trastuzumab was not completely restored by PTEN knock-down in OE19 cells *in vitro*, the anti-proliferative effect of trastuzumab was completely abrogated by PTEN knock-down in xenograft models. These results imply that Akt activation by PTEN loss indeed plays a major role in acquiring resistance to trastuzumab *in vivo*.

The current clinical data indicate that patients with GEA with PTEN loss have a limited response to trastuzumab-based therapy. Recently, Zhang *et al.* examined 48 gastric cancer patients with HER2 overexpression who clinically received anti-HER2 therapy (39 patients received trastuzumab therapy and 9 patients received lapatinib therapy), and showed that patients with PTEN loss demonstrated shorter progression-free survival than those with PTEN positive in gastric cancer [14]. These results and the current data support the finding that PTEN deficiency contributes to trastuzumab resistance.

Several limitations are associated with the current study. First, since the clinical data were retrospectively examined and the sample size was small, the results are only preliminary. However, of note, the findings from our functional experiments suggested that PTEN deficiency contributed to trastuzumab resistance through PI3K pathway activation in GEA cell lines. Second, we did not investigate the influence of PTEN knock-down on the combined effects of trastuzumab with other chemotherapeutic drugs, although trastuzumab is clinically approved for use in combination with other drugs for HER2 overexpressing GEA. However, there have been several studies showing that PTEN deficiency is also associated with increased resistance to multiple chemotherapeutic agents [16, 26]. Collectively, PTEN

loss could be a clinically valuable biomarker of resistance to trastuzumab-based chemotherapy in GEA. It has been suggested that combination therapy with PI3K inhibitors might be helpful in overcoming PTEN loss-mediated trastuzumab resistance in GEA [16] and in breast cancer [7, 27, 28]. These present and previous results suggest that patients with HER2 overexpressing GEA and PTEN loss might require alternative therapy to trastuzumab, or combination therapy with PI3K inhibitors.

In conclusion, HER2 overexpressing GEA includes a considerable subset of patients with PTEN loss, and this is closely associated with a poor response to trastuzumab-based therapy. Further prospective studies are required to validate the predictive value of PTEN loss in trastuzumab-based therapy for patients with HER2 overexpressing GEA.

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Ethical Standards

Human rights statement and informed consent: All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1964 and later versions. Informed consent was obtained from all patients for being included in the study.

Animal studies: All institutional and national guidelines for the care and use of laboratory animals were followed.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Legends for figures

Fig. 1 PTEN expression in gastro-esophageal adenocarcinoma specimens

Representative PTEN scoring by immunohistochemistry (IHC). Immunoreactivity of adjacent stromal cells was used as an internal positive control. **a** Score 0 = no immunostaining is detectable within the tumor cells. **b** Score 1 = the immunostaining is weak. **c** Score 2 = the immunostaining is intermediate between weak and strong. **d** Score 3 = strong immunostaining. PTEN loss was defined as negative staining (score 0) of cells in more than 75 % of the tumor (**a**), and (**b**) – (**d**) were evaluated as PTEN positive. Scale bars represent 100 μm.

Fig. 2 Distribution of IHC-positive cells in tumors

a Distribution of HER2 IHC-positive (2+ and 3+) cells. The open bar indicates HER2 IHC-negative patients. **b** Distribution of PTEN IHC-positive (scores 1, 2 and 3) cells. Open bars indicate patients with PTEN loss.

Fig. 3 PTEN knock-down induces trastuzumab resistance in trastuzumab-sensitive cell lines

a Western blotting analyses of HER2 and PTEN in 9 gastric adenocarcinoma cell lines and 2 esophageal adenocarcinoma cell lines. **b** Cell viability assays with increasing concentration of trastuzumab exposure for 72 hours in 11 cell lines. **c** and **d** PTEN knock-down was performed using 2 distinct siRNAs targeting PTEN (siPTEN #1 and siPTEN #2), and the PTEN expression was analyzed by Western blotting at the indicated time points after the transfection in NCI-N87 cell line (**c**) and in OE19 cell line (**d**). Nt non-transfected control, Ns non-silencing control siRNA. **e** and **f** Cells transfected with Ns, siPTEN #1 or siPTEN #2 were treated with 10 μg/ml of trastuzumab for 72 hours in NCI-N87 cell line (**e**) and in OE19

cell line (**f**). Growth inhibition rates were calculated as a percentage reduction of the cell viability in comparison to the untreated controls. * $p < 0.01$.

Fig. 4 PTEN knock-down reverses trastuzumab-induced Akt suppression, apoptosis, and G1 cell cycle arrest

a Western blotting analyses evaluating the influence of PTEN knock-down on phosphorylation of Akt and ERK. Non-transfected (Nt) control cells and cells transfected with non-silencing siRNA (Ns), siPTEN #1 or siPTEN #2 were untreated or treated with 10 μg/ml of trastuzumab for 24 hours. **b** Cells transfected with Ns, siPTEN #1 or siPTEN #2 were untreated or treated with 10 μg/ml of trastuzumab for 72 hours. The caspase 3/7 activity was measured using a Caspase-Glo 3/7 assay kit. The relative caspase 3/7 activity of trastuzumab treated cells in comparison to that of untreated cells was calculated in each cells. * $p < 0.01$. **c** Flow cytometry analysis detecting the distribution of G1 phase in the cell cycle. Cells transfected with Ns, siPTEN #1 or siPTEN #2 were untreated or treated with 10 μg/ml of trastuzumab for 72 hours. * $p < 0.01$.

Fig. 5 PTEN knock-down induces trastuzumab resistance in xenograft models

a Stable PTEN knock-down clones were selected and the PTEN expression was evaluated by Western blotting. **b** and **c** Representative images of xenograft model in intraperitoneal normal saline injection mice (**b**), and in intraperitoneal trastuzumab (30 mg/kg loading dose followed by 15 mg/kg weekly) injection mice (**c**). Arrow heads represent tumors with scrambled control, white and black arrows represent tumors with shPTEN #1 and shPTEN #2, respectively. Scale bars represent 1 cm. **d - f** Tumor growth curves of xenografts derived from cells with scrambled control (**d**), shPTEN #1 (**e**), and shPTEN #2 (**f**). Mice were treated with weekly intraperitoneal injection of normal saline (NS) or trastuzumab. Data represent

the mean tumor volume + SD. ** $p < 0.05$. **g** HER2 and PTEN immunohistochemistry (IHC) images of scrambled control, shPTEN #1 and shPTEN #2 xenograft tumor specimens after trastuzumab treatment. In PTEN IHC, immunoreactivity of adjacent stromal cells was used as an internal positive control. Scale bars represent 100 μm.

Table 1 Characteristics of patients and tumors based on HER2 status

Variables	HER2 overexpression	
	Positive (n = 29)	Negative (n = 235)
Age		
Median (range)	75 (54 – 87)	69 (29 – 89)
Sex		
Male	26 (89.7 %)	155 (66.0 %)
Female	3 (10.3 %)	80 (34.0 %)
Tumor location		
GE junction	3 (10.3 %)	18 (7.7 %)
Stomach	26 (89.7 %)	217 (92.3 %)
Lauren's classification		
Intestinal	22 (75.9 %)	79 (33.6 %)
Diffuse	1 (3.4 %)	128 (54.5 %)
Mixed	6 (20.7 %)	28 (11.9 %)
pT stage		
2	11 (37.9 %)	89 (37.9 %)
3	12 (41.4 %)	85 (36.2 %)
4	6 (20.7 %)	61 (26.0 %)
pN stage		
0	7 (24.1 %)	93 (39.6 %)
1-3	22 (75.9 %)	142 (60.4 %)

(**Table 1**, continued)

pTNM stage		
I	3 (10.3 %)	53 (22.6 %)
II	15 (51.7 %)	89 (37.9 %)
III	11 (37.9 %)	93 (39.6 %)
PTEN expression		
Loss	10 (34.5 %)	52 (22.1 %)
Positive	19 (65.5 %)	183 (77.9 %)
PIK3CA mutation		
Present	1 (5.6 %)[a]	15 (10.1 %)[a]
Absent	17 (94.4 %)[a]	134 (89.9 %)[a]
Not evaluable	11	86

Abbreviation: GE junction, gastro-esophageal junction

[a] Percentage in evaluable samples

Table 2 PTEN expression and objective response to trastuzumab

Objective response	PTEN expression		*P* value[a]
	Positive (n=18)	Loss (n=5)	
Complete response	1	0	0.142
Partial response	8	0	
Stable disease	7	5	
Progressive disease	2	0	

[a] Fisher's exact test.

Figure 1

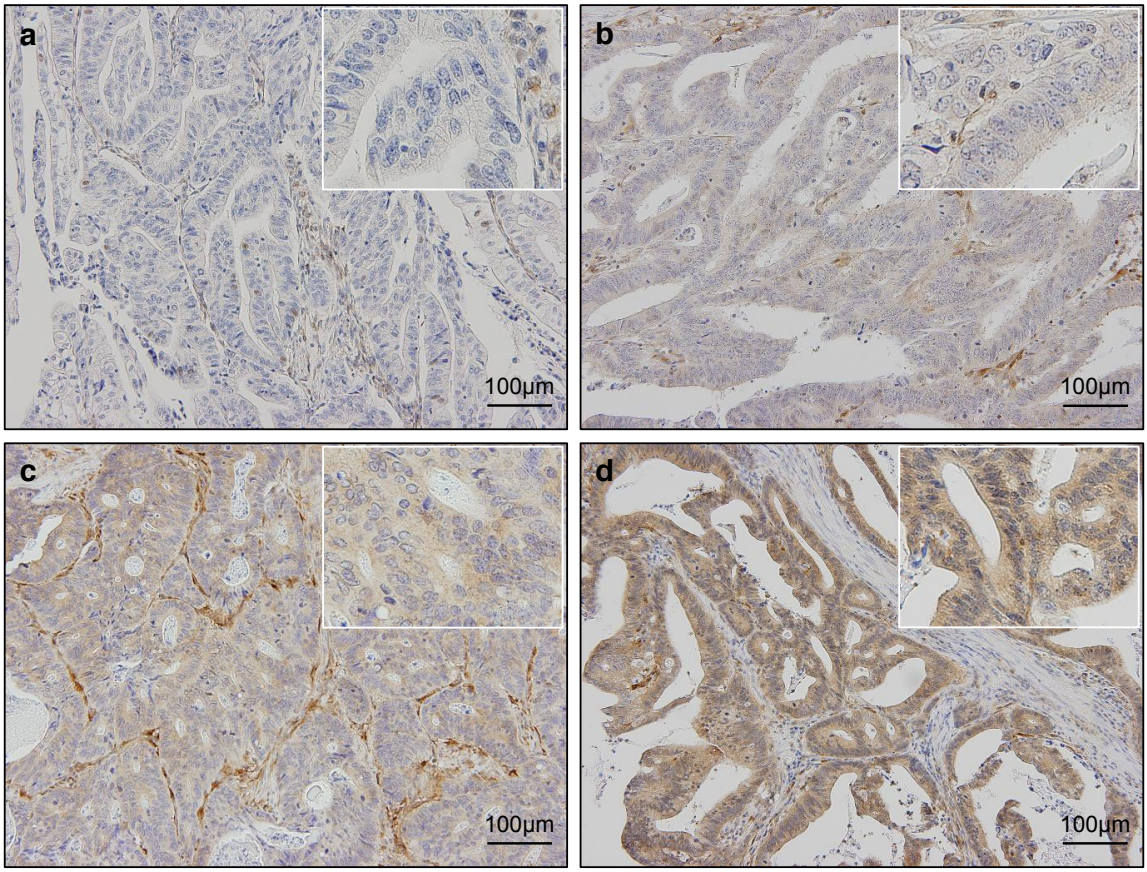


Figure 2

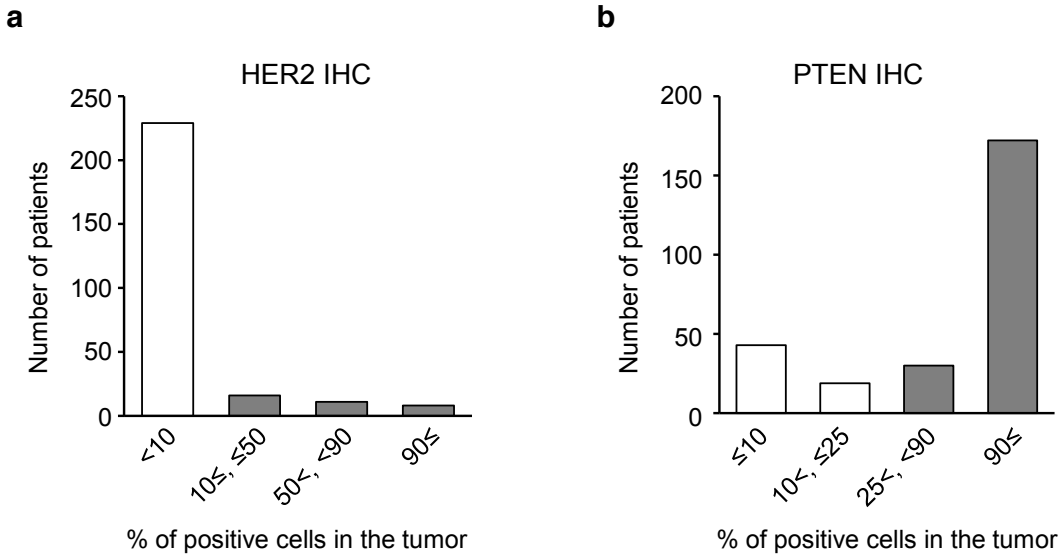

a
HER2 IHC
Number of patients
250
200
150
100
50
0
<10
10≤, ≤50
50<, <90
90≤
% of positive cells in the tumor
b
PTEN IHC
Number of patients
200
150
100
50
0
≤10
10<, ≤25
25<, <90
90≤
% of positive cells in the tumor

Figure 3

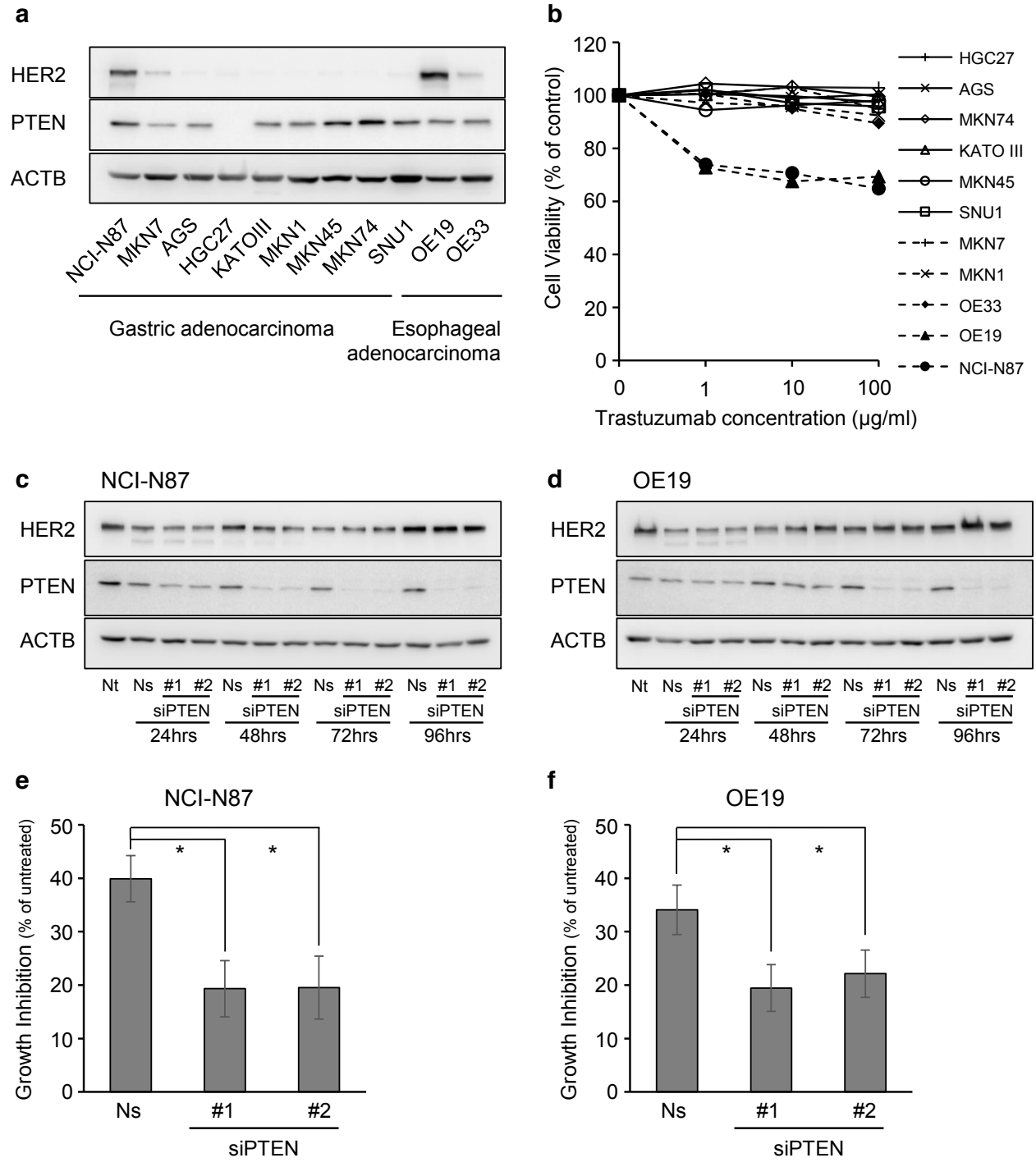

a
HER2
PTEN
ACTB
NCI-N87
MKN7
AGS
HGC27
KATOIII
MKN1
MKN45
MKN74
SNU1
OE19
OE33
Gastric adenocarcinoma
Esophageal adenocarcinoma
b
Cell Viability (% of control)
Trastuzumab concentration (μg/ml)
HGC27
AGS
MKN74
KATO III
MKN45
SNU1
MKN7
MKN1
OE33
OE19
NCI-N87
c
NCI-N87
d
OE19
Nt Ns #1 #2
siPTEN
24hrs
48hrs
72hrs
96hrs
e
NCI-N87
f
OE19
Growth Inhibition (% of untreated)
Ns
#1
#2
siPTEN

Figure 4

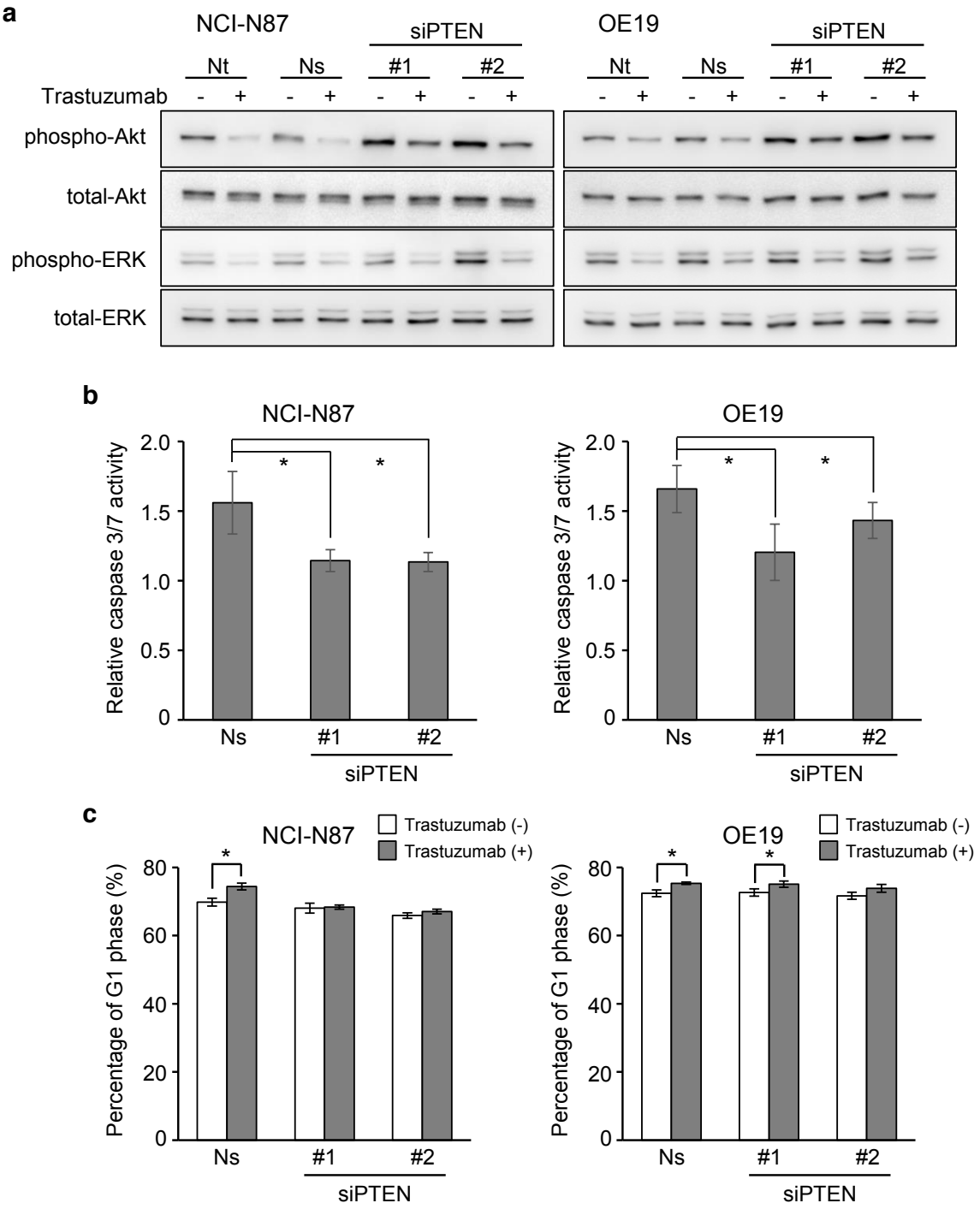
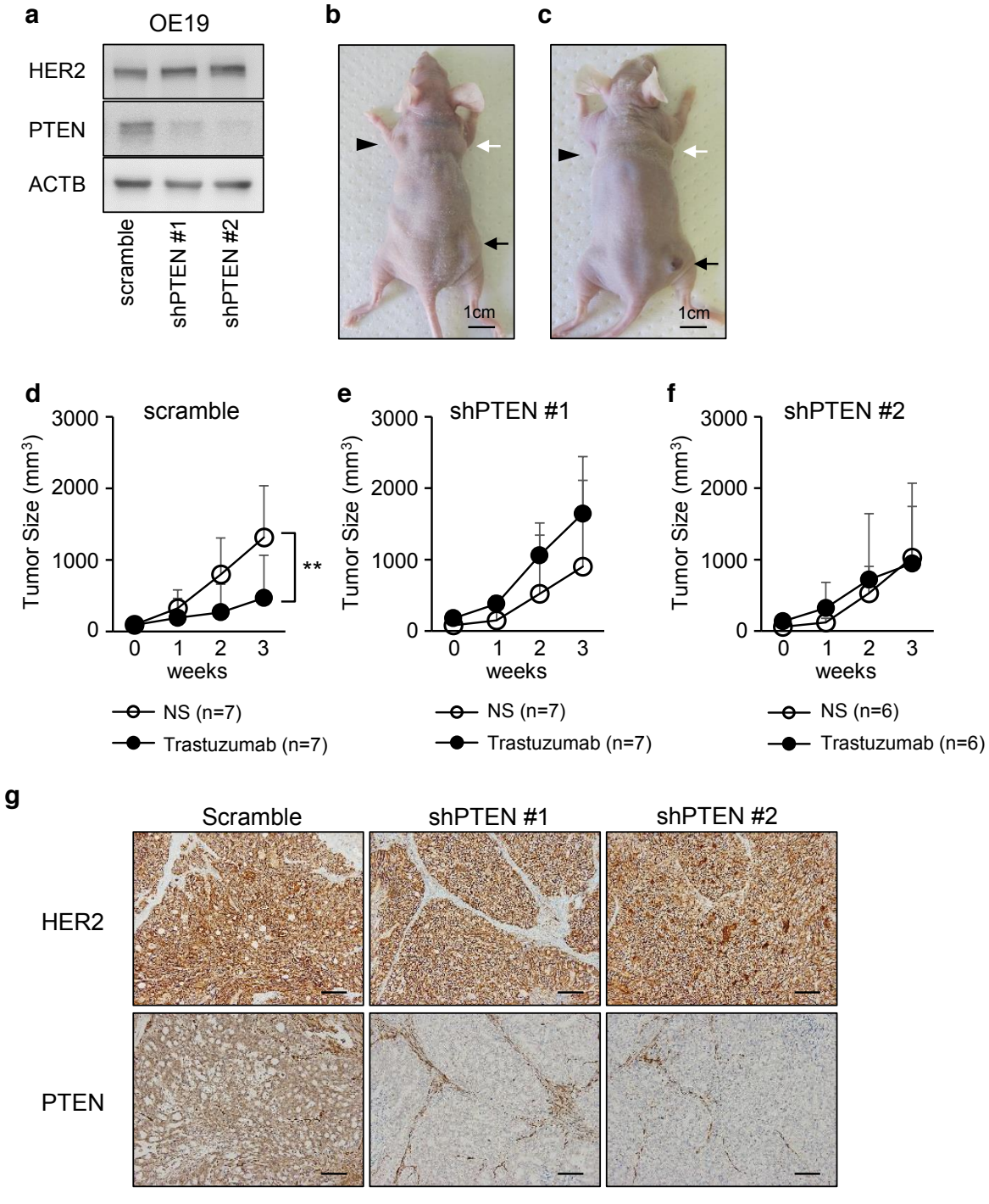

a
NCI-N87
siPTEN
OE19
siPTEN
Nt
Ns
#1
#2
Nt
Ns
#1
#2
Trastuzumab
- + - + - + - + - + - + - + - +
phospho-Akt
total-Akt
phospho-ERK
total-ERK
b
NCI-N87
OE19
Relative caspase 3/7 activity
2.0
1.5
1.0
0.5
0
*
*
Ns
#1
#2
siPTEN
c
NCI-N87
OE19
Trastuzumab (-)
Trastuzumab (+)
Percentage of G1 phase (%)
80
60
40
20
0
*
Ns
#1
#2
siPTEN

Figure 5


a
OE19
HER2
PTEN
ACTB
scramble
shPTEN #1
shPTEN #2
b
1cm
c
1cm
d
scramble
Tumor Size (mm3)
3000
2000
1000
0
0 1 2 3
weeks
**
NS (n=7)
Trastuzumab (n=7)
e
shPTEN #1
Tumor Size (mm3)
3000
2000
1000
0
0 1 2 3
weeks
NS (n=7)
Trastuzumab (n=7)
f
shPTEN #2
Tumor Size (mm3)
3000
2000
1000
0
0 1 2 3
weeks
NS (n=6)
Trastuzumab (n=6)
g
Scramble
shPTEN #1
shPTEN #2
HER2
PTEN