PTEN is a Predictive Biomarker of Trastuzumab Resistance and Prognostic Factor in HER2-overexpressing Gastroesophageal Adenocarcinoma

Daiju Yokoyama
Kyoto University
Shigeo Hisamori (hisamori@kuhp.kyoto-u.ac.jp)
Kyoto University
Yasunori Deguchi
Ijinkai Takeda General Hospital
Tatsuto Nishigori
Kyoto University
Hiroshi Okabe
New Tokyo Hospital
Seiichiro Kanaya
Osaka Red Cross Hospital
Dai Manaka
Kyoto Katsura Hospital
Yoshio Kadokawa
Tenri Hospital
Hiroaki Hata
Kyoto Medical Center
Sachiko Minamiguchi
Kyoto University
Shigeru Tsunoda
Kyoto University
Kazutaka Obama
Kyoto University
Yoshiharu Sakai
Osaka Red Cross Hospital

Research Article

Keywords: PTEN, HER2-overexpressing, gastroesophageal adenocarcinoma, Gastric cancer, prognostic factor

Posted Date: January 29th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-154193/v1

Version of Record: A version of this preprint was published at Scientific Reports on April 27th, 2021. See the published version at https://doi.org/10.1038/s41598-021-88331-3.

Abstract

Introduction

Poor Trastuzumab (Tmab) response in human epidermal growth factor receptor 2-overexpressing gastric or gastroesophageal junction adenocarcinoma (HER2-GEA) is reported to be associated with loss of phosphatase and tensin homolog (PTEN) expression.

Methods

In a multicenter, retrospective observational study, pathological samples of HER2-GEA patients receiving Tmab-combined chemotherapy were immunohistochemically analyzed for PTEN expression. Primary endpoints were disease control rate (DCR), progression-free survival (PFS), and overall survival (OS). The effect of PI3K pathway inhibitors on HER2-GEA cell lines were evaluated in *in-vitro*.

Results

Among 145 HER2-GEA patients, 29 formed the PTEN-loss group and 116 the PTEN-positive group. In patients with target region, DCR was significantly lower in PTEN-loss than in PTEN-positive patients (67% and 87%, respectively, p=0.049). Multivariate analysis demonstrated that PTEN loss was significantly associated with shorter PFS (HR=1.63, p=0.035) and OS (HR=1.83, p=0.022). PTEN knockdown did not affect the cytostatic effect of 5-FU and cisplatin, while Tmab combined with PI3K/mTOR inhibitor NPV-BEZ235 suppressed the proliferation of PTEN knockdown cells which were resistant to Tmab alone.

Conclusion

In HER2-GEA patients, PTEN loss is a predictive biomarker of Tmab resistance and prognostic factor. Molecular-targeted therapy with PI3K/mTOR inhibitor would be an effective treatment option for HER2-GEA with PTEN loss.

Introduction

Gastric cancer, including gastroesophageal junction adenocarcinoma (GEA), is the fifth most frequently diagnosed malignancy and the third leading cause of cancer death worldwide [1]. Many patients with GEA have metastatic disease at the time of diagnosis and are usually considered unresectable oncologically, and using systemic chemotherapy for GEA with distant metastasis or recurrence results in poor prognosis [2].

Identification of human epidermal growth factor receptor 2 (HER2) as an oncogene has enabled the development of molecular-targeted therapies for GEA, as well as breast cancer [3–7]. HER2 amplification and overexpression lead to continuous stimulation of downstream phosphatidylinositol-3 kinase (PI3K) or mitogen-activated protein kinase (MAPK) pathway, triggering uncontrolled cell proliferation, invasiveness, resistance to apoptosis, and angiogenesis [8]. In 2010, systemic chemotherapy combined with trastuzumab (Tmab) demonstrated a significant survival benefit in an international, randomized controlled trial and was recommended for unresectable or recurrent HER2-overexpressing GEA (HER2-GEA) [9]. However, the response rate was only 47%, indicating that 53% of the patients did not have an objective clinical response to Tmab despite HER2 overexpression. Moreover, most patients had intrinsic or developed acquired Tmab resistance within 1 year. Thus, elucidating the molecular mechanisms and identifying a predictive biomarker for Tmab resistance are critical for improving survival in HER2-GEA patients.

In breast cancer, constitutive activation of the PI3K pathway due to phosphatase and tensin homolog (PTEN) deficiency is a major Tmab resistance mechanism [10–12]. By applying this concept to gastric cancer, we recently reported the association between low PTEN expression and Tmab resistance in HER2-GEA[13]. In this previous report, approximately one-third of advanced HER2-GEA patients who underwent gastrectomy showed PTEN loss, which tended to decrease the clinical objective response to Tmab. Moreover, we showed that Tmab-induced growth suppression, apoptosis, and G1 cell cycle arrest were inhibited by PTEN knockdown using gastric or gastroesophageal adenocarcinoma cell lines. These results indicated that PTEN loss could potentially represent a predictive indicator for poor Tmab response, but this association has not been assessed in a large HER2-GEA patient cohort.

Several molecule types have been suggested as potential therapeutics for the Tmab resistant subgroup in breast cancer [14,15]. These molecules potentially inhibit PI3K pathway activation by targeting one or more of its enzymes, including PI3K, protein kinase B (Akt), mammalian Target of Rapamycin Complex 1 (mTORC1), or mTORC2, which intervene upstream or downstream of PTEN [16]. The BOLERO-3 trial, an international, randomized controlled trial, used Tmab in combination with mTORC1 inhibitor everolimus, which significantly prolonged progression-free survival (PFS) in patients with Tmab-resistant HER2-positive advanced breast cancer [17]. Furthermore, the GRANITE-1 study investigated the efficacy and safety of everolimus in advanced gastric cancer that progressed after one or two lines of prior systemic chemotherapy [18]. In this study, everolimus did not improve overall survival (OS) in advanced gastric cancer, compared with that achieved by best supportive care, but a subgroup analysis has not been reported. It is still unclear whether mTOR inhibitors are effective in patients with HER2-overexpressing and Tmab-resistant gastric cancer. To date, there are no reports on other molecular therapies inhibiting the PI3K pathway in this patient subgroup specifically. In this retrospective multicenter study, we investigated the potential of PTEN loss as a predictive biomarker of Tmab response in HER2-GEA patients and explored a new molecular treatment option targeting the signal component of the PI3K pathway.

Results

PTEN loss is associated with poor clinical response to Tmab-CTx in patients with HER2-GEA

A total of 174 HER2-GEA patients were treated with Tmab-combined chemotherapy (Tmab-CTx) from January 2011 to December 2016 in six hospitals, including the Kyoto University Hospital. We excluded 23 patients whose detailed clinical information or pathological tissue samples were not available and 5 patients who were not subjected to a clinical objective evaluation based on Response Evaluation Criteria in Solid Tumors (RECIST version 1.1)[19]. One patient who was not evaluated as HER2 2 + or 3 + in the subsequent assessment was also excluded. Finally, 145 patients were enrolled in this study (Fig. 1). The PTEN-loss group had 29 patients, and the PTEN-positive group had 116.

An assessment of the patient characteristics found no significant differences in age, sex, Charlson comorbidity index (CCI), ECOG-PS, primary site, Lauren classification, macroscopic type, HER2 score, or metastatic situation between the two PTEN expression status groups, whereas the BMI was significantly lower in the PTEN-loss group ($p = 0.041$) than in the PTEN-positive group (Table 1A). The proportion of previous chemotherapy did not significantly vary, but the PTEN-loss group had a significantly lower proportion of previous gastrectomy than the PTEN-positive group (66% vs. 22%, $p < 0.001$).

Tmab is primarily administered in combination with other anticancer agents, which were classified in our Tmab-CTx study as fluoropyrimidine antimetabolites, platinum-based drugs, and others (Table 1B). Fluoropyrimidine

antimetabolites included 5-fluorouracil (5-FU), tegafur/gimeracil/oteracil (S-1), and capecitabine (Cape). Platinum-based drugs included cisplatin (CDDP) and oxaliplatin (L-OHP). The other drugs included docetaxel (DOC), paclitaxel (Pac), and irinotecan (CPT-11). The proportion of Tmab-CTx using fluoropyrimidine antimetabolites and platinum-based drugs was significantly higher in the PTEN-positive group than in the PTEN-loss group (p = 0.013 and 0.004, respectively).

An assessment of the objective clinical response to Tmab-CTx for GEA indicated that the disease control rate (DCR) was lower in the PTEN-loss group than in the PTEN-positive group (72.4% and 86.2%, respectively; p = 0.094), while there was no significant difference in the response rate between the PTEN-loss and PTEN-positive groups (34.5% and 44.8%, respectively; p = 0.402) (Table 2A). Among the cases with target lesions, DCR was significantly lower in the PTEN-loss than in the PTEN-positive group (66.7% and 86.6%, respectively; p = 0.049), whereas the response rate did not differ between the PTEN-loss and PTEN-positive groups (47.6% and 52.6%, respectively; p = 0.811) (Table 2B). The PTEN-loss group had a relatively shorter stable disease (SD) duration than the PTEN-positive group (8.3 months vs. 13.6 months, respectively; p = 0.063) (Table 2C).

PTEN loss is associated with a significantly shorter PFS and OS in patients with HER2-GEA receiving Tmab-CTx

With an overall median follow-up duration of 14.3 months, the median PFS and OS of all patients with HER2-GEA were 8.9 and 19.2 months, respectively. Patients with PTEN loss had a significantly shorter PFS period than those who were PTEN positive (Fig. 2a; 6.4 vs. 10.0 months, respectively; p = 0.018). Furthermore, patients with PTEN loss had a significantly shorter OS than patients with PTEN positive (Fig. 2b; 13.3 vs. 21.0 months, respectively; p = 0.021).

PTEN loss has prognostic significance and is a predictive factor for shorter OS and PFS in patients with HER2-GEA receiving Tmab-CTx

To identify predictive factors for OS and PFS in Tmab-CTx, we subjected various clinicopathological and molecular characteristics to univariate and multivariate analyses (Table 3). We found that two or more lesions of metastases and PTEN loss were significantly related to shorter PFS in HER2-GEA based on univariate analysis (p = 0.003 and p = 0.020, respectively) and multivariate analysis (p = 0.002 and p = 0.035, respectively). For OS, platinum-containing regimen, two or more lesions of metastases, and PTEN loss were significantly related to shorter OS in HER2-GEA based on univariate analysis (p = 0.049, p = 0.002, and p = 0.023, respectively). In the multivariate analysis, macroscopic type 4, two or more lesions, and PTEN loss were significantly related to shorter OS (p = 0.038, 0.001, and 0.022, respectively).

Inhibitory effect of Tmab on cell growth is specifically suppressed by PTEN knockdown in HER2-GEA cell lines

To investigate the drug sensitivities in relation to the PTEN expression status in HER2-GEA, both NCI-N87 and OE19 cells were used to establish two types of stable PTEN-knockdown clones and a scrambled control clone by lentiviral infection of short hairpin RNAs (shRNAs) (referred to as “shPTEN#1” and “shPTEN#2” for each cell line; Supplementary Fig. 1). The cell viability assay showed that the tumor growth inhibition rate of Tmab was significantly decreased in the PTEN knockdown clones of both cell lines (Fig. 3a, b), as demonstrated in a previous report [13]. In contrast, the tumor growth inhibition rates of 5-FU and CDDP did not significantly vary in either cell line with or without PTEN knockdown.

Molecular-targeted drugs inhibiting downstream factors in the PI3K pathway have an antiproliferative effect in PTEN knockdown HER2-GEA cell lines

To identify effective molecular targets in addition to Tmab treatment for PTEN knockdown in HER2-GEA, we tested the growth inhibitory activity of PI3K pathway inhibitors: LY294002, PI3K inhibitor; everolimus, mTORC1 inhibitor; MK-2206, Akt inhibitor; and NVP-BEZ235, potential inhibitor of PI3K, mTORC1, and mTORC2 (Supplementary Fig. 2). LY294002 had a limited growth inhibitory effect in both PTEN knockdown cell lines of NCI-N87 and OE19 with or without Tmab. Growth inhibition by erverolimus combined with Tmab was weak in NCI-N87 cells. MK-2206 had a relatively high growth inhibitory effect in both PTEN knockdown lines of NCI-N87 and OE19 with or without Tmab, and the inhibitory effect was augmented in combination with Tmab. The growth inhibitory effect of NVP-BEZ235 with Tmab in both PTEN knockdown NCI-N87 and OE19 cells was more than 70%, compared with that in the control cells (Fig. 3c, d, e, f).

To examine the pharmacokinetics of Tmab or PI3K inhibitors, the phosphorylation status of Akt, S6, and extracellular-signal-regulated kinase (ERK), which are critical downstream factors in the PI3K and MAPK pathways, was examined by western blotting (Fig. 3g). All original images, including controls, are in the supplementary information. The levels of phospho-Akt, phospho-S6, and phospho-ERK were increased in PTEN knockdown lines of NCI-N87, compared with control cells transfected with nonsilencing small interfering RNAs (siRNAs). Tmab alone suppressed the levels of phosho-Akt, phospho-S6, and phospho-ERK, but their levels were increased using Tmab alone in PTEN knockdown cell lines. LY294002 did not affect the levels of phospho-Akt, phospho-S6, and phospho-ERK in PTEN knockdown cells. Everolimus, in combination with Tmab, lowered the phospho-S6 level but did not affect the levels of phospho-Akt and ERK in PTEN knockdown cells. MK-2206 suppressed the levels of phospho-Akt and phospho-S6 but did not affect the phospho-ERK level in PTEN knockdown cells, even when tested in combination with Tmab. NVP-BEZ235 alone reduced the levels of phospho-Akt and phospho-S6 but did not affect the phospho-ERK level in PTEN knockdown cells. However, the combination of NVP-BEZ235 and Tmab reduced the levels of phospho-Akt, phospho-S6, and phospho-ERK in PTEN knockdown cells.

Discussion

This multicenter retrospective study demonstrated that PTEN loss was associated with a poor response to Tmab in HER2-GEA patients. Although we conducted a retrospective study, the clinical objective evaluation and PTEN immunohistochemistry (IHC) were performed under certain conditions and assessed by multiple physicians and a pathologist who were blinded for the clinical outcome, which makes the results exceptionally reliable. So far, there have been a couple of reports in which HER2-GEA patients were divided into two groups according to the therapeutic effect of Tmab, and their histological characteristics and gene expression pattern were evaluated [20,21]. In both reports, PTEN loss was focused on as an important factor of Tmab resistance. Our study is the first report in which the expression pattern of PTEN was analyzed in blind manner to the patient outcomes with a central judgment and the pathological effect and prognosis of patients were evaluated based on the expression pattern of PTEN. Moreover, we found that combining Tmab with PI3K pathway inhibitors might overcome Tmab resistance in HER2-GEA with PTEN loss. This result suggested the possibility of developing a new therapeutic approach for patients with HER2-GEA.

We previously reported that the continuous activation of HER2 downstream signaling pathways by PTEN loss could be a potential mechanism for lowering the efficacy of Tmab in HER2-GEA [13]. One important suggestion from the current study is to provide guidance for improving personalized medicine in gastric cancer treatment. The PTEN expression status confirmation in HER2-GEA should allow the early identification of patient subsets with effective Tmab treatment response and the advance planning of additional treatment for patient subsets with a less effective Tmab treatment response. In the GRANTE-1 trial, everolimus (mTOR1 inhibitor) failed to show statistically significant efficacy in all gastric cancer patients, but the study details revealed that everolimus was clearly effective in a

subpopulation [18]. Furthermore, the SWOG phase II trial demonstrated the potential efficacy of Akt inhibitor MK2206 in patients with GEA [22]. It is possible that these molecular-targeted drugs are effective in a subpopulation of HER2-GEA patients identified by biomarkers.

We used the best overall response of each patient according to RECIST v1.1 to evaluate the clinical objective response. Almost all study subjects underwent Tmab treatment combined with conventional therapy. We retrospectively demonstrated that CDDP and 5-FU had at least a transient and constant effect on HER2-GEA regardless of PTEN expression. The primary endpoint of our study was the DCR, defined as the period during which Tmab suppressed the disease progression of HER2-GEA. Importantly, we determined that the duration of the best overall response by Tmab-CTx was longer in the PTEN-positive than in the PTEN-loss group, and this tendency was more pronounced in patients with target lesions. Furthermore, the PTEN-loss group had a shorter SD duration than the PTEN-positive group. These outcomes are consistent with the Tmab mechanism based on blocking cell proliferation by inhibiting intracellular signaling[23,24].

Our multivariate analysis indicated that PTEN loss was a predictive factor for significantly shorter PFS and OS periods, indicating that PTEN loss in HER2-GEA was associated with insufficient Tmab treatment response, resulting in a poor OS. Although the ratios of platinum-based and 5-FU-based combinations with Tmab differed significantly between the PTEN-positive and the PTEN-loss group, only the number of patients who received platinum-based treatment was included in the multivariate analysis. This value was selected because most cases from both study groups were treated with 5-FU and platinum chemotherapy. Originally, our study included patients who were diagnosed as HER2-positive and treated with Tmab. Since important factors, such as the number of metastatic sites, macroscopic type, and Lauren classification, were also adjusted for confounding, we assumed that our results were sufficiently reliable.

However, it is still unclear whether PTEN loss is an independent prognostic factor for all HER2-GEA patients because this study did not include HER2-GEA patients without Tmab treatment. Moreover, the relationship between PTEN expression and patient prognosis in HER2-negative GEA remains unclear. Since a recent report demonstrated that PTEN negative was a poor prognostic factor for all gastric cancer patients [25], it might be necessary to consider personalized treatment for PTEN-negative gastric cancer regardless of HER2 expression. Further clinical studies are needed to determine which treatment strategies are required for PTEN-negative GEA patients.

Our *in vitro* experiments suggested that combining Tmab with BEZ235 (dual inhibitor of PI3K and mTOR) was more effective in HER2-GEA with PTEN loss, compared to Tmab combinations with other PI3K pathway inhibitors. Under the PTEN loss status, the PI3K pathway is activated downstream of HER2 by a complex feedback mechanism; thus, the suppression of a single factor may not be sufficient for growth suppression in HER2-GEA. Interestingly, BEZ235 with Tmab inhibited the expression of pERK, which is a key downstream factor of the MAPK pathway. As described in previous reports, complex interactions between the PI3K and MAPK pathways make it difficult to explain why a PI3K/mTOR inhibitor affects the expression of pERK [26]. However, our results implied that concurrent inhibition of upstream and downstream factors of the PTEN action site could effectively inhibit growth of HER2-GEA. Dual PI3K-mTOR inhibitors have been investigated as a new therapeutic option [27], but the risk of adverse effects should be assessed for future clinical applications.

The current study has several limitations. Firstly, since this was a retrospective study, we could not rule out the effects of unmeasured confounders and case selection bias. For example, there were differences in the combination regimens with Tmab, as well as second- or third-line therapies, including empirical treatment. Secondly, since we conducted a multicenter retrospective study, the follow-up interval and the timing of the following imaging study

depended on each attending doctor. However, most of the patients were subjected to a diagnostic imaging examination within 3 months from the Tmab-CTx initiation, and the physiological or image analysis findings for each patient were reviewed by two independent physicians, which likely made these assessments sufficiently reliable. Thirdly, the expression pattern of HER2 and PTEN in gastric cancer is potentially heterogeneous[28–32], and IHC of HER2 and PTEN proteins can only be confirmed by biopsy or surgical specimens. Thus, it is unclear how HER2 and PTEN are expressed when the disease progresses or a new recurrence appears. Establishing a treatment strategy for recurrence with multidrug resistance will remain a critical issue in gastric cancer therapy.

Based on our retrospective study, a considerable subset of HER2-GEA patients with PTEN loss is potentially resistant to Tmab and could benefit from additional molecular-targeted therapies suppressing the activation of the PI3K pathway. Our study could provide guidance for overcoming a terrible GEA outcome by pioneering precision medicine with molecular-targeted therapy.

Methods

Patients, clinical data, and pathological tissue sample collection

This study was approved by the review board of each hospital, including Kyoto University (confirmation No. R1230), and patients provided informed consent for sample use and data analysis through opt-out agreements. This multicenter, retrospective observational study was conducted at Kyoto University, Osaka Red Cross Hospital, Kyoto Medical Center, Kyoto Katsura Hospital, Tenri Hospital, and Otsu Municipal Hospital. The study protocol was approved by the ethics committee of Kyoto University and each hospital. This study was conducted in accordance with the Ethical Guidelines for Medical and Health Research Involving Human Subjects and the Declaration of Helsinki.

We retrospectively enrolled 174 patients who received systemic Tmab-CTx for HER2-GEA between January 2011 and December 2016 in any of the participating hospitals. Clinical data and pathological tissue samples were collected from patients at each hospital. We included patients 1) whose HER2 score was 2 plus and the fluorescence in situ hybridization result was positive or whose HER2 score was 3 plus, and 2) who had received Tmab-CTx. The exclusion criteria were as follows: 1) pathological tissue samples or detailed clinical information could not be obtained, and 2) could not be evaluated for the objective response to chemotherapy. Using the database of each hospital, we obtained the clinical information of these patients since they received Tmab-CTx retrospectively. Comorbidity was assessed using the CCI.

Pathological tissue samples with formalin-fixed, paraffin-embedded sections were obtained from the specimens of surgeries or endoscopic biopsy when the case was diagnosed as HER2-GEA. These samples were processed at the pathological department of each hospital.

PTEN expression analysis

Tissue sections were analyzed for PTEN by IHC with PTEN monoclonal antibody (clone 138G6, #9559, Cell Signaling Technology, Danvers, MA; diluted 1:200) at Kyoto University, as reported previously [13,33]. PTEN IHC was subjectively scored as absent (0) without detectable immunostaining in the whole cancer specimen, as weak (1+) with low cytoplasmic staining, as moderate (2+) with intermediate staining (between weak and strong), and as strong (3+) with intense staining (Supplementary Fig. 3). PTEN loss was defined as negative staining (score 0) of cells in more than 75 % of the tumor [13]. Interpretation was performed by three independent observers, including a specialist of pathology, and discrepancies were discussed to obtain a final result.

Clinical response to Tmab-CTx

We evaluated the clinical objective response to Tmab-CTx according to the RECIST version 1.1 [19]. The best overall response to Tmab-CTx was recorded as the objective response of each patient. This evaluation was based on radiological images, including computed tomography results, along with magnetic resonance imaging and relevant physical examination findings, all of which were reviewed retrospectively in a blinded manner by at least two clinicians.

The sum of the proportion of complete response (CR) and partial response (PR) represented the response rate used to compare the PTEN-loss with the PTEN-positive group. Then, the DCR, which was defined as the sum of the proportions of CR, PR, SD, and non-CR/non-PD, was used as the study's primary endpoint for comparing these two groups. In addition, DCR was evaluated in patients with target lesions. The duration of SD, which represented the period from the Tmab-CTx initiation date to the progressive disease (PD) diagnosis date, was also evaluated and compared between the two groups.

OS and PFS

OS was defined as the period from the Tmab-CTx initiation date to the date of the patient's death. PFS was defined as the period from the Tmab-CTx initiation date to the date of disease "progression" onset based on an objective evaluation of the patient chart or to the date of the patient's death.

Cell lines, cell culture, and reagents

Human gastric adenocarcinoma cell line NCI-N87 (RRID: CVCL_1603) was purchased from the American Type Culture Collection (Manassas, VA). Human esophageal adenocarcinoma cell line OE19 (RRID: CVCL_1622) was purchased from the European Collection of Cell Cultures (Salisbury, UK). These cell lines were confirmed by STR profiling, and were mycoplasma-free. Cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 µg/mL; Life Technologies) and were incubated at 37°C in a humidified chamber containing 5% CO_2. Tmab was provided by Chugai Pharmaceutical (Tokyo, Japan) for nonclinical investigations; 5-FU was purchased from Wako (Tokyo, Japan); CDDP was provided by YAKULT HONSHA (Tokyo, Japan). LY294002 and NVP-BEZ235 *were purchased from Cayman Chemical (Ann Arbor, MA).* Everolimus (RAD001) and MK2206 were purchased from Selleck Chemicals (*Houston, TX).*

Small interfering RNA and short hairpin RNA

Two distinct siRNA species targeting PTEN (siPTEN #1, Hs_PTEN_6 FlexiTube siRNA, SI00301504; siPTEN #2, Hs_PTEN_8 FlexiTube siRNA, SI03048178) and nonsilencing control siRNA (AllStars negative control siRNA, SI03650318) were purchased from Qiagen and transfected with Lipofectamine RNAiMAX (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's protocol.

The shRNA vector (PTEN Human shRNA Plasmid Kit, TL320498) was purchased from Origene (Rockville, MA). The Plasmid kit contained a non-effective 29-mer scrambled shRNA and four unique 29-mer shRNA constructs, #A #B #C #D, in the lentiviral GFP vector, from which we selected #C and #D shRNA because they had better PTEN knockdown efficacy. The packaging vector (psPAX2, plasmid 12260) and envelope vector (pMD2.G, plasmid 12259) were purchased from Addgene (Cambridge, MA). The shRNA lentivirus containing shRNA constructs were prepared according to the manufacturer's protocol, and cell lines were infected with them. We selected stable knockdown clones by flow cytometry to collect GFP-positive cells.

Western blotting

Cells were lysed in sodium dodecyl sulfate lysis buffer supplemented with a protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan) and phosphatase inhibitor cocktail (Nacalai Tesque, Kyoto, Japan). A total of 20 μg of whole-cell lysate was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Merck Millipore). Membranes were probed with specific primary antibodies against HER2 (polyclonal, #2242), PTEN (clone 138G6, #9559), pan-Akt (clone C67E7, #4691), phosphorylated Akt (Ser473, clone D9E, #4060), p44/p42 mitogen-activated protein kinase [MAPK; ERK1/2] (clone 137F5, #4695), phosphorylated p44/p42 MAPK (ERK1/2) (Thr202/Tyr204, clone D13.14.4E, #4370), S6 ribosomal protein (clone 5G10, #2217), phosphorylated S6 ribosomal protein (Ser235/236, clone D57.2.2E, #4858) (Cell Signaling Technology), and horseradish peroxidase (HRP)-conjugated secondary antibody (Dako, Santa Clara, CA). HRP-conjugated β-actin antibody (Sigma-Aldrich, St. Louis, MO) was used as a loading control. Bands were visualized using a Pierce Western blotting substrate kit (Thermo Scientific).

Cell viability and cell growth inhibition assay

Cell viability was measured by the WST-8 colorimetric assay using the Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan). Exponentially growing cells (2500–3500/100 μL/well) were seeded in triplicate into 96-well plates and cultured in reagent-containing medium for 120 h. Then, 10 μL of CCK-8 was added to each well, and incubation continued for 3 h. The absorbance at 450nm was measured with a GloMax-Multi detection system (Promega, Madison, WI) to calculate the number of viable cells per well. The working concentrations of the reagents were in the range in which the tumor growth inhibition rate was 30%–50%. We investigated the tumor inhibition rate of each reagent in the original cell lines at various concentrations (Supplementary Fig. 4). The optimized reagent concentrations in NCI-N87 and OE19 cell cultures were derived from preliminary experiments: Tmab, 10 μg/mL in both cell lines; 5-FU, 1 and 10 μM in NCI-N87 and OE19, respectively; CDDP, 1 μM in both cell lines; LY294002, 5 and 10 μM in NCI-N87 and OE19, respectively; everolimus, 10 nM in both cell lines; MK-2206, 500 nM and 5 μM in NCI-N87 and OE19, respectively; NVP-BEZ235, 50 and 500 nM in NCI-N87 and OE19, respectively.

Statistical analysis

All values are expressed as mean ± standard deviation. All *in vitro* experiments were repeated at least three times. Categorical data were analyzed using Fisher's exact test. Continuous variables were analyzed using Student's t-test. To investigate factors associated with OS or PFS, multivariate logistic regression analysis was used for factors that were included in the model with $p<0.20$ in univariate analysis. Survival curves were calculated according to the Kaplan-Meier method and analyzed using the log-rank test. All analyses were two-sided, and differences with $p<0.05$ were considered statistically significant. Statistical analyses were performed using JUMP software, version 14.0, Statistical Discovery (SAS, Cary, NC).

Declarations

Data Availability Statement

The data that support the findings of our study are available upon reasonable request from the corresponding author.

Acknowledgments

The authors are deeply grateful to Prof. Dr. Masakazu Toi for providing helpful comments and thankful to Daisuke Nishizaki for providing critical comments regarding statistical analyses and review, and Fumie Uemura for technical assistance.

Authors' Contributions

- Conception and design: Y. Deguchi, H. Okabe and S. Hisamori
- Development of methodology: D. Yokoyama and S. Hisamori
- Acquisition of data (clinical data and pathological samples of the patients, acquired and managed patients, provided facilities, etc.): D. Yokoyama, H. Okabe, S. Kanaya, D. Manaka, Y. Kadokawa, H. Hata and S. Hisamori,
- Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D. Yokoyama, T. Nishigori, S. Minamiguchi, S. Tsunoda and S. Hisamori
- Writing, review, and/or revision of the manuscript: D. Yokoyama, T. Nishigori, S. Tsunoda, K. Obama, Y. Deguchi, H. Okabe and S. Hisamori
- Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): D. Yokoyama, T. Nishigori, S. Tsunoda, K. Obama and S. Hisamori
- Study supervision: K. Obama and Y. Sakai

Conflict of interest

There are no conflicts of interest to declare.

Funding

This research was funded by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan KAKENHI Grant Number 17K10585.

References

1. Bray, F. *et al.* Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* **68**, 394-424, doi:10.3322/caac.21492 (2018).
2. Van Cutsem, E., Sagaert, X., Topal, B., Haustermans, K. & Prenen, H. Gastric cancer. *Lancet* **388**, 2654-2664, doi:10.1016/s0140-6736(16)30354-3 (2016).
3. Gravalos, C. & Jimeno, A. HER2 in gastric cancer: a new prognostic factor and a novel therapeutic target. *Ann Oncol* **19**, 1523-1529, doi:10.1093/annonc/mdn169 (2008).
4. Slamon, D. J. *et al.* Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science (New York, N.Y.)* **235**, 177-182, doi:10.1126/science.3798106 (1987).
5. Slamon, D. J. *et al.* Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *The New England journal of medicine* **344**, 783-792, doi:10.1056/nejm200103153441101 (2001).
6. Piccart-Gebhart, M. J. *et al.* Trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer. *The New England journal of medicine* **353**, 1659-1672, doi:10.1056/NEJMoa052306 (2005).
7. Smith, I. *et al.* 2-year follow-up of trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer: a randomised controlled trial. *Lancet* **369**, 29-36, doi:10.1016/s0140-6736(07)60028-2 (2007).

8. Ross, J. S. *et al.* The HER-2 receptor and breast cancer: ten years of targeted anti-HER-2 therapy and personalized medicine. *The oncologist* **14**, 320-368, doi:10.1634/theoncologist.2008-0230 (2009).
9. Bang, Y. J. *et al.* Trastuzumab in combination with chemotherapy versus chemotherapy alone for treatment of HER2-positive advanced gastric or gastro-oesophageal junction cancer (ToGA): a phase 3, open-label, randomised controlled trial. *Lancet* **376**, 687-697, doi:10.1016/S0140-6736(10)61121-X (2010).
10. Berns, K. *et al.* A functional genetic approach identifies the PI3K pathway as a major determinant of trastuzumab resistance in breast cancer. *Cancer Cell* **12**, 395-402, doi:10.1016/j.ccr.2007.08.030 (2007).
11. Nahta, R., Yu, D., Hung, M. C., Hortobagyi, G. N. & Esteva, F. J. Mechanisms of disease: understanding resistance to HER2-targeted therapy in human breast cancer. *Nat Clin Pract Oncol* **3**, 269-280, doi:10.1038/ncponc0509 (2006).
12. Nagata, Y. *et al.* PTEN activation contributes to tumor inhibition by trastuzumab, and loss of PTEN predicts trastuzumab resistance in patients. *Cancer Cell* **6**, 117-127, doi:10.1016/j.ccr.2004.06.022 (2004).
13. Deguchi, Y. *et al.* PTEN loss is associated with a poor response to trastuzumab in HER2-overexpressing gastroesophageal adenocarcinoma. *Gastric Cancer* **20**, 416-427, doi:10.1007/s10120-016-0627-z (2017).
14. Lee, J. J., Loh, K. & Yap, Y. S. PI3K/Akt/mTOR inhibitors in breast cancer. *Cancer Biol Med* **12**, 342-354, doi:10.7497/j.issn.2095-3941.2015.0089 (2015).
15. Pohlmann, P. R., Mayer, I. A. & Mernaugh, R. Resistance to Trastuzumab in Breast Cancer. *Clin Cancer Res* **15**, 7479-7491, doi:10.1158/1078-0432.CCR-09-0636 (2009).
16. McKenna, M., McGarrigle, S. & Pidgeon, G. P. The next generation of PI3K-Akt-mTOR pathway inhibitors in breast cancer cohorts. *Biochim Biophys Acta Rev Cancer* **1870**, 185-197, doi:10.1016/j.bbcan.2018.08.001 (2018).
17. Andre, F. *et al.* Everolimus for women with trastuzumab-resistant, HER2-positive, advanced breast cancer (BOLERO-3): a randomised, double-blind, placebo-controlled phase 3 trial. *Lancet Oncol* **15**, 580-591, doi:10.1016/S1470-2045(14)70138-X (2014).
18. Ohtsu, A. *et al.* Everolimus for previously treated advanced gastric cancer: results of the randomized, double-blind, phase III GRANITE-1 study. *J Clin Oncol* **31**, 3935-3943, doi:10.1200/JCO.2012.48.3552 (2013).
19. Eisenhauer, E. A. *et al.* New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1). *Eur J Cancer* **45**, 228-247, doi:10.1016/j.ejca.2008.10.026 (2009).
20. Pietrantonio, F. *et al.* Biomarkers of Primary Resistance to Trastuzumab in HER2-Positive Metastatic Gastric Cancer Patients: the AMNESIA Case-Control Study. *Clin Cancer Res* **24**, 1082-1089, doi:10.1158/1078-0432.Ccr-17-2781 (2018).
21. Kim, C. *et al.* PTEN loss and level of HER2 amplification is associated with trastuzumab resistance and prognosis in HER2-positive gastric cancer. *Oncotarget* **8**, 113494-113501, doi:10.18632/oncotarget.23054 (2017).
22. Ramanathan, R. K. *et al.* Phase 2 study of MK-2206, an allosteric inhibitor of AKT, as second-line therapy for advanced gastric and gastroesophageal junction cancer: A SWOG cooperative group trial (S1005). *Cancer* **121**, 2193-2197, doi:10.1002/cncr.29363 (2015).
23. Hudis, C. A. Trastuzumab--mechanism of action and use in clinical practice. *The New England journal of medicine* **357**, 39-51, doi:10.1056/NEJMra043186 (2007).
24. Valabrega, G., Montemurro, F. & Aglietta, M. Trastuzumab: mechanism of action, resistance and future perspectives in HER2-overexpressing breast cancer. *Ann Oncol* **18**, 977-984, doi:10.1093/annonc/mdl475 (2007).
25. Fan, J. P., Qian, J. & Zhao, Y. J. The loss of PTEN expression and microsatellite stability (MSS) were predictors of unfavorable prognosis in gastric cancer (GC). *Neoplasma*, doi:10.4149/neo_2020_200422N427 (2020).

26. Rozengurt, E., Soares, H. P. & Sinnet-Smith, J. Suppression of feedback loops mediated by PI3K/mTOR induces multiple overactivation of compensatory pathways: an unintended consequence leading to drug resistance. *Mol Cancer Ther* **13**, 2477-2488, doi:10.1158/1535-7163.MCT-14-0330 (2014).
27. Zhu, Y. *et al.* Dual PI3K/mTOR inhibitor BEZ235 exerts extensive antitumor activity in HER2-positive gastric cancer. *BMC Cancer* **15**, 894, doi:10.1186/s12885-015-1900-y (2015).
28. Lee, H. E. *et al.* Clinical significance of intratumoral HER2 heterogeneity in gastric cancer. *Eur J Cancer* **49**, 1448-1457, doi:10.1016/j.ejca.2012.10.018 (2013).
29. Kurokawa, Y. *et al.* Multicenter large-scale study of prognostic impact of HER2 expression in patients with resectable gastric cancer. *Gastric Cancer* **18**, 691-697, doi:10.1007/s10120-014-0430-7 (2015).
30. Kim, K. C. *et al.* Evaluation of HER2 protein expression in gastric carcinomas: comparative analysis of 1,414 cases of whole-tissue sections and 595 cases of tissue microarrays. *Annals of surgical oncology* **18**, 2833-2840, doi:10.1245/s10434-011-1695-2 (2011).
31. Nishida, Y. *et al.* A novel gene-protein assay for evaluating HER2 status in gastric cancer: simultaneous analyses of HER2 protein overexpression and gene amplification reveal intratumoral heterogeneity. *Gastric Cancer* **18**, 458-466, doi:10.1007/s10120-014-0394-7 (2015).
32. Wakatsuki, T. *et al.* Clinical impact of intratumoral HER2 heterogeneity on trastuzumab efficacy in patients with HER2-positive gastric cancer. *Journal of gastroenterology* **53**, 1186-1195, doi:10.1007/s00535-018-1464-0 (2018).
33. Bettstetter, M. *et al.* Epidermal growth factor receptor, phosphatidylinositol-3-kinase catalytic subunit/PTEN, and KRAS/NRAS/BRAF in primary resected esophageal adenocarcinomas: loss of PTEN is associated with worse clinical outcome. *Hum Pathol* **44**, 829-836, doi:10.1016/j.humpath.2012.08.005 (2013).

Tables

Table 1
(A) Characteristics of patients divided according to the phosphatase and tensin homolog (PTEN) status. (B) Chemotherapy regimen with trastuzumab.

(A)

Variables		PTEN loss		PTEN positive		
		n = 29		n = 116		
		n		n		P value[a]
Age	mean (SD)	68.4	(10.8)	68.2	(8.9)	0.915
Sex	Male	21	(72%)	81	(70%)	1.000
	Female	8	(28%)	35	(30%)	
BMI	mean (SD)	19.5	(3.2)	21	(3.5)	*0.041*
Charlson Comorbidity Index	0 or 1	23	(79%)	99	(85%)	0.407
	2≤	6	(21%)	17	(15%)	
ECOG-PS	0 or 1	22	(76%)	86	(74%)	1.000
	2 or 3	7	(24%)	30	(26%)	
Primary Site	Stomach	27	(93%)	113	(97%)	0.261
	GE junction	2	(7%)	3	(3%)	
Lauren classification	intestinal	15	(52%)	53	(46%)	0.692
	diffuse	8	(28%)	30	(26%)	
	Mixed	6	(21%)	33	(28%)	
Macroscopic type	type 4	1	(3%)	14	(12%)	0.305
	other	28	(97%)	102	(88%)	
HER2 score	2+	4	(14%)	20	(17%)	0.785
	3+	25	(86%)	96	(83%)	
Liver metastasis	yes	9	(31%)	49	(42%)	0.298
Lung metastasis	yes	5	(17%)	21	(18%)	1.000
Para-aorta LN metastasis	yes	10	(34%)	45	(39%)	0.831
Bone metastasis	yes	0	0%	9	(8%)	0.203
peritoneal dissemination	yes	14	(48%)	34	(29%)	0.076
Number of metastatic lesion	0–1	18	(62%)	73	(63%)	1.000
	1<	11	(38%)	43	(37%)	
Previous chemotherapy	yes	8	(28%)	16	(14%)	0.094
Previous gastrectomy	yes	19	(66%)	26	(22%)	*< 0.001*

(B)

Variables	PTEN loss		PTEN positive		
	n=29		n=116		
	n		n		P value[a]
Regimen					
Trastuzumab alone	2	(7%)	1	(1%)	0.102
5-FU or S-1 or Cape	22	(76%)	108	(93%)	*0.013*
CDDP or L-OHP	19	(66%)	103	(89%)	*0.004*
others[b]	6	(21%)	10	(9%)	0.092

Abbreviations: PTEN, phosphatase and tensin homolog; GE, gastroesophageal; HER2, human epidermal growth Factor Type2; 5-FU, fluorouracil; S-1, tegafur/gimeracil/oteracil; Cape, capecitabine; CDDP, cisplatin; L-OHP, oxaliplatin.
a Fisher's exact test and Student's t-test were used for categorical items and continuous variables, respectively.

b Others include docetaxel, paclitaxel, and irinotecan.

Table 2
Clinical response to trastuzumab combined chemotherapy

(A)					
Variables	PTEN loss		PTEN Positive		
	n = 29		n = 116		
	n		n		P value[a]
Clinical response					0.114
CR	0	(0%)	3	(3%)	
PR	10	(34%)	49	(42%)	
SD	4	(14%)	33	(28%)	
Non-CR/non-PD	7	(24%)	15	(13%)	
PD	8	(28%)	16	(14%)	
Disease control rate[b]	21	(72%)	100	(86%)	0.094
Response rate[c]	10	(34%)	52	(45%)	0.402
(B)					
Variables	PTEN loss		PTEN Positive		
	n = 21		n = 97		
	n		n		P value[a]
Clinical response					0.142
CR	0	(0%)	2	(2%)	
PR	10	(48%)	49	(51%)	
SD	4	(19%)	33	(34%)	
PD	7	(33%)	13	(13%)	

(A). Objective response rate and disease control rate in whole 145 patients. (B). Objective response rate and disease control rate in 118 patients with target lesions. (C). Duration of stable disease.

abbreviations: CR: complete response, PR: partial response, SD: stable disease, PD: progressive disease.

a Fisher extract test was used for categorical items and Student t test was used for continuous variables, respectively.

b Disease control rate: the sum of the proportion of CR and PR and SD and Non-CR/non-PD in (A).

c Response rate: the proportion of complete response and partial response in (A) and (B).

d Disease control rate: the sum of the proportion of CR and PR and SD in (B).

e Duration of stable disease: the length from the date when trastuzumab-combined therapy was first conducted to the date when PD was determined.

(A)					
Disease control rate[d]	14	(67%)	84	(87%)	*0.049*
Response rate[c]	10	(48%)	51	(53%)	0.811
(C)					
Variables	PTEN loss		PTEN Positive		
	months		months		P value[a]
Duration of stable disease[e]					
mean (SD)	8.3	(7.9)	13.6	(14.7)	0.063

(A). Objective response rate and disease control rate in whole 145 patients. (B). Objective response rate and disease control rate in 118 patients with target lesions. (C). Duration of stable disease.

abbreviations: CR: complete response, PR: partial response, SD: stable disease, PD: progressive disease.

a Fisher extract test was used for categorical items and Student t test was used for continuous variables, respectively.

b Disease control rate: the sum of the proportion of CR and PR and SD and Non-CR/non-PD in (A).

c Response rate: the proportion of complete response and partial response in (A) and (B).

d Disease control rate: the sum of the proportion of CR and PR and SD in (B).

e Duration of stable disease: the length from the date when trastuzumab-combined therapy was first conducted to the date when PD was determined.

Table 3
Univariable and multivariable analysis of overall survival progression free survival

(A)														
	Progression Free Survival													
	Univariate analysis									Multivariate analysis				
	Median Survival[a]			HR (95%CI)				P value[b]		HR (95%CI)				P value[b]
Age (> 65 vs. ≤ 65)	8.4	vs.	9.6	1.05	(0.70	-	1.56)	0.827						
Sex (Male vs. Female)	9.3	vs.	8.0	0.97	(0.65	-	1.44)	0.870						
BMI (≤ 18.5 vs. >18.5)	8.4	vs.	8.9	1.16	(0.77	-	1.72)	0.479						
Charlson Comorbidity Index (2 ≤ vs. 0 or 1)	10.4	vs.	8.4	0.99	(0.61	-	1.63)	0.984						
Lauren classification (diffuse or Mixed vs. intestinal)	8.4	vs.	8.9	0.98	(0.68	-	1.42)	0.922						
Macroscopic type (type 4 vs. other)	5.0	vs.	8.9	1.39	(0.78	-	2.48)	0.263						
HER2 score (3 + vs. 2+)	8.7	vs.	8.4	0.85	(0.52	-	1.38)	0.503						
Regimen (Platinum drug (+) vs. Platinum drug (-))	9.5	vs.	5.8	0.68	(0.42	-	1.10)	0.119	0.72	(0.36	-	1.46)	0.366	
Number of metastatic lesion (2 ≤ vs. 0–1)	7.6	vs.	10.3	1.77	(1.22	-	2.56)	*0.003*	1.80	(1.23	-	2.62)	*0.002*	
Previous chemotherapy (yes vs. no)	6.0	vs.	9.3	1.40	(0.87	-	2.24)	0.165	0.99	(0.50		1.97)	0.983	
Previous gastrectomy (yes vs. no)	6.5	vs.	9.8	1.21	(0.82	-	1.78)	0.339			-			

Abbreviations: HR, Hazard Ratio; 95%CI, Confidence interval; Platinum drug, Capecitabine or cisplatin; PTEN, phosphatase and tensin homolog; HER2, Human Epidermal Growh Factor Type2.

a months.

b logistic regression model.

(A)													
PTEN (loss vs. positive)	6.5	vs.	10.0	1.70	(1.09	-	2.65)	*0.020*	1.63	(1.04	-	2.57)	*0.035*
(B)													
	Overall Survival												
	Univariate analysis								Multivariate analysis				
	Median Survival[a]			HR (95%CI)				P value[b]	HR (95%CI)				P value[b]
Age (> 65 vs. ≤ 65)	20.6	vs.	17.7	0.96	(0.61	-	1.51)	0.863					
Sex (Male vs. Female)	19.6	vs.	16.9	0.98	(0.62	-	1.56)	0.937					
BMI (≤ 18.5 vs. >18.5)	17.7	vs.	20.3	1.24	(0.79	-	1.94)	0.349					
Charlson Comorbidity Index (2 ≤ vs. 0 or 1)	21.0	vs.	19.2	1.00	(0.56	-	1.81)	0.988					
Lauren classification (diffuse or Mixed vs. intestinal)	16.6	vs.	20.3	1.15	(0.75	-	1.77)	0.507					
Macroscopic type (type 4 vs. other)	12.6	vs.	20.1	1.60	(0.80	-	3.19)	0.185	2.13	(1.04	-	4.34)	*0.038*
HER2 score (3 + vs. 2+)	18.2	vs.	22.6	1.06	(0.59	-	1.92)	0.839					
Regimen (Platinum drug (+) vs. Platinum drug (-))	20.3	vs.	12.0	0.59	(0.35	-	1.00)	0.049	0.63	(0.37	-	1.07)	0.089
Number of metastatic lesion (2 ≤ vs. 0–1)	13.7	vs.	21.1	1.99	(1.30	-	3.04)	*0.002*	2.05	(1.34	-	3.15)	*0.001*
Previous chemotherapy (yes vs. no)	14.5	vs.	19.6	1.39	(0.83	-	2.34)	0.212					

Abbreviations: HR, Hazard Ratio; 95%CI, Confidence interval; Platinum drug, Capecitabine or cisplatin; PTEN, phosphatase and tensin homolog; HER2, Human Epidermal Growh Factor Type2.

a months.

b logistic regression model.

(A)														
Previous gastrectomy (yes vs. no)	19.2	vs.	20.6	1.07	(0.69	-	1.67)	0.752				-		
PTEN (loss vs. positive)	13.3	vs.	21.0	1.78	(1.08	-	2.94)	*0.023*	1.83	(1.09	-	3.07)	*0.022*	
Abbreviations: HR, Hazard Ratio; 95%CI, Confidence interval; Platinum drug, Capecitabine or cisplatin; PTEN, phosphatase and tensin homolog; HER2, Human Epidermal Growh Factor Type2.														
a months.														
b logistic regression model.														

Figures

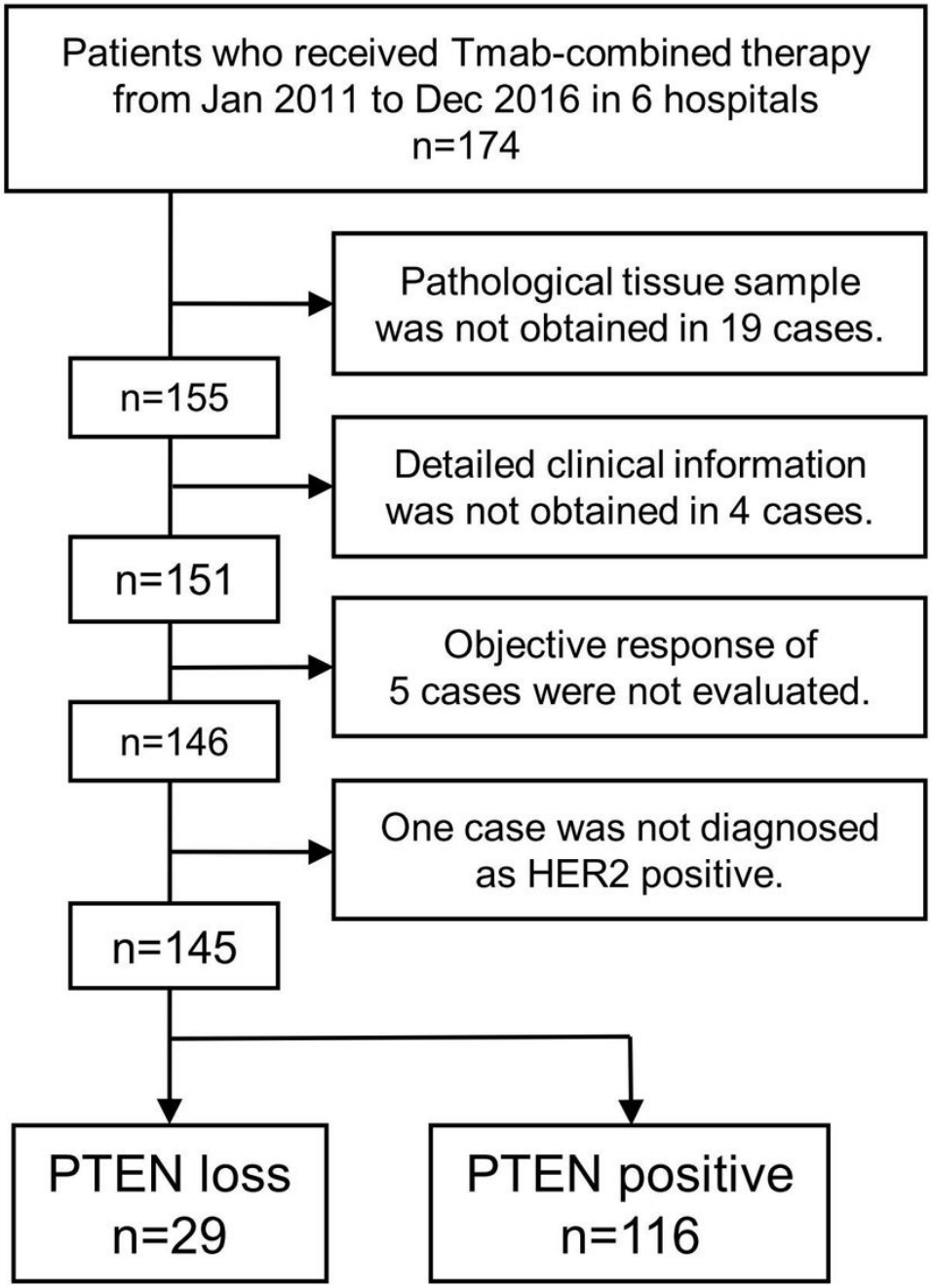

Figure 1

Flow diagram of study selection. A total 145 patients were assigned to either the PTEN-loss or the PTEN-positive group, based on the PTEN IHC result. The PTEN-loss group had 29 patients, the PTEN-positive group had 116 patients.

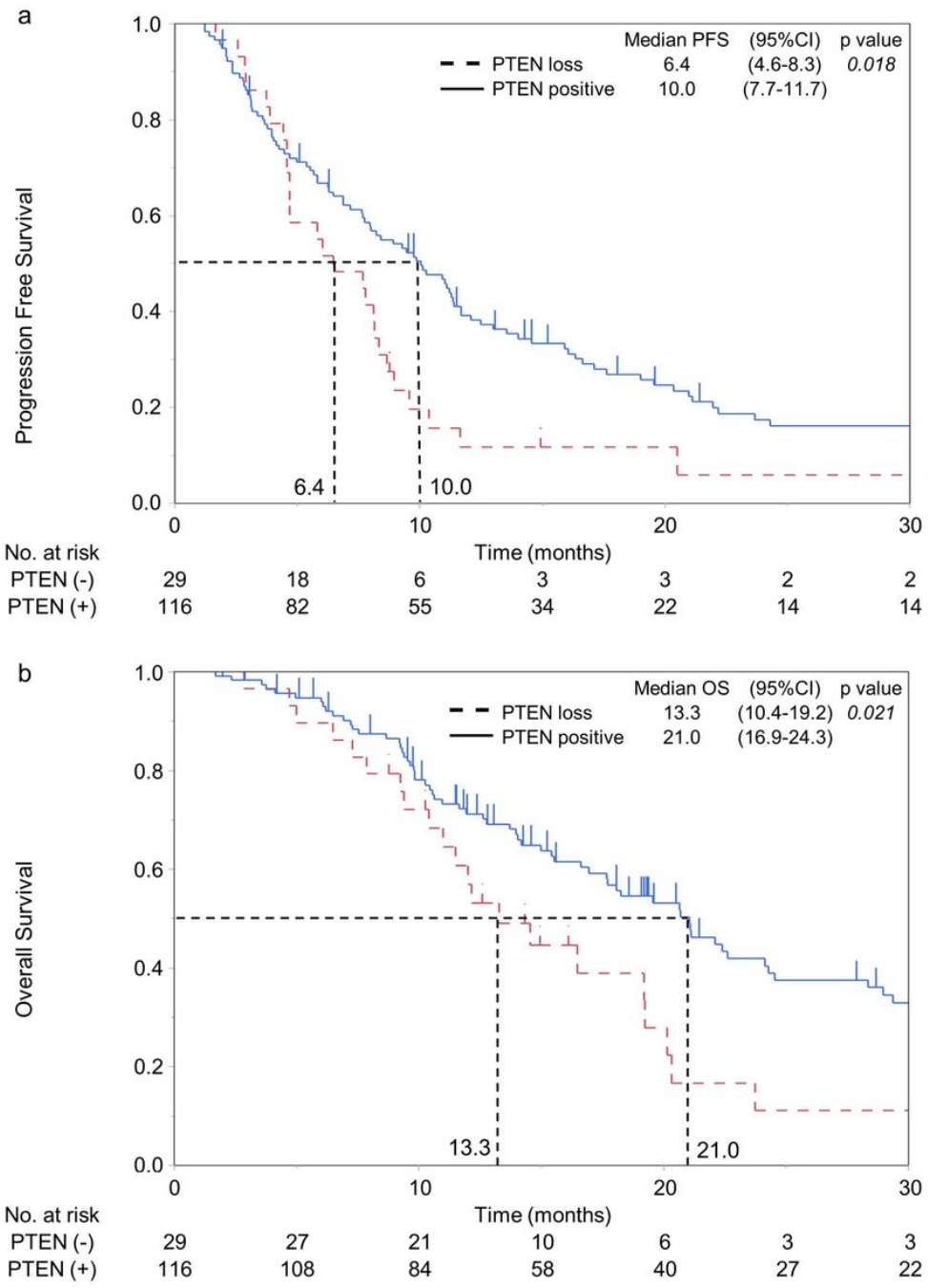

Figure 2

Overall survival (OS) and progression-free survival (PFS) of Tmab-combined chemotherapy (Tmab-CTx). (a) OS was calculated from the date of Tmab-based therapy was first conducted. (b) PFS was defined as the period from the date when Tmab-CTx was first conducted to the date when an objective evaluation as “progression” was determined from the review of the patient chart or to the patient death. Survival curves were calculated according to the Kaplan-Meier method and analyzed using the log-rank test.

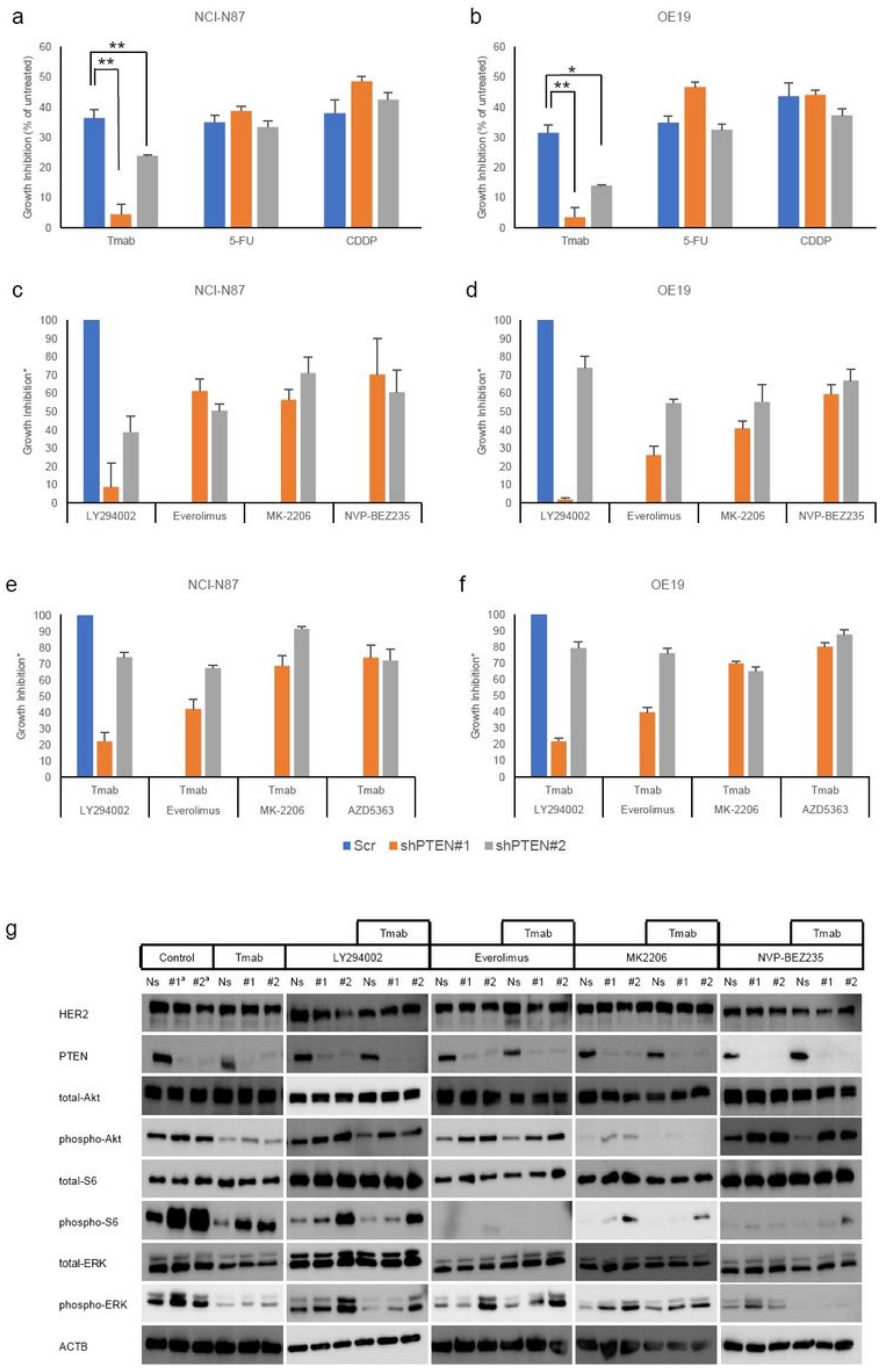

Figure 3

Cell growth inhibition. (a) N87 cells treated with Tmab, 5-FU, and CDDP. (b) OE19 cells treated with Tmab, 5-FU, and CDDP. (c) NCI-N87 cells treated with PI3K inhibitors. (d) OE19 cells treated with PI3K inhibitors. (e) NCI-N87 cells treated with PI3K inhibitors and Tmab. (f) OE19 cells treated with PI3K inhibitors and Tmab. The cell viability was measured with the WST-8 colorimetric assay. PTEN knockdown was conducted with shRNA (shPTEN#1 and shPTEN#2). The reagent exposure was performed for 120 h using the following conditions: Tmab (10 ug/ml), 5-FU(1 μM for NCI-N87 cells and 10 μM for OE19 cells), CDDP (1 μM), LY294002 (5 μM for NCI-N87 cells and 10 μM for OE19 cells), everolimus (10 nM), MK-2206 (500 nM for NCI-N87 cells and 5 μM for OE19 cells), and NVP-BEZ235 (50 nM for NCI-N87 cells and 500 nM for OE19 cells). Cell growth inhibition was calculated with the following formula: [1 - experimental absorbance (treated well)/control absorbance (untreated well)] × 100; except for (a) and (b), all values

were expressed as a ratio with the value of the scrambled cells set as 100%. n=3 in each group. (g) Influence of PI3K inhibitors on NCI-N87 cells. Western blotting analyses of PI3K and MAPK proteins. PTEN knockdown was achieved using siRNA (siPTEN#1 and siPTEN#2). The reagent exposure duration was 24 h, and concentrations were the same as above. *p<0.05, **p<0.001 by Student's t-test. #1a was siPTEN#1, #2a was siPTEN#2.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- 20210127SupplementaryMaterial.docx