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HER2 loss in HER2-positive gastric or gastroesophageal cancer after trastuzumab therapy: Implication for further clinical research

F. Pietrantonio[1], M. Caporale[1], F. Morano[1], M. Scartozzi[2], A. Gloghini[3], F. De Vita[4], E. Giommoni[5], L. Fornaro[6], G. Aprile[7], D. Melisi[8], R. Berenato[1], A. Mennitto[1], C. C. Volpi[3], M. M. Laterza[4], V. Pusceddu[2], L. Antonuzzo[5], E. Vasile[6], E. Ongaro[7], F. Simionato[8], F. de Braud[1,9], V. Torri[10] and M. Di Bartolomeo[1]

[1] Medical Oncology Department, Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy
[2] Medical Oncology Department, Azienda Ospedaliera Universitaria Cagliari, Presidio Policlinico Universitario "Duilio Casula", Cagliari, Italy
[3] Diagnostic pathology and laboratory medicine Department, Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy
[4] Medical Oncology Department, Seconda Università degli studi di Napoli, Naples, Italy
[5] Medical Oncology 1, Azienda Ospedaliera Universitaria Careggi, Florence, Italy
[6] U.O. Oncologia Medica 2 Universitaria, Ospedale S. Chiara - Azienda Ospedaliero-Universitaria Pisana, Istituto Toscano, Tumori, Italy
[7] Medical Oncology Department, University and General Hospital, Udine, Italy
[8] Medical Oncology, Università degli studi di Verona, Italy
[9] Oncology Department, University of Milan, Italy
[10] Oncology Department, IRCCS-Mario Negri Institute for Pharmacological Rresearch, Milan, Italy

Mechanisms of acquired resistance to trastuzumab-based treatment in gastric cancer are largely unknown. In this study, we analyzed 22 pairs of tumor samples taken at baseline and post-progression in patients receiving chemotherapy and trastuzumab for advanced HER2-positive [immunohistochemistry (IHC) 3+ or 2+ with *in-situ* hybridization (ISH) amplification] gastric or gastroesophageal cancers. Strict clinical criteria for defining acquired trastuzumab resistance were adopted. Loss of HER2 positivity and loss of HER2 over-expression were defined as post-trastuzumab IHC score <3+ and absence of ISH amplification, and IHC "downscoring" from 2+/3+ to 0/1+, respectively. HER2 IHC was always performed, while ISH was missing in 3 post-progression samples. Patients with initial HER2 IHC score 3+ and 2+ were 14 (64%) and 8 (36%), respectively. Loss of HER2 positivity and HER2 over-expression was observed in 32 and 32% samples, respectively. The chance of HER2 loss was not associated with any of the baseline clinicopathological variables. The only exception was in patients with initial IHC score 2+ versus 3+, for both endpoints of HER2 positivity (80 *vs*. 14%; $p = 0.008$) and HER2 over-expression (63 *vs*. 14%; $p = 0.025$). As already shown in breast cancer, loss of HER2 may be observed also in gastric cancers patients treated with trastuzumab-based chemotherapy in the clinical practice. This phenomenon may be one of the biological reasons explaining the failure of anti-HER2 second-line strategies in initially HER2-positive disease.

Trastuzumab was the first targeted agent approved for the treatment of advanced gastric or gastroesophageal junction (GEJ) cancers. The ToGA phase III study demonstrated a significant overall survival (OS) advantage when adding trastuzumab to cisplatin and fluoropyrimidine-based doublet first-line chemotherapy in HER2-positive disease.[1] In the *post-hoc* exploratory analysis of the study, the results were not statistically significant in the HER2 low expression subset [as defined by immunohistochemistry (IHC) 0/1+ with fluorescent *in-situ* hybridization (FISH) amplification] and were outstanding in the HER2 over-expressing subset (IHC 2+ with FISH amplification or IHC 3+).

Regarding the second-line setting, phase III trials investigating anti-HER2 targeted treatment failed to demonstrate any OS advantage for HER2-positive gastric or GEJ cancer.[2,3] In the TyTAN trial,[2] the addition of lapatinib to second-line paclitaxel was not superior to placebo plus paclitaxel, although a statistically significant OS gain was observed in the IHC 3+ subgroup. Notably, in this Asian trial most of the patients received prior chemotherapy alone without biological agents. In the recently presented GATSBY trial,[3] trastuzumab emtansine was not superior to a taxane

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Correspondence to: Dr Filippo Pietrantonio, Department of Medical Oncology, Medical Oncology Unit, Fondazione IRCCS Istituto Nazionale dei Tumori, *Via* Venezian 1, 20133 Milan, Italy, Tel.: [390223903807], E-mail: filippo.pietrantonio@istitutotumori.mi.it

What's new?

Patients with cancers positive for human epidermal growth factor receptor 2 (HER2) generally fail to respond to second-line treatments, particularly when first-line therapy included use of the HER2-targeted agent trastuzumab. Post-progression changes in HER2 expression, however, have not been studied extensively, and as a consequence, their therapeutic relevance is unclear. In this study, HER2 loss was associated with acquired resistance to trastuzumab in almost one-third of patients with gastric or gastroesophageal cancers that initially were HER2-positive. The findings suggest that after failure of trastuzumab, HER2 status should be reassessed prior to inclusion in clinical trials with targeted agents.

monochemotherapy in previously treated HER2-positive, advanced gastric or GEJ cancers. In this study, 77% of the patients included had received first-line anti-HER2 targeted agents, mainly trastuzumab. Several clinical and biological reasons may explain these disappointing and unexpected findings. Under a clinical point of view an undesirable interaction between lapatinib and paclitaxel in terms of toxicity and activity might have negatively influenced patients' global outcome, as previously shown in breast cancer.[4] However, acquired resistance to first-line trastuzumab-based treatment might have induced resistance to subsequent anti-HER2-based therapies. In breast cancer, the selective pressure of treatments—either trastuzumab-based or even cytotoxics alone—may preferentially eradicate HER2-overexpressing cancer clones, while the HER2-negative ones may emerge and drive tumor progression.[5–7] This phenomenon may be observed also for gastric cancer, being even more relevant because of its marked heterogeneity.

In our study, we aimed at assessing changes in HER2 status following disease progression on trastuzumab-based first-line treatment for HER2-positive advanced gastric or GEJ cancer.

Material and Methods

Patient population

Patients with HER2-positive advanced gastric or GEJ cancer who received trastuzumab-based first-line therapy until progressive disease (PD) at 7 Italian Institutions were eligible in case a new tumor sample was taken at the time of disease progression and retested for HER2 status.

Eligible patients received trastuzumab in combination with cisplatin and fluoropyrimidines according to the Italian Regulatory Agency [IHC score 3+, or 2+ with *in-situ* hybridization (ISH) amplification]. Patients were included only in case of acquired clinical resistance to trastuzumab defined as: prior RECIST objective response (or stable disease lasting at least 9 months) to trastuzumab plus doublet chemotherapy, and PD occurring after $\geq$3 months of single agent maintenance trastuzumab. All PDs to trastuzumab were required within 6 weeks from last dose administered. At the time of PD, tumor rebiopsy had to be performed at primary tumor site or, in case of previous gastrectomy, at the most accessible site of metastasis.

All living patients signed a written informed consent and agreed to participate to an observational prospective cohort study.

HER2 IHC and *in-situ* hybridization

HER2 immunoreactivity was evaluated according to previously described scoring criteria[8,9] and *HER2* gene amplification was defined as positive when *HER2*/CEP17 ratio was $\geq$2 or when *HER2* gene copy number was of >6.[9] HER2 positivity was defined by *HER2* gene amplification by ISH or IHC score of 3+. In postprogression samples, two main variables were selected: (*i*) loss of HER2 positivity was defined as presence of IHC score <3+ and absence of ISH amplification; (*ii*) loss of HER2 over-expression was defined as IHC "downscoring" from 2+/3+ to 0/1+ (independently of ISH status).

HER2 status was tested in surgical samples or biopsies depending on what clinically available. In case of HER2 determination in tumor biopsies only cases with 6–8 biopsies of the tumoral area were included.[8,9]

No central pathologic review was carried out for the purpose of this article, but all analyses were performed in referral centers.

Statistical analysis

The association of HER2 status and its changes after treatment with baseline categorical variables (patients' and disease characteristics) were summarized by frequencies and proportion of changed cases and assessed by Mantel Haenzel test. Progression-free survival (PFS) was measured from date of treatment start until date of progression or death from any cause or last follow up for alive patients without PD; OS was measured from the date of treatment start to the time of death from any cause, or last follow up for alive patients. Survival curves were plotted by the Kaplan-Meier method and compared by log-rank test according to HER2 status and its changes. A cut-off value of 0.05 was used for selecting variables to be excluded from the final model.

The study was based on a convenience sample of patients and no attempt was made sample size estimation. This hypothesis-generating purpose will be confirmed by further research.

Results

Patients

From a prospective, multi-institutional database including 274 HER2-positive advanced gastric or GEJ cancer patients treated with trastuzumab-based first-line therapy from

Table 1. Patients demographics and disease characteristics, in the overall population and according to initial HER2 status (IHC 2+ compared with 3+)

	Overall population ($n = 22$)		HER2 IHC 2+/FISH+ ($n = 8$)		HER2 IHC 3+ ($n = 14$)		
	N	%	*N*	%	*N*	%	p^1
Median age (range)	61.5 (32–70)		63 (54–70)		58.5 (32–69)		0.09[2]
Sex							
Male	18	82	7	88	11	79	0.61
Female	4	18	1	12	3	21	
Primary Tumor							
Gastric	9	41	2	25	7	50	0.26
GEJ	13	59	6	75	7	50	
Histotype							
Diffuse	6	27	3	38	3	22	0.43
Intestinal	16	73	5	62	11	78	
ECOG PS							
0	15	68	5	62	10	72	0.67
1	7	32	3	38	4	28	
Metastatic sites							
1	12	55	3	38	9	64	0.24
>1	10	45	5	62	5	36	

Abbreviations: IHC, immunohistochemistry; GEJ, gastroesophageal junction; PS, performance status.
[1]Mantel Haenzel 2 test.
[2]Wilcoxon test.

February 2011 to July 2015, 22 cases met the inclusion criteria. Patients' demographics and disease characteristics in the overall population are summarized in Table 1. Moreover, Table 1 shows the absence of significant differences of such characteristics according to initial HER2 IHC score (3+ *vs.* 2+). Regarding survival outcomes, median PFS and OS were 8.8 (95% CI, 7.0–12.9) and 21.2 (95% CI, 13.8–43.0) months, respectively. As expected, patients with IHC score 2+ had a worse outcome when compared with those with 3+ (median PFS: 8 *vs.* 11 months; HR = 0.31 [95% CI, 0.11–0.89]; $p = 0.02$; and median OS: 14 *vs.* 32 months; HR = 0.44 [95%CI, 0.10–1.96]; $p = 0.27$).

Changes in HER2 status

Patients with initial HER2 IHC score 3+ and 2+ were 14 (64%) and 8 (36%), respectively. Table 2 shows baseline and post-progression status according to IHC score or ISH, and according to the two main variables as defined in the methods section: (*i*) loss of HER2 positivity and loss of HER over-expression. Since ISH analysis was missing in 3 post-progression samples, loss of HER positivity was assessable in 19 (86%) cases. Loss of HER2 positivity and HER2 over-expression was observed in 6/19 (32%) and 7/22 (32%) samples, respectively. Figures 1a and 1b show an example of baseline and post-progression samples with loss of HER2 over-expression. As shown in Table 3, the probability of loss of HER2 positivity was significantly higher in patients with initial IHC score 2+ versus 3+ (80 *vs.* 14%; OR = 24.0; 95%CI, 1.7–344.8; $p = 0.008$). The same was true when considering the probability of loss of HER2 over-expression (62 *vs.* 14%; OR = 10.0; 95%CI, 1.3–79.4; $p = 0.025$). Finally, there no significant differences in terms of patients' demographics and disease characteristics were found according to HER2 concordance versus loss of HER2 positivity (Supporting Information Table S1, online only) or loss of HER2 over-expression (data not shown). Similarly, there were no differences in median PFS, OS and postprogression survival in patients with HER2 concordance versus those with HER2 loss.

Discussion

Understanding the biological mechanisms of trastuzumab resistance is crucial to monitor, prevent and/or overcome resistance to HER2 inhibition in advanced HER2-positive gastric or GEJ cancer. However, data on primary or acquired resistance are few and mainly preclinical, and seem to suggest the activation of alternative tyrosine kinase receptors (such as EGFR, HER3, FGFR2 and MET) or signalling pathways (such as Src and Notch1), often leading to epithelial-mesenchymal transition.[10–13] In our study, we showed that HER2 loss is a possible mechanism of acquired resistance to trastuzumab in advanced HER2-positive gastric or GEJ cancer. Since none of the patients received second-line anti-HER2 agents, it is not possible to demonstrate whether HER2 loss may have impacted patients outcomes after progression on trastuzumab.

Table 2. Change in HER2 status after trastuzumab-based chemotherapy

Pretreatment tumor HER2 status ($N = 22$)			Post–treatment tumor HER2 status ($N = 22$)		
	N	%		*N*	%
Positive	22	100	Positive	13	59
			Negative	6	27
			Not assessable	3	14
Overexpressed	22	100	Overexpressed	15	68
			Loss of HER2 overexpression	7	32
Immunohistochemical analysis					
HER2 3+	14	64	HER2 3+	9	64
			HER2 2+	3	22
			HER2 1+	1	7
			HER2 0	1	7
HER2 2+	8	36	HER2 3+	1	12
			HER2 2+	2	25
			HER2 1+	2	25
			HER2 0	3	38
ISH analysis					
Positive	18	82	Positive	9	50
			Negative	5	28
			Not assessed	4	22
Not assessed	4	18	Positive	2	50
			Negative	2	50

Abbreviations: ISH, *in-situ* hybridization.

In breast cancer, high HER2 concordance between primary tumors and distant metastases has been shown by several studies.[14,15] In the discordant cases, HER2-positive metastases with negative primary tumors are more frequent than the opposite. This phenomenon could correlate with enhanced tumor aggressiveness or with an underestimation of HER-2 protein overexpression in the primary tumor by the pathologist. A further factor of variability has been introduced by recent ASCO and the College of American Pathologists recommendations for HER2 testing that substantially upgrade the score of 1+ to a score of 2+.[16] This means that it might be more probable to find higher scores in metastases than in primary tumors that have been previously tested, increasing the rate of discordance. Therefore, current practice guidelines recommend that metastatic disease at the first recurrence should be biopsied as a part of a workup for patients with recurrent or stage IV disease, and HER2 status should be reevaluated if it is unknown, negative, or not over-expressed.[16] Several previous studies showed that chemotherapy with or without anti-HER2 agents, including trastuzumab, influences HER2 expression.[5–7] HER-2 loss may be due to changes of HER2 status during tumor progression, differential effects of treatments on clonal subsets, and heterogeneity of HER2 expression, or even technical errors. In gastric or GEJ cancers, this issue has not been extensively explored so far. The GASTHER1 study investigated the role of HER2 reassessment in primary and metastatic or recurrent sites in patients whose primary tumor was initially HER2-negative.[17] The results showed a HER2 positivity rescue of 8.7%, thus confirming the relevant heterogeneity of receptor status in gastric cancer and the possibility of missing trastuzumab treatment in patients with potential benefit. However, the opposite phenomenon, that is, the loss of HER2 positivity and/or over-expression is largely unknown. Dynamic changes of HER2 status may be potentially induced by chemotherapy with or without trastuzumab, although postprogression rebiopsies, with reassessment of HER2

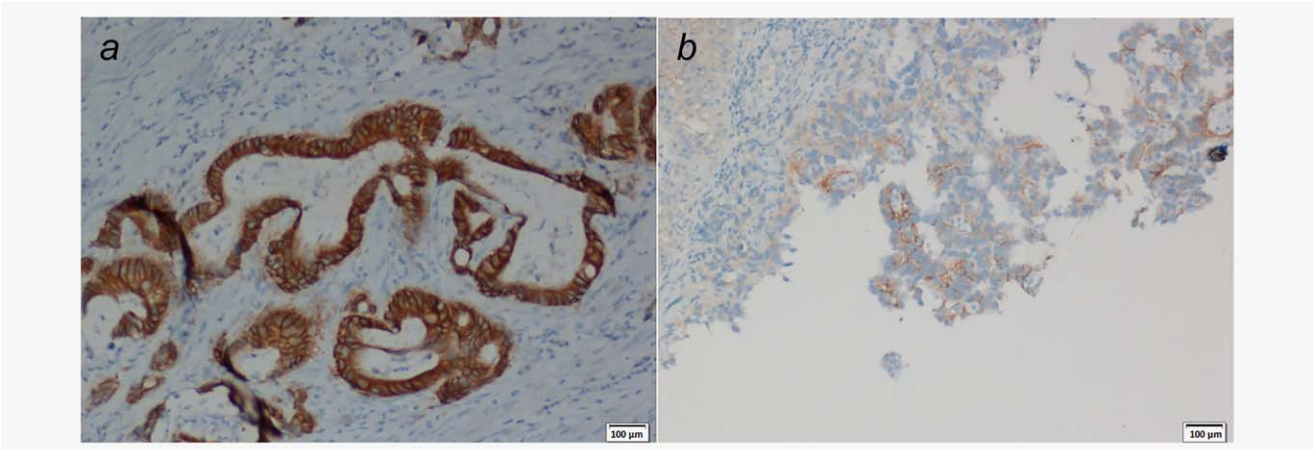

Figure 1. HER2 Immunoreactivity in baseline (*a*) and post-progression samples (*b*) in a patient receiving trastuzumab in association to cisplatin and 5-fluorouracil followed by trastuzumab maintenance until disease progression. [Color figure can be viewed at wileyonlinelibrary.com]

Table 3. HER2 status changes according to definition of HER2 positivity (IHC + ISH) and HER2 overexpression (IHC only)

	HER2 positivity				HER2 over-expression			
	Concordance		Loss		Concordance		Loss	
	N	%	*N*	%	*N*	%	*N*	%
Baseline HER2 IHC score	1	20	4	80	3	38	5	62
2+								
3+	12	86	2	14	12	86	2	14
All	13	68	6	32	15	68	7	32
p values[1]	0.008				0.025			

Abbreviations: IHC, immunohistochemistry; ISH, *in-situ* hybridization.
[1]Mantel–Haenszel test.

status in primary and/or metastatic sites, are not routinely performed. Two case reports described patients with loss of HER2 over-expression in surgical specimens obtained in disease responding to trastuzumab-based chemotherapy.[18,19] A study on 23 cases showed that about one third of HER2 positive gastric cancer may undergo loss of HER2 when considering ISH or IHC.[20] Differently from our study, combined assessment of the two methodologies was not reported, as well as significant association of HER2 loss with initial IHC score 2+.

Our data are important for several reasons. First, we emphasize that tumor rebiopsies taken prior to enrolment in second-line trials with targeted agents may be crucial in the near future. In fact, clonal evolution of gastric cancer under the selective pressure of treatments, considering the intrinsically and highly heterogeneous disease, may lead to changes in the molecular landscape during the disease course. A better knowledge of the biological profile in the setting of acquired drug resistance may help to better refine the molecular selection, and increase the chance of positive results from randomized clinical trials. Intriguingly, the negative results of the GATSBY trial[3] may have been influenced by the loss of HER2 over-expression emerged as a mechanism of acquired resistance to trastuzumab-based therapy, since the assessment of HER2 status was performed in archival tumor tissue. Thus, the unique administration of an anti-HER2 targeted treatment, that is, the antibody-drug conjugate trastuzumab emtansine—without a cytotoxic backbone—may have missed the opportunity to treat HER2 negative clones potentially selected following PD. Since we observed that loss of HER2 preferentially occurs in cases with initial IHC 2+, a subgroup analysis of the GATSBY trial according to the extent of HER2 expression is warranted, since 34,2% had IHC 2+/ISH positive and 10.4% had IHC 3+/ISH negative. In TyTAN,[2] even if the addition of lapatinib to second-line paclitaxel was not beneficial in the overall population, a clinically and statistically significant OS benefit was observed in the initially IHC 3+ subgroup. We speculate that also first-line chemotherapy alone (even if without trastuzumab) may have induced the loss of HER2 over-expression and this effect may have been exerted preferentially in patients with IHC 2+.

Our work has some limitations. First, the small sample size limits the accuracy of statistical results. Above all, loss of HER2 might have been influenced by intratumor heterogeneity or discordance between tumor sites. In fact, heterogeneity of HER2 expression in gastric or GEJ cancers is markedly increased when compared to breast cancer.[8,9,21,22] Therefore, there is still the possibility that part of our results may be due to intralesional and interlesional variations of HER2 expression. Moreover, the mechanisms of acquired resistance to anti-HER2 treatment may be heterogeneous by themselves—being multiple and potentially concomitant within the same individual. Obviously, there must be other mechanisms of importance requiring further clinical and possibly basic research, as recently shown for FGFR3 activation.[23] Therefore, a comprehensive assessment of putative resistance mechanisms including HER2 loss are still to be considered an unmet clinical need in this research field. From this point of view, liquid biopsy may be a helpful tool to encompass tumor heterogeneity by providing a comprehensive description of acquired resistance to trastuzumab.[24]

In conclusion, HER2 loss was identified as a mechanism of acquired resistance to trastuzumab and chemotherapy in advanced HER2-positive gastric or GEJ cancer. This study may prompt further development of personalized medicine in the second-line setting in this hard-to-treat disease.

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