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ORIGINAL ARTICLE

Loss of HER2 positivity after anti-HER2 chemotherapy in HER2-positive gastric cancer patients: results of the GASTric cancer HER2 reassessment study 3 (GASTHER3)

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Abstract

Background Although discordance in HER2 positivity between primary and metastatic lesions is well established, changes in HER2 positivity after anti-HER2 therapy have not been well evaluated in gastric cancer. We aimed to evaluate whether HER2 expression in gastric cancer is affected by trastuzumab therapy.

Methods We enrolled 48 HER2-positive advanced gastric cancer patients treated with trastuzumab-containing first-line chemotherapy and had paired biopsies at baseline and after progression.

Results At baseline, HER2 was positive, with immunohistochemistry (IHC) 2+ and in situ hybridization (ISH)+ in five patients, and with IHC 3+ in 43 patients. Fourteen patients (29.1%) exhibited loss of HER2 positivity on post-progression biopsy: 10 with IHC 0 or 1+, and four with IHC 2+/ISH−. HER2 remained positive on second biopsy in 34 patients: four with IHC 2+/ISH+, and 30 with IHC 3+. Median *H*-scores decreased from 225 to 175 ($p = 0.047$). *HER2* genetic heterogeneity was defined in one of 34 ISH-assessable patients (2.9%) at baseline and seven of 32 (21.9%) at second biopsy. Among 13 patients who received second-line trastuzumab emtansine, three showed HER2-negative conversion; they had no objective response and short progression-free survival (1.2, 1.3, and 3.4 months). Patients with stable HER2 status had a 44% response rate and median progression-free survival of 2.7 (0.4–36.8) months.

Conclusion A substantial portion of HER2-positive patients showed HER2-negative conversion with increased *HER2* genetic heterogeneity after failure of trastuzumab-containing chemotherapy. Loss of HER2 positivity could be predictive of second-line anti-HER2 treatment, suggesting a need to reexamine HER2 status before initiating second-line anti-HER2 therapy.

Keywords HER2 · Advanced gastric cancer · Heterogeneity · Trastuzumab · T-DM1

Introduction

Advanced gastric cancer (GC) is the third-leading cause of cancer-related deaths worldwide in males and the fifth in females [1]. Although various chemotherapeutic agents have been investigated to improve the prognosis of metastatic GC, including 5-fluorouracil, platinum, anthracyclines, and taxanes [2–5], the clinical outcomes of advanced GC remain disappointing with median overall survival (OS) of less than 1 year. Early studies suggested that the addition of targeted agents to conventional cytotoxic chemotherapy may lead to better treatment responses, and trastuzumab-based chemotherapy has been a standard approach for HER2-positive GC after exhibiting a survival benefit in the ToGA trial [6]. However, not all patients with HER2-positive GC respond to trastuzumab-containing regimens; overall response rates

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(ORRs) range from 47 to 68% [6, 7]. Furthermore, most patients develop resistance to trastuzumab as their disease progresses. Mechanisms of primary or acquired resistance to trastuzumab have not yet been demonstrated in patients with HER2-positive GC.

Based on previous experience in patients with breast cancer, lapatinib and T-DM1 were explored for the treatment of patients with advanced GC who experienced progression during or after trastuzumab therapy. Unlike with HER2-positive breast cancer, lapatinib (an oral anti-HER2 small molecule agent) plus paclitaxel did not improve survival when compared to paclitaxel alone [8] and T-DM1 (an antibody-drug conjugate) also did not show better efficacy than taxanes as a second-line treatment in HER2-positive GC [9]. Acquired resistance to anti-HER2 therapy after exposure to trastuzumab is a possible explanation of these disappointing results, although the resistance mechanism remains largely unknown. Selective pressure against HER2-overexpressing clones might induce loss of HER2 positivity, which was observed in patients with resectable breast cancer who received trastuzumab-based neoadjuvant therapy [10, 11].

Changes in HER2 expression after trastuzumab treatment are not well established in GC. There have been two case studies that reported complete loss of HER2 positivity on surgical specimens from patients with metastatic GC after noticeable responses to trastuzumab-based chemotherapy [12, 13]. Recently, two study groups demonstrated a considerable occurrence of consecutive HER2 loss after trastuzumab treatment in metastatic GC [14–16]. However, although both studies surmised that the loss of HER2 positivity might influence the response to second-line anti-HER2 treatment, the data on second-line anti-HER2 treatment were not available.

In this study, we aimed to investigate the changes of HER2 status in patients with HER2-positive metastatic or locally advanced GC who received first-line trastuzumab-based chemotherapy, and the impact of the loss of HER2 positivity on second-line anti-HER2 treatment.

Methods

Patient population and clinical data

Between August 2011 and February 2016, patients with pathologically confirmed HER2-positive locally advanced, recurrent, or metastatic GC who received trastuzumab-containing first-line chemotherapy were included in a prospective, observational registry of the Asan Medical Center, Seoul, Korea. The analysis included all patients from the registry who underwent pre-treatment and post-progression biopsies. Clinical data, including disease status and survival outcomes, were updated in July 2016. This study protocol adhered to the guidelines established by the Declaration of Helsinki and was approved by the Center's institutional review board. All living patients provided written informed consent.

HER2 immunohistochemistry (IHC) and in situ hybridization (ISH)

HER2 IHC was conducted with Ventana anti-Her2/neu (4B5) rabbit monoclonal primary antibody (Ventana Medical System, Tucson, AZ) and HER2 immunoreactivity was assessed on a scale of 0 to 3 using the GC consensus panel recommendations [17, 18]. H-scores were calculated based on the formula, (% tumor IHC 1+) + 2 (% tumor IHC 2+) + 3 (% tumor IHC 3+), meaning possible *H*-score values range from 0 to 300 [19].

ISH was performed in cases with IHC 2+ at diagnosis and in all available cases at the time of analysis. *HER2* gene amplification was evaluated by counting signals in the 20 non-overlapping tumor cells with the highest gene counts. ISH was interpreted as positive if the *HER2*/CEP17 ratio was >2.2 and negative if the *HER2*/CEP17 ratio was <1.8. When the results were equivocal (1.8–2.2), 20 additional tumor cells were counted, and an *HER2*/CEP17 ratio ≥ 2 was considered positive. Based on the criteria of genetic heterogeneity in *HER2*-amplified breast cancer, genetic heterogeneity was defined as 5% to $<50\%$ of tumor nuclei with an *HER2*/CEP17 ratio of ≥ 2.0 by ISH [20].

HER2 positivity was defined as IHC 3+, or as IHC 2+ with *HER2* gene amplification by ISH. Loss of HER2 positivity was defined as IHC score 0/1+, or as IHC 2+ with no amplification by ISH on post-progression biopsies. All pathological samples were reviewed, and the IHC score and ISH results were confirmed by one specialized gastrointestinal pathologist (Y Park).

Statistical analysis

Categorical variables were evaluated using the Chi-square test or Fisher's exact test, and quantitative variables were analyzed with the Mann–Whitney *U* test or the Wilcoxon signed-rank test, as appropriate. For defining optimal *H*-score and ISH ratio cutoff points to predict loss of HER2-positivity, we used receiver operating characteristic (ROC) curve analysis. With the optimal cutoff point, we performed logistic regression to estimate odds ratios.

We assessed objective tumor response according to Response Evaluation Criteria in Solid Tumors (RECIST) v1.1 [21]. Progression-free survival (PFS) was defined as the time that elapsed from the start of treatment until documented tumor progression or death due to any cause. OS was determined from the date of chemotherapy initiation to death from any cause. Data were censored if patients were free of

progression or alive at the last follow-up. The Kaplan–Meier method was used to estimate PFS and OS. Survival curves were compared by log-rank test according to loss of HER2 positivity. The Cox proportional hazards model was used to estimate HRs for survival outcomes.

All statistical analyses were performed using the Statistical Package for the Social Sciences (IBM Corp., Armonk, NY, USA) version 21 and statistical software package R version 3.0.2 (http://www.r-project.org/). All tests were two sided with a 5% significance level.

Results

Patient characteristics and treatment outcomes

During the study period, 251 patients who received first-line trastuzumab-containing chemotherapy for locally advanced, recurrent, or metastatic HER2-positive GC were included in the observational registry. Among these, 185 patients were confirmed to have experienced disease progression while on first-line chemotherapy. Fifty-two patients underwent repeat biopsies after progression, and 48 of these biopsies included enough tissue for the re-evaluation of HER2 status by both IHC and ISH for inclusion in this analysis.

The baseline characteristics of the 48 eligible patients are summarized in Table 1. The median age was 57 years (range 29–79). At baseline, HER2 was positive, with IHC 2+ and ISH+ in five patients, and with IHC 3+ in 43 patients. The median H-score was 225 (range 50–300). Seventeen patients (35.4%) had tumors with poorly differentiated histology at baseline. Except for disease status, the baseline characteristics of the 48 enrolled patients were not significantly different from the entire observational registry ($N=251$) (Supplementary Table 1). Recurrent GC was more prevalent in the entire registry than in this study patients who met the inclusion criteria (25.1% vs. 8.3%).

Thirty-nine patients were treated with capecitabine, cisplatin/oxaliplatin, and trastuzumab (XPT); seven patients received XPT plus pertuzumab or placebo as part of the JACOB study [22]. The other two patients received XPT plus pertuzumab as part of the JOSHUA study [23]. Among 34 patients with measurable lesions, the ORR was 76.5% (26 patients) and the disease control

Table 1 Baseline characteristics ($N=48$)

	Characteristic	*N*	(%)
Gender	Male	38	79.2
Age	Median (range)	57 (29–79)	
	> 60	20	41.7
ECOG performance score	0 or 1	44	93.7
Borrmann type at initial diagnosis	I–III	31	64.6
	IV	5	10.4
	Not available	12	25
Histology	WD/MD	31	64.6
	PD/SRC	17	35.4
Disease status	Initially metastatic	43	89.6
	Recurred	4	8.3
	Locally advanced	1	2.1
Metastatic organ	Peritoneum	21	43.8
	Liver	22	45.8
	Lung	6	12.5
	Intra-abdominal distant LN	34	70.8
	Extra-abdominal distant LN	11	22.9
	Bone	2	4.2
HER2 IHC	IHC 2+	5	10.4
	IHC 3+	43	89.6
Cycles of trastuzumab	≤ 10 cycles	24	50
	> 10 cycles	24	50
Use of pertuzumab[a]	No	39	81.3
	Yes	2	4.2

ECOG Eastern Cooperative Oncology Group, *WD* well differentiated, *MD* moderately differentiated, *PD* poorly differentiated, *SRC* signet ring cell, *LN* lymph node, *IHC* immunohistochemistry

[a]Seven patients were blinded to pertuzumab or placebo in a clinical trial (JACOB)

rate was 97.1% (33 patients). With a median follow-up duration of 16.2 months (range 2.9–59.2), the median PFS and OS were 8.3 months [95% confidence interval (CI) 6.1–10.5] and 16.3 months (95% CI 11.6–21.1), respectively. The median PFS and OS were not significantly different between the total observational registry patients and the eligible study patients; the ORR, PFS, and OS of the total observation registry patients were 76.8%, 8.5 months, and 16.9 months, respectively. Thirty patients experienced disease progression during combination therapy with trastuzumab and cytotoxic chemotherapy, whereas the other 18 patients showed disease progression while on trastuzumab-only maintenance therapy (Table 2).

Change in HER2 positivity and genetic heterogeneity

The baseline and post-progression HER2 IHC and ISH results are summarized in Table 3. There were 43 HER2 IHC 3+ patients and five HER2 IHC 2+ with ISH + patients. Fourteen patients' samples (29.2%) were interpreted as HER2 negative on post-progression biopsies. Among 43 HER2 IHC 3+ patients, 28 patients (65.1%) maintained the IHC 3+ score, whereas six (14.0%) and nine (20.9%) patients changed to IHC 2+ and IHC 0 or 1+, respectively. There were two patients (40%) who had HER2 IHC 2+ on initial biopsy and then IHC 3+ on the second biopsy, while the three other HER2 IHC 2+ patients (60%) remained in the range of IHC 0 to 2+.

Table 2 Change of HER2 positivity and clinicopathologic factors

		Persistence of HER2 positivity	Loss of HER2 positivity	*p*
Gender	Male	26 (76.5)	12 (85.7)	0.701
	Female	8 (23.5)	2 (14.3)	
Age	≤ 60	21 (61.8)	7 (50)	0.452
	> 60	13 (38.2)	7 (50)	
ECOG performance score	0–1	32 (94.1)	14 (100)	0.578
	2–4	2 (5.9)	0 (0)	
Primary site	Gastroesophageal junction	1 (14.3)	2 (2.9)	0.200
	Gastric	33 (85.7)	12 (97.1)	
Borrmann type at diagnosis	I/II/III	21 (84.0)	10 (90.9)	0.664
	IV	4 (16.0)	1 (9.1)	
Histologic differentiation	WD/MD	22 (64.7)	9 (64.3)	1.00
	PD/SRC	12 (35.3)	5 (35.7)	
Disease status	Initially metastatic	32 (94.1)	11 (78.6)	0.216
	Recurred	2 (5.9)	2 (14.3)	
	Locally advanced	0 (0)	1 (7.1)	
HER2 IHC	IHC 2+	2 (5.9)	3 (21.4)	0.140
	IHC 3+	32 (94.1)	11 (78.6)	
H-score	> 170	23 (67.6)	5 (35.7)	0.041
	≤ 170	11 (32.4)	9 (64.3)	
HER2 ISH ratio[a]	> 4.36	23 (85.2)	4 (44.4)	0.026
	≤ 4.36	4 (14.8)	5 (55.6)	
Changes of genetic homogeneity	Stable homogeneity	19 (100)	1 (16.7)	< 0.001
	Change to heterogeneity	0 (0)	5 (83.3)	
First line chemotherapy	Capecitabine + cisplatin + trastuzumab	24 (70.6)	11 (78.6)	0.066
	Capecitabine + oxaliplatin + trastuzumab	4 (11.8)	0 (0.0)	
	Capecitabine + cisplatin + trastuzumab + pertuzumab (JOSHUA)	0 (0.0)	2 (14.3)	
	Capecitabine + cisplatin + trastuzumab ± pertuzumab (JACOB)	6 (17.6)	1 (7.1)	
Progression time point	During combination of anti-HER2 and cytotoxic chemotherapy	22 (64.7)	8 (57.1)	0.623
	During anti-HER2 maintenance therapy alone	12 (35.3)	6 (42.9)	

ECOG Eastern Cooperative Oncology Group, *WD* well differentiated, *MD* moderately differentiated, *PD* poorly differentiated, *SRC* signet ring cell, *IHC* immunohistochemistry, *ISH*, in situ hybridization

[a]Initial HER2 ISH ratio data were available for 36 patients

Table 3 Change in HER2 immunohistochemistry and in situ hybridization results after trastuzumab-based chemotherapy

Pre-treatment			Post-progression		
	N	(%)		*N*	(%)
Positive	48	100	Positive	34	70.8
			Negative	14	29.2
Immunohistochemistry					
HER2 3+	43	89.6	IHC 0 or 1+	9	20.9
			IHC 2+	6	14.0
			IHC 3+	28	65.1
HER2 2+	5	10.4	IHC 0 or 1+	1	20
			IHC 2+	2	40
			IHC 3+	2	40
In situ hybridization					
Positive	39	81.3	Positive	24	61.5
			Negative	9	23.1
			NA	6	15.4
Negative	1	2.1	NA	1	100
NA	8	16.7	Positive	6	75
			Negative	0	0
			NA	2	25
Genetic heterogeneity[a]					
No	33	70.8	No	20	60.6
			Yes	5	15.2
			NA	8	24.2
Yes	1	2.1	Yes	1	100
NA	14	27.1	No	5	35.7
			Yes	1	7.1
			NA	8	57.1

NA not assessed

[a]Genetic heterogeneity was defined as 5% to < 50% of tumor nuclei with a *HER2*/CEP17 ratio of ≥ 2.0 by in situ hybridization

The median H-score for the post-progression biopsies was 175 (range 0–300), which was significantly lower than the median H-score of 225 for the baseline biopsies ($p = 0.047$). All patients who experienced loss of HER2 positivity also had a lower H-score at post-progression biopsy than at baseline; the median H-score among these patients changed from 135 to 15 ($p = 0.001$). In contrast, patients who maintained HER2 positivity showed mixed H-score changes, both lower and higher; the median H-score among them changed from 240 at baseline to 230 for the post-progression biopsies ($p = 0.373$) (Fig. 1). Figure 2a, b presents examples of pre- and post-progression microscopic findings in the persistent HER2-positivity group, whose H-scores were unchanged (300 on both first and second biopsies), while Fig. 2c, d shows examples of loss of HER2 positivity, with H-scores from 290 to 0. Among 40 patients who had baseline ISH results, 24 (60%) were ISH+; nine of these (22.5%) were ISH- after progression (Table 3). The ISH ratio of patients in the persistent HER2-positivity group was similar between pre- and post-progression samples; the median ISH ratio changed from 5.41 to 5.82 ($p = 0.224$), while most patients in the loss of HER2-positivity group showed decreased ISH ratios on post-progression biopsies; the median ISH ratio changed from 4.36 to 1.23 ($p = 0.028$).

We conducted ROC curve analysis to find the optimal cutoff points for H-score and ISH ratio to predict loss of HER2 positivity. The cutoff points for H-score and ISH were > 170 [area under the curve (AUC) 0.655; sensitivity 67.6%; specificity 64.3%] and > 4.36 (AUC 0.663; sensitivity 85.2%; specificity 55.6%), respectively (Supplementary Fig. 1). With a logistic regression model, the odds ratio (OR) for predicting loss of HER2 positivity for an initial H-score ≤ 170 was 3.20 (95% CI 0.88–11.63; $p = 0.077$). The OR for predicting loss of HER2 positivity for an initial ISH ratio ≤ 4.36 was 7.19 (95% CI 1.33–38.95; $p = 0.022$).

In terms of genetic heterogeneity, 33 of 34 assessable samples showed genetic homogeneity at initial biopsy. After trastuzumab-based treatment, five patients' samples (15.2%) changed to heterogenous status, whereas 20 patients (60.6%) maintained their genetic homogeneity (Table 3).

Loss of HER2 positivity and clinical characteristics

We evaluated differences in various clinical characteristics according to HER2 status change after treatment. The clinical factors including sex, age, performance status, Borrmann type, histologic differentiation, and disease status were not significantly associated with HER2 changes after trastuzumab-based treatment (Table 2).

In contrast, although initial IHC 2+ status was not associated with loss of HER2 positivity, a low initial H-score (≤ 170) was significantly associated with a loss of HER2 positivity ($p = 0.041$). Furthermore, among 36 patients who had available ISH ratio data, there were significant differences in loss of positivity rates between low ISH ratio (≤ 4.36) and high ISH ratio (> 4.36) groups ($p = 0.026$). All five patients whose genetic homogeneity changed to heterogeneity showed loss of HER2 positivity. Furthermore, although there were only two patients who were treated with a pertuzumab combination regimen, both of these patients showed loss of HER2 positivity.

Impact of HER2 changes on treatment outcomes

According to univariate analysis, loss of HER2 positivity was not significantly associated with worse PFS [hazard ratio (HR) 1.34; 95% CI 0.70–2.55; $p = 0.379$] and OS (HR 1.67; 95% CI 0.84–3.32; $p = 0.142$) in patients who received first-line trastuzumab-containing chemotherapy for locally advanced, recurrent, or metastatic HER2-positive GC (Supplementary Fig. 2). Moreover, initial HER2 IHC scores of

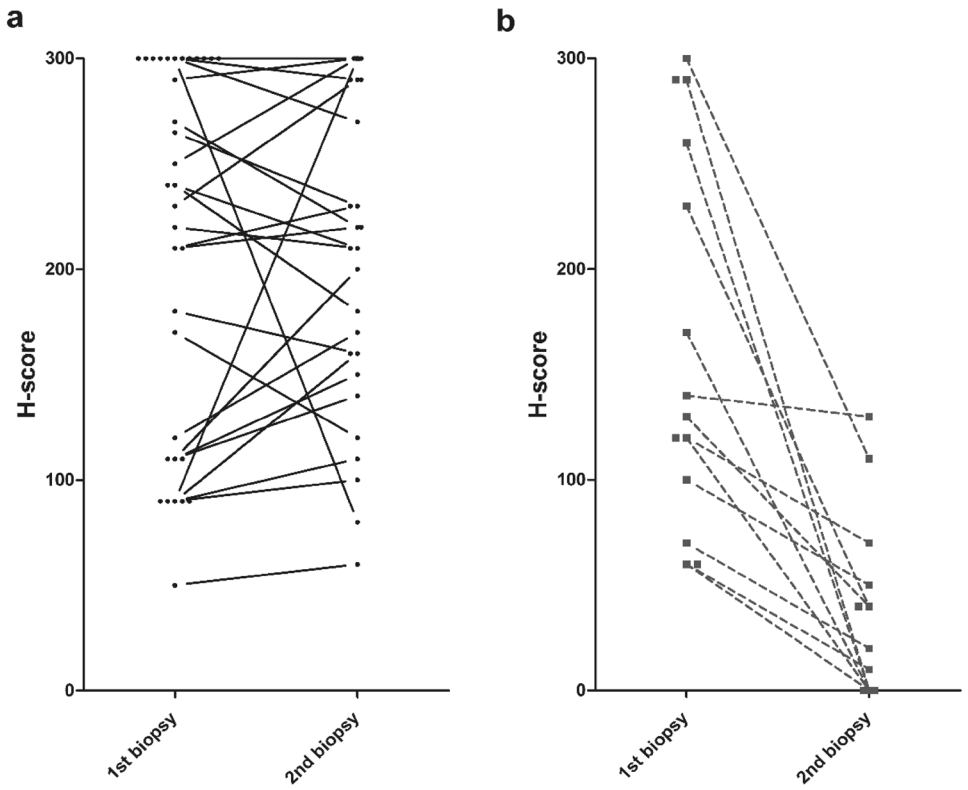

Fig. 1 Changes in H-score after trastuzumab-based chemotherapy. **a** Persistent HER2-positivity group, **b** loss of HER2-positivity group

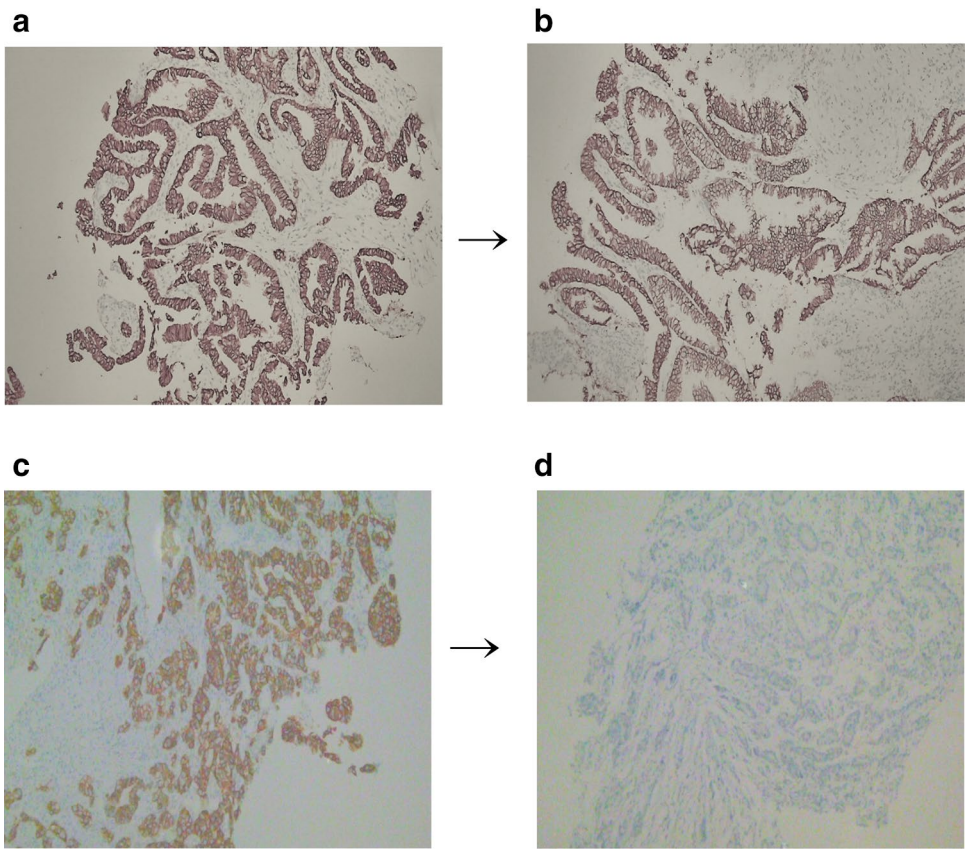

Fig. 2 Microscopic findings of HER2 status. **a** Pre-treatment and **b** post-progression HER2 immunohistochemistry of patient in the persistent HER2-positivity group, **c** pre-treatment and **d** post-progression HER2 immunohistochemistry of patients in the loss of HER2-positivity group

2+ versus 3+ were not predictive prognostic factors for PFS (HR 0.90; 95% CI 0.35–2.30; $p=0.830$) and OS (HR 1.17; 95% CI 0.45–3.05; $p=0.748$). A previously reported prognostic factor, HER2/CEP17 ratio ≥ 4.48, was not associated with PFS (HR 1.37; 95% CI 0.69–2.74; $p=0.367$) or OS (HR 1.60; 95% CI 0.76–3.38; $p=0.214$) in our study population [24].

For second-line treatment, 13 patients received T-DM1, ten of whom were included in the persistent HER2-positivity group. The other three patients were included in the loss of HER2-positivity group; no objective response was observed in this group. Furthermore, these three patients had a short PFS (1.2, 1.3, and 3.4 months, respectively), whereas the patients with persistent HER2 positivity had a 44% ORR and a median PFS of 2.7 months (95% CI 0.0–7.6). These different responses to second-line anti-HER2 chemotherapy were not statistically significant ($p=0.169$), although the Kaplan–Meier estimation curve showed a distinct tendency for worse prognosis in the loss of HER2-positivity group (Fig. 3).

Discussion

We determined that loss of HER2 positivity could occur in about one-third of patients with HER2-positive advanced GC after trastuzumab-based treatment. Additionally, loss of HER2 positivity might be a mechanism promoting resistance to subsequent anti-HER2 therapy. In particular, we assessed the impact of the loss of HER2 expression on the efficacy of second-line T-DM1 therapy. The patients who lost HER2 positivity tended to have a poor response to second-line T-DM1 and a worse PFS. This is the first study to demonstrate the predictive role of HER2-positivity changes on second-line anti-HER2 treatment.

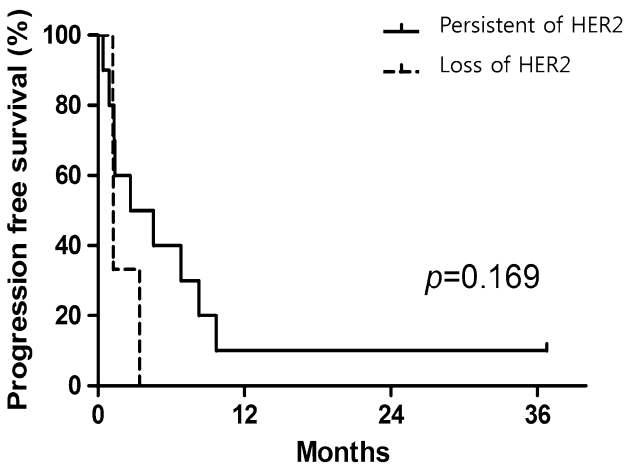

Fig. 3 Impact of HER2 status changes on progression-free survival in patients treated with second-line T-DM1 therapy

The loss of HER2 positivity between primary and metastatic sites in GC has been investigated in several studies [25–27]. Furthermore, we previously demonstrated that a 6% extra gain in HER2 positivity could be possible by reassessment of HER2 status in metastatic sites (GASTHER-1) [28]. However, there is limited published evidence about consecutive HER2 changes after trastuzumab-based treatment and the impact such changes might have on tumor response to second-line anti-HER2 therapy in HER2-positive advanced GC. Previously, two study groups reported that 24–35% of patients who received trastuzumab-based chemotherapy for HER2-positive GC experienced loss of HER2 positivity [14–16], which is consistent with our incidence of loss of HER2 positivity (29.2%).

As opposed to previous studies, in our study, one pathologist reviewed all samples included in the analysis, which could have led to more consistent results. Furthermore, a registry from a single center is advantageous for survival analysis because it reduces the bias associated with different treatment strategies used by different centers. According to our results, a low initial H-score (≤ 170) and ISH ratio (≤ 4.36) were associated with serial HER2 discordance. While loss of HER2 positivity was observed more in patients with IHC2+ than IHC3+ in a previous study [14], there was no significant difference in this regard between IHC2+ and IHC3 + patients in our study. As our cohort contained only five HER2 IHC2+ patients (11.4%), the predictive role of HER2 IHC 2+ for loss of HER2 positivity may not be conclusive from our results. Theoretically, the relationship between HER2 IHC 2+ and loss of HER2 positivity seems to be reasonable considering the predictive role of low initial H-score and ISH ratio for loss of HER2 positivity.

Genetic heterogeneity increased from 2.9% on pre-treatment samples to 21.9% on post-treatment samples. Furthermore, the changes from genetic homogeneity to heterogeneity were significantly associated with consecutive HER2 loss, and most patients included in the loss of HER2-positivity group showed decreased H-score and ISH ratio values on post-progression biopsies. These findings could be partially explained by a simultaneous process of loss of HER2 positivity.

In breast cancer, various mechanisms of resistance to anti-HER2 treatment have been suggested, such as prevention of trastuzumab binding to HER2, upregulation of HER2 downstream pathways, signaling through alternative pathways, and failure to trigger an immune-mediated mechanism to remove tumor cells [29]. Recent preclinical data also suggested potential mechanisms of acquired resistance to trastuzumab in GC, including activation of the PI3K-AKT pathway, overexpression of IQGAP1 protein, and upregulation of HER3 or HER4 [30–32]. Despite these in vitro studies, the

mechanism of trastuzumab resistance has not been clearly established, and strategies to overcome this resistance are yet to be put into practice. From this study, it would be reasonable to assume that the selection pressure of trastuzumab led to the elimination of all HER2-positive clones and, therefore, loss of HER2 positivity, which has been observed in both cell-line experimental data and clinical data in breast cancer [10, 11]. Considering intratumoral heterogeneity, it is not clear whether HER2-positive tumor cells changed into HER2-negative tumor cells or only HER2-negative clones survived after elimination of all HER2-positive clones. Regardless of which hypothesis is correct, the HER2-positivity change appears to be one of the mechanisms of acquired resistance to first-line trastuzumab in GC.

In the present study, we first assessed the relationship between post-progression HER2 loss and second-line T-DM1 response. From a randomized phase II/III trial, which compared T-DM1 and taxanes in patients with HER2-positive GC who progressed during or after first-line XPT (GATSBY trial), T-DM1 did not show an efficacy benefit over taxanes [9]. Similar to the GATSBY trial, the combination of lapatinib and paclitaxel did not improve OS compared with paclitaxel alone as a second-line treatment in patients with HER2-positive GC [8]. Loss of HER2 positivity after first-line trastuzumab-based treatment could affect these negative results, and we propose that re-biopsy for confirming HER2 status facilitates more appropriate patient selection for second-line anti-HER2 treatment.

HER2-positivity loss was not associated with ORR, PFS, or OS in patients who received first-line trastuzumab-containing chemotherapy for locally advanced, recurrent, or metastatic HER2-positive GC. In contrast to our findings, a study on HER2-positive breast cancer patients receiving neoadjuvant trastuzumab-based therapy revealed that the median recurrence-free survival was significantly better among patients with persistent HER2 amplification than those with a loss of HER2 amplification after neoadjuvant chemotherapy [11]. However, two previous case reports described dramatic trastuzumab-based chemotherapy responses in patients with HER2-positive metastatic GC in whom loss of HER2 positivity after chemotherapy was confirmed by surgical resection [12, 13]. Biological and disease status differences between resectable breast cancer and metastatic GC could explain this disagreement on the prognostic role of HER2-positivity loss. It may be possible that elimination of all HER2-positive clones by anti-HER2 therapy could induce a good treatment response and loss of HER2 positivity initially, but eventually may promote resistance to anti-HER2 treatment by proliferating HER2-negative clones.

There are some inevitable limitations in this study. First, due to the difficulty and risk of performing a re-biopsy, this study had a relatively small number of subjects and it might not necessarily represent all patients with HER2-positive gastric cancer. However, our current study utilized one of the largest datasets among studies of a similar topic. Furthermore, because the baseline characteristic and main outcomes of analyzed datasets were not significantly different with the total observational registry, we believe that the analyzed dataset exhibits similar findings from the total HER2-positive registry population. Second, since the ISH ratio and genetic heterogeneity data were not available for all enrolled patients, we could only partially assess these results. Lastly, intralesional and intratumoral heterogeneity, which has been established in GC, could have affected the pathologic interpretation.

In summary, we confirmed that a substantial portion of initially HER2-positive advanced GC patients could lose HER2 positivity with increased heterogeneity of *HER2* amplification after trastuzumab-containing chemotherapy. Loss of HER2 positivity is a potential predictive factor for subsequent anti-HER2 treatment responses. This study suggests the need to reexamine HER2 status before initiating second-line anti-HER2 treatment. Further validation of our hypothesis is warranted.

Compliance with ethical standards

Conflict of interest Y-K Kang discloses a consultant role for Ono, Taiho, Daehwa, Roche, Novartis, Bayer, Blueprint, and Merck. The remaining authors declare no conflict of interest.

Research involving human and/or animal rights All procedures followed were in accordance with the ethical standards of the Institutional Review Board of Asan Medical Center and with the Helsinki Declaration of 1964 and later versions.

Informed consent Informed consent or a substitute for it was obtained from all patients included in the study.

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