HER2 in gastric cancer: a digital image analysis in pre-neoplastic, primary and metastatic lesions

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The assessment of human epidermal growth factor receptor 2 (HER2) status in gastric cancer is crucial in selecting patients who may benefit from targeted therapy, yet heterogeneous expression could represent an important drawback for HER2 testing. We aimed to analyze (i) HER2 heterogeneity in primary gastric cancers, pre-neoplastic and metastatic lesions and (ii) HER2 prognostic role. We studied 292 surgically resected primary gastric carcinomas and constructed 21 tissue microarrays including tumor tissue cores, invasive front, paired lymph node metastasis, low- and high-grade dysplasia. Microarrays were immunohistochemically stained with HER2 antibody and digitally scanned. Novel digital analysis algorithms were developed to score HER2 expression. Fluorescence *in situ* hybridization was performed on equivocal cases. HER2-positive cases were 13% and heterogeneous HER2 expression was observed in 71% of positive samples. Analysis of HER2 status in tumor and tumor invasive front demonstrate concordance in 177 cases (88%). Comparison of HER2 expression in primary cancer and synchronous lymph node metastasis exhibited discordant status in 14% of cases. Dysplastic epithelium surrounding the tumor showed immunohistochemical score 2 or 3 in 19% of high-grade and in 9% of low-grade dysplastic samples. HER2 status was significantly associated with intestinal-type carcinomas ($P=0.018$) and prognosis since patients with primary HER2-positive tumor showed decreased overall survival ($P=0.006$). Intratumoral HER2 expression heterogeneity and variable lymph node metastases status strongly suggest evaluating more than one sample and, if available, metastatic foci for routinely HER2 testing. *Modern Pathology* (2013) 26, 816–824; doi:10.1038/modpathol.2012.228; published online 25 January 2013

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Gastric cancer is the second most common cause of cancer-related deaths globally and the fourth most commonly diagnosed tumor.¹ Its prognosis is still poor since most patients present with advanced or metastatic disease and few therapeutic options are available.²

Recently, treatment with human epidermal growth factor receptor 2 (HER2) inhibitor, Trastuzumab, has been demonstrated to increase overall

survival in HER2-positive, inoperable locally advanced or metastatic gastric carcinoma.^{3–6}

ERBB2 is a key oncogene in many human cancers since its receptor tyrosine kinase activity triggers crucial intracellular signaling events for cell growth and survival.⁷

In gastric cancer, ERBB2 positivity ranges from 7 to 27% of the tumors,^{8–13} with high concordance rate between overexpression in immunohistochemistry and gene amplification tested by fluorescence *in situ* hybridization or chromogenic *in situ* hybridization.^{3,14,15} It is now recommended that all gastric cancers should be tested for ERBB2 status at the time of diagnosis in order to ensure patient eligibility for treatment with Trastuzumab. Most frequently immunohistochemistry is performed first, followed by

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fluorescence *in situ* hybridization in cases with equivocal expression.³

HER2 overexpression in gastric neoplasia is heterogeneous and comprises a minority of tumor cells: less than 10% of gastric carcinomas display HER2 immunoreactivity in more than 5% of tumor cells.¹⁶ Heterogeneity could represent an important drawback for HER2 testing and must be taken into account when selecting patients eligible for targeted therapy. Conversely, recent studies have documented high concordance regarding HER2 status between primary and metastatic tumor.^{17,18}

ERBB2 alterations in gastric cancer have been associated with disease stage and patient survival.^{19,20} However, several studies failed to prove these associations and the prognostic role of HER2 overexpression in gastric carcinoma remains controversial.^{16,21,22}

Due to the increasing importance of HER2 testing in gastric cancer and the controversial issues outlined, we evaluated by immunohistochemistry the HER2 expression in a large series of gastric cancers. The series comprises pre-neoplastic lesions, primary carcinomas, and lymph nodes metastases. We developed a novel digital image analysis algorithm, according to gastric cancer-specific HER2 testing protocols.^{15,23–25}

We aimed to document the frequency and distribution of HER2 positivity in primary gastric cancer and its correlation with clinicopathological features. The HER2 status in the development (pre-neoplastic lesion) and progression (nodal metastases) of gastric cancer were also investigated.

Materials and methods

Patients and Tissue Specimens

We analyzed a series of 292 surgically resected primary gastric carcinomas. Cases were retrospectively collected from the files of A.O. San Paolo Hospital and

Fondazione IRCCS Ca' Granda—Ospedale Maggiore Policlinico in Milan, Italy, from 1996 to 2005. All patients provided informed, written consent.

All medical records were reviewed to obtain patients' data, including age at diagnosis, sex, tumor location, tumor size, histological classification according to World Health Organization and Laurén classification and pathological stage (pTNM-stage) according to the seventh edition of American Joint Committee on Cancer staging manual. Esophageal cancer staging was used for gastroesophageal junction carcinomas.

Clinical–pathological features are summarized in Table 1.

Follow-up data were available for 95 patients until 2008. Mean follow-up time was 40.6 months. Patients lost during follow-up or resulting in death from a cause other than gastric carcinoma were censored for survival analysis.

Tissue Microarray Construction

Routinely prepared formalin-fixed paraffin-embedded blocks were used to construct 21 paraffin-embedded tissue microarrays. We sampled a mean of 3.1 tumor tissue cores (from 2 to 7 cores), including the tumor invasive front, for each patient. Moreover, we collected, when available, paired lymph node metastasis, low-grade and high-grade gastric dysplasia (from one to two cores each).

One-mm diameter cores were generated using a semi-automatic arrayer (Alphelys Minicore2, Plaisir, France) and each tissue microarray block contained up to 140 cores with a total of 2121 spots.

Four-microns-thick sections were cut from each tissue microarray block and stained with hematoxylin and eosin and subsequently used for immunohistochemistry.

Table 1 HER2 expression and clinical–pathological features

	<i>nr</i>	<i>pos (%)</i>		<i>nr</i>	<i>pos (%)</i>	<i>nr</i>	<i>pos (%)</i>
<i>Sites</i>			<i>pT</i>				
GEJ	30	3 (10)	Tis	2	0	0	0
CAR	10	2 (20)	T1	47	8 (32)	3	0
B-F	78	9 (12)	T2	71	11 (16)	4	1 (25)
ANT	174	23 (13)	T3	75	9 (12)	20	2 (10)
<i>Laurén classification</i>			T4	67	6 (21)	3	0
INT	185	30 (16)	pN				
NO_INT	107	7 (6)	N0	89	12 (14)	5	0
<i>WHO</i>			N1	39	2 (5)	4	2 (50)
ADC	185	30 (16)	N2	43	7 (16)	13	1 (8)
SRC	47	2 (4)	N3	87	11 (25)	8	0
MIX	48	5 (10)					
UND	12	0					
					Gastric cancer		GEJ cancer

nr: number of cases; pos: positive cases; M: male; F: female; GEJ: gastroesophageal junction; CAR: cardia; B-F: body-fundus; ANT: antrum; INT: intestinal-type carcinoma; NO_INT: non-intestinal-type carcinoma; ADC: adenocarcinoma; SRC: signet ring cells carcinoma; MIX: mixed carcinoma; UND: undifferentiated carcinoma; pos: cases with HER2 score 2 and 3.

HER2 Immunohistochemical Staining

Tissue microarray slides and whole tissue tumor sections for any of the heterogeneous cases were deparaffinized in fresh xylene and rehydrated through sequential graded ethanol. Antigen retrieval was performed using citrate buffer incubation (10 mmol/l, pH 6) with a microwave oven (20 min). Slides were cooled (20 min), incubated with 3% hydrogen peroxide (5 min), washed with phosphate-buffered saline-0.1% Triton X-100 (pH 7.6), blocked with 20% normal goat serum (20 min), and incubated (30 min) with anti-HER2 antibody (Dako HercepTest, Glostrup, Denmark). Slides were washed with phosphate-buffered saline-0.1% Triton X-100, incubated and developed (30 min) with Novolink detection system (Novocastra Leica Microsystems, Wetzlar, Germany). Slides were subsequently counterstained with hematoxylin.

Evaluation of Immunostaining

HER2-stained tissue microarray slides and hematoxylin and eosin sections were digitally scanned using the NanoZoomer 2.0-HT (Hamamatsu Photonics, Higashi-ku, Japan) with 40 $\times$ magnification.

The Definiens TissueStudio Composer segmentation and classification tool (Definiens AG, Munich, Germany) was used to identify the tumor regions of interest (Figure 1). Both the membranous and nuclear features of the cells were identified and scored using four multiparametric training algorithm adjustment sliders.

The settings for cell count and differentiation between stroma and neoplasia included: segmentation, nuclear curvature threshold, intensity thresholds, size, roundness, compactness, and elongation. The main parameters for membrane staining

evaluation included segmentation, intensity thresholds, cell size, nuclear size, cell radius, roundness compactness, and elongation.

We performed the final analysis using two new algorithms distinguishing neoplastic and non-neoplastic counterparts of the tumor, and including parameters for the intestinal and the diffuse type gastric carcinoma.

Percentage of stained tumor cells and their membrane staining distribution and intensity were recorded by means of a quantitative scoring novel algorithm developed by our group using Definiens TissueStudio 2.0 image analysis software (Definiens AG, Munich, Germany).

According to validated score criteria,^{15,23} 10% of complete, lateral or basal-lateral membrane HER2 positivity in the neoplastic component of the core was the cutoff value. HER2 was graded on a scale of 0–3 (0 for no reactivity or membranous reactivity in <10% of cells; 1+ for faint/barely perceptible membranous reactivity in >10% of cells or reactivity detected in only part of the cell membrane; 2+ for weak-to-moderate complete or basolateral membranous reactivity in >10% of tumor cells; 3+ for strong complete or basolateral membranous reactivity in >10% of tumor cells). Cases with scores of 2+ or 3+ were considered positive (Figure 1).

An expert pathologist validated results by blindly evaluating all 1+ (209), 2+ (141), and 3+ spots (109) and 10% of zero-scored spots (166). Subsequently, a second expert pathologist blindly and randomly reviewed 10% of 1+ (21) and 3+ spots (11), 20% of 2+ (28) scored spots.

Fluorescence *In Situ* Hybridization

ERBB2 fluorescence *in situ* hybridization analysis was performed on immunohistochemically scored

Figure 1 Digital evaluation of 3+ (a) and 2+ (b) score. In blue is shown the total cell count, in green the neoplastic cell count, in red the strong complete or basolateral membranous reactivity, in orange the weak/moderate complete or basolateral membranous immunoreactivity.

2+ tumors (24 cases), using the PathVysion ERBB2 DNA probe kit (Abbott, North Chicago, IL, USA), according to the manufacturer's protocol.

Briefly, standard 4- μ m-thick sections were baked overnight at 56 °C, deparaffinized and dehydrated. Pre-treatment steps included 0.2 N HCl (room temperature (RT), 20 min), 1 N NaSCN (80 °C, 10 min) and protease digestion (37 °C, 20–30 min).

Dual-color probe for ERBB2 (Spectrum Orange) and chromosome centromere (CEP) 17 (Spectrum Green) was applied on the section, cover slipped and sealed with rubber cement. Specimens were denaturated (74 °C, 4 min) and hybridized (37 °C, overnight) in a hot-plate (HyBrite chamber Vysis, Abbott, North Chicago, IL, USA). Slides were washed ($2 \times$ SSC/0.3% NP40, first 1 min at RT, thereafter 2 min at 72 °C) and counterstained with 4',6-diamidino-2-phenylindole (DAPI).

Results were analyzed under a fluorescence microscope (Zeiss, Oberkochen, Germany) using recommended filters. In each specimen at least 60 cells were counted for both ERBB2 and CEP17 signals.

ERBB2 was considered amplified when the ratio of ERBB2 to CEP17 average copy number was ≥ 2 .

Statistical Analysis

SPSS statistical software program (SPSS, Chicago, IL, USA) was used. All correlation analyses were performed using χ^2 or Fisher exact test, according to variable number. Overall survival was estimated by Kaplan–Meier method and evaluated using log-rank (Mantel–Cox) test.

Multivariate survival time analysis was performed using multiple Cox regression model.

All the probability values were two-sided, with an overall significance level of 0.05.

Results

HER2 Expression in Primary Gastric Cancer

HER2-positive tumors (score 2 and 3) were 51/292 (17%) cases, and specifically 27 tumors (9%) were classified as score 3+, 24 tumors (8%) as score 2+. Negative cases included 29 (10%) score 1+ carcinomas and 212 (73%) score 0 tumors.

Among 51 positive cases, 15 (30%) showed immunoreactive tumor cells in all neoplastic spots whereas heterogeneous HER2 expression was observed in 36 patients (70%), as detailed in Table 2. Whole tissue section were evaluated in heterogeneous cases and 13 (36%) tumors were classified as negative (score 0 and 1). Within this group, 10 cases displayed only one positive spot in TMA analysis.

Comparative analysis of HER2 expression in tumor and tumor invasive front (202 cases) showed discordance in 20 (65%) of the 31 score 2 or 3 cases. Four tumors showed positive HER2 tumor cells only at the tumor infiltrative front. Analysis of the whole

Table 2 Heterogeneous HER2 expression

Nr	Neoplastic spots	Positive spots (%)
8	2	1 (50)
7	3	1 (33)
3	3	2 (67)
7	4	1 (25)
1	4	2 (50)
2	5	1 (20)
6	6	1 (17)
2	6	4 (67)
36		

nr: number of cases.

sections of heterogeneous tumors displayed tumor and tumor invasive front discordance in 50% of positive cases.

ERBB2 Amplification in Primary Gastric Cancer

Fluorescence *in situ* hybridization analysis showed amplification in 3/24 (13%) cases. The remaining carcinomas included 14 non-amplified tumors, 4 non-evaluable cases for inadequate quantity of tumor, 3 inadequate cases for technical problems.

Considering together immunohistochemistry and fluorescence *in situ* hybridization results, positive cases (immunohistochemistry 3+ score and immunohistochemistry 2+ score/fluorescence *in situ* hybridization amplified) were 37/292 (13%).

HER2 Status and Clinicopathological Features

HER2 status was significantly associated with Laurén classification, since HER2 positivity was associated with intestinal-type cancer ($P=0.018$), whereas no statistically significant association was observed with World Health Organization classification, tumor site, that is, gastroesophageal junction, cardial, fundic or antral location, as well as pathological T- and N-stage categories.

No significant relationships were found between HER2 expression and gender and age.

Correlations between HER2 status and clinicopathological features are summarized in Table 1.

HER2 Expression in Lymph Node Metastasis and Dysplasia

Comparative analysis of HER2 expression in primary cancer and synchronous lymph node metastasis was performed in 154 cases. Discordant immunohistochemistry score was seen in 22/154 (14%) cases. Considering negative primary cancers (126 cases, score 0 or 1), we found 6 (5%) discordant HER2-positive metastasis cases (score 2 or 3). Among the primary tumors scoring 2 (12 cases) and 3 (17 cases), we observed a decrease of

immunohistochemistry score in the matched lymph node metastasis (score 0 or 1) in 9 (75%) and 7 (41%) cases, respectively (Table 3).

Evaluation of dysplastic epithelium surrounding the tumor showed score 2 or 3 immunohistochemistry in 12/63 (19%) high-grade dysplastic samples and in 7/75 (9%) low-grade dysplastic samples. Considering paired neoplasms, high-grade dysplasia showed concordance in 3/7 score 2 (43%) and 4/9 score 3 cases (44%). We found only one concordant HER2-positive low-grade dysplasia case (Table 3).

Survival Analysis

Survival analysis indicated that HER2 status was related to patient survival. As shown in Figure 2, significant association was found between HER2 positivity and poor survival with 14.1 and 40.8 months median overall survival for positive and negative cases, respectively ($P=0.006$). Considering Laurén types, stage and lymph node status separately, this association was retained only in intestinal tumors ($P=0.002$) (Figure 3) and node-positive cases ($P=0.03$).

Patient survival was significantly associated with Laurén classification (intestinal vs non-intestinal; $P=0.034$) and with pathological staging ($P<0.001$ for both pT and pN) (data not shown).

Multivariate analysis conducted including stage, Laurén classification, and HER2 status showed that HER2 is not an independent prognostic factor ($P=0.188$).

Discussion

The assessment of HER2 status is crucial in selecting patients who benefit from targeted therapy with

Trastuzumab, as recently demonstrated by Trastuzumab for Gastric Cancer (ToGA) trial.³ However, some issues still require additional data, including the distribution of HER2 overexpression and/or ERBB2 amplification in gastric cancer, the concordance rate between primary gastric cancer and lymph node metastasis, the prevalence of HER2 expression in precancerous lesions, and the prognostic significance of HER2 status.

Overexpression of HER2 protein in gastric cancer, detected by immunohistochemistry, was first

Figure 2 Kaplan–Meier survival analysis of HER2 status in gastric cancer.

Table 3 HER2 expression in primary gastric cancer, corresponding lymph node metastasis, and dysplastic counterpart

HER2 score	Primary gastric cancer (%)			
	0	1	2	3
<i>Lymph node metastasis (%)</i>				
0	104 (68)	12 (8)	9 (6)	6 (4)
1	3 (2)	0	0	1 (1)
2	3 (2)	2 (1)	2 (1)	2 (1)
3	1 (1)	0	1 (1)	8 (5)
<i>High-grade dysplasia (%)</i>				
0	29 (46)	4 (6)	4 (6)	3 (5)
1	5 (8)	4 (6)	0	2 (3)
2	3 (5)	1 (2)	1 (2)	0
3	1 (2)	0	2 (3)	4 (6)
<i>Low-grade dysplasia (%)</i>				
0	45 (60)	7 (9)	4 (5)	2 (3)
1	5 (7)	2 (3)	2 (3)	1 (1)
2	6 (8)	0	0	0
3	0	0	0	1 (1)

Figure 3 Kaplan–Meier survival analysis of HER2 status in intestinal-type gastric cancer.

described in 1986.²⁶ Since then, a wide range of HER2 immunoreactivity, from 6 to 34%, has been observed.^{8,12,14,19,27–34} This variability required to determine HER2 scoring guidelines, necessary for patients selection for targeted therapy,^{15,23} similarly to what was done previously for breast cancer.³⁵

The image analysis algorithm we set up to analyze tissue microarray slides was built by a skilled pathologist translating HER2 testing protocols^{15,23} to machine codes.³⁶

Under supervision of expert pathologists, digital image analysis can be a useful diagnostic support tool. Automated algorithms could help in standardizing and maintaining high-quality HER2 scoring in patients with gastric and gastroesophageal junction cancer, particularly in equivocal cases.³⁷

We observed HER2 immunoreactivity (2+ or 3+) in 17% of cases, close to the values reported by most recent studies (15–28%).^{18,38,39} However, considering fluorescence *in situ* hybridization results, we found a lower rate of positivity (13%), since 70% of 2+ tumors did not display ERBB2 amplification.

Our analysis documented the association between HER2 positivity and intestinal-type cancer, as recently reported.^{1,12,13,21} This finding supports the concept that there are substantial molecular differences between the different histologic tumor types, which likely develop through different pathways.

We found no statistically significant correlation among HER2 overexpression and tumor site. Although in previous studies a distinction in tumor location is not mentioned,^{8,9,12,17,20,40–44} the majority of the present literature makes a distinction between gastric and gastroesophageal junction cancers.^{3,38,45,46} Moreover, some works showed a higher HER2 expression and/or ERBB2 amplification in gastroesophageal junction tumors.^{3,38} It must be noted that the high prevalence of antral cancers in our cohort limited the statistical evaluation of differences between tumors arising in the gastroesophageal junction and all others.

Our tissue microarrays included core samples of tumor, at least two and up to seven per case, from the superficial part to the invasive front, low- and high-grade dysplasia, lymph node metastasis.

In contrast to breast carcinoma, heterogeneity of HER2 overexpression and ERBB2 amplification is frequently described in gastric cancer.^{16,45–47} This phenomenon could represent a major drawback to sampling tumors for targeted cancer therapy.^{2,5,45,46,48,49} In colon and lung cancer, intratumoral heterogeneity of KRAS mutation status is reported and crucial in selecting patients for anti-EGFR therapy.^{50,51} It is described that testing DNA from a single sample of colorectal cancer will wrongly assign wild-type status to 10% patients,⁵⁰ suggesting multiple sampling in order to improve mutations detection.

In this study, we found heterogeneous HER2 expression in 36/51 (71%) positive scored gastric

primary tumor, with 22 cases showing positive score in less than 50% of the neoplastic spots. Similarly to colon cancer, these results, together with whole tissue section analysis, show how multiple sampling may reduce the number of false negative cases.

Although it has been previously reported that HER2 staining has no preferential distribution inside the tumor, with negligible variation between tumor mucosa surface and tumor invasive front,⁵² we found high rate of heterogeneity of HER2 expression in tumor and tumor edge spots, with 65% positive tumor displaying negative score in their invasive front.

The therapeutic implications of heterogeneous HER2 status are yet to be ascertained, although we could hypothesize that only the HER2-positive neoplastic cells would be sensitive to Trastuzumab.⁵³ Whether HER2 heterogeneity adversely affects treatment response requires further clinical prospective studies. However, the possibility of heterogeneous HER2 expression has important implications for HER2 testing, requiring preferably the analysis of more than one tumor block in surgical resections and multiple biopsy specimens in unresectable advanced tumors.

An additional facet linked to patients' selection for targeted therapy is the different HER2 expression between primary tumor and metastatic site. Several papers investigated this issue in breast cancer with a concordance range between primary tumor and corresponding synchronous lymph node metastasis of 89–100%.^{54–58}

Previously, few studies evaluated the expression of HER2 in gastric cancer in lymph node and distant metastasis, showing high concordance (95%).^{18,59} Our comparative analysis of HER2 expression in primary gastric cancer and synchronous lymph node metastasis showed a concordance rate of 86%. However, we found six cases with positive nodes and negative primary tumors and 16 positive primary tumors that lost HER2 immunoreactivity in the corresponding metastatic nodes. These observations underline the therapeutic importance of HER2 evaluation in both primary and metastatic site, when feasible, and suggest that in gastric cancer HER2 status could change during the metastatic process and tumorigenesis.

We were interested to evaluate low-grade and high-grade dysplasia for HER2 immunoreactivity since, in breast cancer, HER2 is overexpressed in ductal carcinoma *in situ* and not in benign and atypical proliferative lesions.^{60–64} Few studies have described the upregulation of HER2 in gastric dysplastic lesions.^{53,65–69} To our knowledge, this is the first time that HER2 status is assessed in a wide series of gastric precancerous and invasive lesion. High-grade dysplasia showed concordant negative (0 and 1) and positive (2 and 3) scores in 42 and 7 cases, respectively. Moreover, in nine cases with positive neoplastic cores, paired dysplasia was negative and, more intriguing, five

cases showed negative neoplastic spots, whereas high-grade precursor lesions displayed HER2 immunoreactivity.

The biological and clinical significance of HER2 in gastric dysplasia is unknown.

In breast carcinoma, HER2 overexpression in ductal carcinoma *in situ* has been associated with the risk of invasive progression^{18,70} and with several poor prognostic features.⁶² Moreover, the increased rate of HER2 overexpression in ductal carcinoma *in situ* compared with invasive breast cancer has been cited as evidence that HER2 expression may represent a transient phenomenon. HER2 may be characteristic of discrete stage of tumor that is upregulated as *in situ* progress to invasive tumors and downregulated in more advanced disease.⁷⁰ Alternative hypothesis is that HER2 downregulation is an epiphenomenon of disease progression and invasive cancer may develop *de novo* by mechanisms independent of HER-2/neu.^{62,70}

Present data suggest that HER2 overexpression phenomenon may appear quite early also in gastric carcinogenesis and need further analysis to better understand its biological and clinical significance.

Another controversial issue is the prognostic significance of HER2 overexpression in gastric cancer, with some reports showing a negative effect^{19,20,42,71} and others unable to prove HER2 prognostic value.⁴⁶

We found no statistically significant HER2 status association with pathological T-stage categories,^{16,17} nevertheless survival analysis demonstrated that HER2 status is related to poor prognosis. Patients with primary HER2-positive tumor showed decreased overall survival, in particular considering the intestinal-type tumor and node-positive subgroup. However, multivariate analysis indicated that HER2 is not an independent prognostic factor, as previously reported.^{16,17,72–79}

In conclusion, HER2 targeted therapy is now included in the management of the metastatic gastric cancer patient. However, the heterogeneous expression in primary tumor and the variable lymph node metastasis status bring up the questions about what type and how many samples to use for HER2 evaluation. Our data suggest that it could be important to expand the analysis to more than one sample and, if feasible, to specifically evaluate the metastatic foci. Although our data demonstrated a strong correlation between HER2 status and patients survival, there is still a need to improve the knowledge about the link between HER2 expression and the other diverse molecular signatures of this tumor, to better understand its role in gastric cancer tumorigenesis and progression.

Disclosure/conflict of interest

The authors declare no conflict of interest.

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