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Title: HER2 expression and its clinicopathological features in resectable gastric cancer

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Short running head: HER2 in resectable gastric cancer

Abstract

Background: A recent randomized controlled trial (ToGA study) established the standard scoring criteria of human epidermal growth factor receptor 2 (HER2) for gastric cancer and demonstrated efficacy of trastuzumab for metastatic gastric cancer. The aim of this study was to evaluate the frequency of HER2-positive cases by application of the standard criteria in patients with resectable gastric cancer and to examine the relationships between HER2 expression and prognosis, mucin phenotype , p53 status and clinicopathologic features.

Methods: A total of 213 patients were included in this retrospective study. All tumor samples were examined for HER2 expression by immunohistochemistry, HER2 amplification by *in situ* hybridization, and mucin and p53 expression by staining for CD10, MUC2, MUC5AC, MUC6, and p53.

Results: HER2-positive tumors were identified in 25 patients (11.7%). HER2-positive cases were more frequently found in male, older patients and the intestinal histologic type (P=0.0048, P=0.0309, and P<0.0001 respectively). Although no association was found between HER2 overexpression and mucin phenotype, expression of CD10 and p53 was significantly correlated with HER2 positivity (P=0.0079 and P=0.013). Overall survival of HER2-negative and -positive patients was not significantly different. However, in patients with stage III/IV, overall survival was worse in HER2-positive patients (P=0.0149). In comparison between dual *in situ* hybridization (DISH) and fluorescence *in situ* hybridization (FISH), four IHC2+/3+ cases that were DISH positive were judged as negative by FISH.

Conclusions: Our study indicated that HER2 expression in resectable gastric cancer is less frequent than metastatic gastric cancer. The impact of HER2 expression on survival is limited. DISH was superior for evaluating cases with limited HER2 expression.

Mini-abstract: HER2 expression in resectable gastric cancer is less frequent than metastatic or recurrent gastric cancer. The impact of HER2 expression on survival is limited, especially in earlier stages.

Key words: human epidermal growth factor receptor 2, gastric cancer, immunohistochemistry, *in situ* hybridization, mucin phenotype

Introduction

The clinical benefit of trastuzumab was shown in the international phase III randomized controlled trial in patients with inoperable or metastatic human epidermal growth factor receptor 2 (HER2)-positive advanced gastric or gastroesophageal junction cancer (Trastuzumab for Gastric Cancer study, ToGA study) [1]. Although many studies have previously evaluated HER2 status in gastric cancer, the patient cohorts and scoring criteria have varied, resulting in discrepancies in HER2 positivity that have ranged from 8.2 to 53.4% [2]. Frequent heterogeneity of HER2 status in gastric adenocarcinoma has also made the diagnosis of HER2 overexpression difficult and irreproducible. To solve these problems, the ToGA study employed the new set of immunohistochemistry (IHC) scoring criteria, which was developed based on the study by Hofmann and colleagues and considers biological features of gastric cancer [3]. Using the new criteria, HER2-positive tumors were found in 22.1% of metastatic gastric cancer cases.

The efficacy of trastuzumab for metastatic gastric cancer has been clearly demonstrated in the ToGA study, suggesting that anti-HER2 therapy is also promising for resectable HER2-positive gastric cancer. However, the frequency of HER2-positive tumors by the new criteria in resectable gastric cancer has not been examined. To design a proper trial protocol of neoadjuvant or adjuvant therapy using trastuzumab for resectable HER2-positive gastric cancer, the frequency of HER2 positivity in resectable gastric cancer needs to be determined.

Some studies have reported that HER2 expression is associated with poorer prognosis in gastric cancer [4-12], while its direct correlation has not been proven [13-19]. Interpretation of these controversial results is difficult, because each study used a different definition of HER2 overexpression or amplification. Regarding clinicopathological features of HER2-positive gastric cancer, HER2 expression and intestinal histologic type has shown high correlation. When focusing on the cellular origin or differentiation of gastric adenocarcinoma, expression of different types of mucins are used as epithelial

differentiation markers: MUC5AC and MUC6 as gastric cell markers, CD10 and MUC2 as intestinal markers [20]. Development of HER2-positive tumors could be linked to the particular type of differentiation. However, no study has investigated the relationship between HER2-positive gastric cancer and mucin phenotype. The overexpression of mutated p53 gene is a major genetic event in the gastric carcinogensis [21]. Although some studies have shown the correlation between p53 nuclear staining and HER2 expression, little is known about the relationship between p53 overexpression and HER2 positivity or mucin phenotype [17, 22].

The purpose of this study was to evaluate the frequency of HER2-positive gastric cancer, by applying the standard scoring criteria in patients with curatively resected gastric cancer. The relationships between HER2 expression and prognosis, mucin phenotype, p53 overexpression and other clinicopathological features were also examined. Finally, we discuss heterogeneity of HER2 overexpression in gastric cancer with careful review of the cases with discordance of HER2 overexpression and gene amplification, or the two different hybridization methods, fluorescence *in situ* hybridization (FISH) and dual color *in situ* hybridization (DISH).

Patients and methods

Patients

Among patients who underwent curative resection for primary gastric cancer at the Kyoto University Hospital between January 2001 and December 2007, 242 patients were diagnosed with pathological TNM stage IB to IV. Excluding 29 patients who received neoadjuvant chemotherapy, a total of 213 patients were included in this retrospective study. The study protocol was approved by the institutional review board. Clinicopathologic parameters, including age, gender, tumor location, histological classification, pathological TNM stage, and lymphovascular invasion status was retrieved from medical charts or pathologic reports. Histological classification was determined according to Lauren’s

classification, and the World Health Organization (WHO) classification. In the WHO classification, tubular adenocarcinoma with a poorly differentiated variant in more than half part of the tumor was defined as mixed carcinoma, and if less than half, tubular adenocarcinoma. There were 60 patients with adjuvant chemotherapy.

Evaluation of HER2 expression and amplification

All tissues were fixed with 10% buffer formalin for 24-72 h, and then paraffin-embedded. Sections of 3-μm thick were cut from a paraffin block of each specimen and applied to DISH, hematoxylin and eosin staining, and IHC of HER2. Among 213 cases, 32 with IHC2+/3+ or DISH+, and 43 randomly selected from IHC 0/1+ cases, were evaluated by FISH. IHC staining of HER2 with PATHWAY® HER2/neu (4B5) antibody (Ventana Medical Systems) was performed using an automated slide stainer (Bench-Mark XT; Ventana Medical Systems). As 4B5 stains show invariably extensive cytoplasmic background staining of the gastric foveolar layer and intestinal metaplasia, HER2 IHC was evaluated according to the stepwise process proposed by Rüschoff and colleagues [23]. For IHC scoring, the scoring scheme of the ToGA was employed [1].

DISH was performed using the INFORM Dual ISH HER2 kit (Ventana). HER2 IHC and DISH were evaluated by an investigator (YK) and a pathologist (SM). Positivity for HER2 was defined as either IHC3+ or IHC2+ with DISH+. FISH analysis was carried out using the PathVysion HER-2 DNA Probe Kit (Abbott) after pretreatment with the Paraffin Pretreatment Kit (Abbott). FISH was evaluated by an investigator (YK). Nuclei of invasive tumor cells were scored using Biozero 8000 microscope (Keyence) equipped with DAPI/Green/Orange triple bandpass filters. In DISH and FISH, the HER2/chromosome 17 (Chr17) ratio was determined by counting the HER2 signals and Chr17 signals in 20 nuclei. Amplification of the HER2 gene was defined as a HER2/Chr17 ratio of higher than 2.2. Negativity for HER2 amplification was defined as a HER2/Chr17 ratio < 1.8. When a ratio was between 1.8 and 2.2, signals in another 20 nuclei were counted, and a HER2/Chr17 ratio in a total of 40

nuclei was determined. When a ratio was ≥ 2.0, amplification was defined as positive; otherwise it was defined as negative.

Mucin phenotype and p53 expression

Mucin and p53 IHC staining was performed by the tyramide signal amplification-avidin-biotin complex method [24]. We used monoclonal antibodies against MUC5AC (Novocastra, Newcastle-upon-Tyne, UK; diluted 1:100) as a marker for gastric foveolar cells, MUC6 (Novocastra; 1:100) as a marker for gastric mucous neck cells and pyloric glands, MUC2 (Novocastra; 1:100) as a marker for intestinal goblet cells, CD10 (Novocastra; 1:100) as a marker for the small intestinal brush border, and p53 (Novocastra, NCL-p53-Do7).

The expressions of CD10, MUC2, MUC5AC,and MUC6 were regarded as positive when more than 10% of the area was positively stained [20]. Overexpression of p53 was regarded as positive when more than 10% of tumor cells displayed nuclear immunostaining [25]. The phenotypes were classified into four categories according to the combination of the expressions of CD10 (brush border), MUC2 (goblet cells), MUC5AC (gastric foveolar epithelium), and MUC6 (mucous neck cells, pyloric glands). The intestinal (I) phenotype exhibited expression of either CD10 or MUC2 but not of MUC5AC or MUC6. The gastrointestinal (GI) phenotype exhibited expression of either CD10 or MUC2, in addition to expression of either MUC5AC or MUC6. The gastric (G) phenotype exhibited expression of either MUC5AC or MUC6 but not of CD10 or MUC2. The unclassified (U) phenotype exhibited no expression of CD10, MUC2, MUC5AC, or MUC6.

Recurrence patterns

Recurrence patterns were classified as locoregional, peritoneal, or hematogenous[26]. Locoregional recurrence was defined as any cancer recurrence at the resection margin or LNs (including regional nodes as well as retroperitoneal, retropancreatic, para-aortic and

Virchow's nodes). Peritoneal recurrence was defined as any cancer recurrence within the abdominal cavity due to intraperitoneal distribution including rectal shelf. Hematogenous recurrence was defined as any metastatic lesion detected in liver, lung, ovary, adrenal gland and bone.

Statistical analysis

All statistical analyses were conducted using the JMP 9.0.0 statistical software program (SAS Institute Inc.). The Pearson chi-square test and Wilcoxon test were performed to assess the correlation of clinicopathologic parameters with HER2 positivity. All P values were two-sided and $P<0.05$ were considered statistically significant. Survival curves were plotted using the Kaplan–Meier method, and the significance of differences between survival curves was evaluated using the log-rank test.

Results

HER2 positivity and clinical factors

The results for HER2 IHC and DISH in 213 patients are shown in Table 1. When HER2 positivity was defined as IHC3+, or IHC2+ and DISH+, 25 out of 213 patients (11.7%) were diagnosed as HER2 positive. The association of HER2 positivity and clinicopathologic features is summarized in Table 2. HER2-positive cases were more frequently found in male ($P=0.0048$) and older ($P=0.0309$) patients. The HER2-positive tumor was twice as often found in the upper third compared to the lower third lesion (13.8% vs 8.6%). Notably, HER2-positive tumor was more frequently found among tumors invading to the esophagus; four out of 12 tumors (33.3% vs 10.4%, $P=0.0167$). In terms of histology, HER2-positive gastric cancer showed a predominantly papillary, tubular, and mixed adenocarcinoma according to the WHO classification ($P<0.0001$). According to Lauren's classification, HER2 overexpression was more often detected in the intestinal histologic type (22.3%), than in the mixed (15.4%) or diffuse (0%) type. All four mixed type HER2-positive cases displayed HER2 IHC positivity in the intestinal component, supporting the strong correlation between

HER2 expression and intestinal histologic type. HER2-positive tumors tended to present venous invasion, although this tendency was not statistically significant (P=0.2150). No correlation was found between T, N-factor, as well as TNM stage and HER2 positivity.

HER2 and mucin phenotypic classification

The results of the expression analysis of four mucin markers and phenotypic classification based on mucin expression are shown in Table 3. Among the four markers, expression of CD10 was significantly correlated with HER2 positivity (P=0.0079). The representative example of a case positive for HER2 and CD10 is shown in Figure 1. There was no correlation between the other three mucin markers and HER2 overexpression. When the mucin phenotype was classified into four subtypes, the HER2 positive ratio in the I, G, and GI phenotypes was 12.2%, 13.9%, and 13.2%, whereas only one case among the U phenotype exhibited HER2 positivity (3.2%); however, the difference was not significant. Expression of any type of mucin or mucin phenotype was not associated with overall survival (OS) or recurrence-free survival (RFS) of patients (data not shown).

HER2 and p53 overexpression

Overexpression of p53 was detected in 75 (35.2%) of the cases, being significantly correlated with HER2 positivity (P=0.013); 16 out of 25 cases (64.0%) in HER-2 positive tumor, and 59 out of 188 cases (31.4%) in HER-2 negative tumor. It was also expressed more in intestinal than diffuse/mixed type (54.3% vs 20.2%: P<0.0001). Regarding the relationship between p53 and markers for mucin phenotype, p53 overexpression tends to be found more frequently in CD10-positive than in CD10-negative tumors (46.9% vs 31.7%: P=0.0501).

HER2 and survival

Kaplan-Meier curves for OS and RFS according to the different HER2 status are shown

in Figure 2a and 2b. Although survival curves of HER2-positive patients were slightly worse than HER2-negative patients, the difference was not significant (OS: P=0.2203, RFS: P=0.1996). When patients were stratified into TNM stage IB/II and III/IV, no correlation was found between HER2 status and OS in the stage IB/II group (P=0.6060). However, in patients with stage III/IV, OS of patients with a HER2-positive tumor was significantly worse than patients with a HER2-negative tumor (P=0.0149) (Figure 2c and 2d).

In the HER2-positive patients with stage III/IV, the most common pattern of recurrence was hematogenous (3 cases, 60%), followed by locoregional (1 case, 20%) and peritoneal (1 case, 20%) recurrence. In the HER2-negative patients with stage III/IV, the most common pattern was locoregional (15 cases, 35.7%), followed by peritoneal (14 cases, 33.3%) and hematogenous (13 cases, 31.0%) recurrence. Patterns of recurrence were not significantly different between the two groups (P=0.4316).

Diagnosis of HER2 positivity and HER2 heterogeneity

The summary of the assessment of IHC scoring and HER2 amplification by *in situ* hybridization in 75 cases is shown in Table 4. When IHC2+/3+ was defined as IHC positive, and IHC1+/0 was defined as IHC negative, the overall concordance rate between IHC and DISH was 96.7%. Among 13 tumors with equivocal IHC results (IHC2+), DISH was positive in seven tumors (53.8%). When these IHC2+ cases were excluded, DISH was positive in all IHC3+ patients, and only one patient among IHC-negative patients showed HER2 amplification. Of 75 samples, four could not be assessed by FISH due to technical difficulties (2 cases with IHC0/DISH-, 1 case with IHC2+/DISH+, and 1 case with IHC0 and DISH+). By comparing the results between DISH and FISH analyses, four cases (5.6%) of inconsistency were identified. Three IHC3+ cases and one IHC2+ case positive for DISH were judged as negative by FISH.

Table 5 shows the ratio of HER2-stained cells with IHC2+ and 3+ in the HER2-positive cases. Among 25 tumors, only two (8%) stained 100% positive for HER2. Comparing the

area of HER2 expression and HER2 amplification, in the majority of HER2-positive cases, HER2 amplification was observed in the positively stained area by IHC for HER2. However, HER2 amplification in the HER2-negative area was occasionally identified.

Discussion

This study included 213 patients with curative resection of primary gastric cancer, and HER2 expression was assessed using the scoring scheme employed in the ToGA study [1]. We defined HER2 positivity as IHC3+, or IHC2+ and DISH+, because the clinical benefit of trastuzumab in this subgroup was evident. When the same definition is applied to the patients in the ToGA study, the HER2 positive ratio was estimated to be 17.1%, whereas the current study identified 25 HER2-positive cases, accounting for 11.7% of all included cases. The difference of HER2-positive ratio may be attributed to the different backgrounds of patients. That is, the ToGA study only included metastatic or recurrent gastric cancer patients, while the present study included curatively resected gastric cancer. A recent study has reported similar a HER2-positive ratio of 8.1% for curatively resected gastric cancer [16]. Taken together, HER2-positive gastric cancer might be less frequent in resectable gastric cancer than in metastatic cases.

There have been four large studies that have assessed HER2 expression and survival in gastric cancer patients, two of which have shown no association [14, 16]. The other two studies analyzed only intestinal type gastric cancer and demonstrated poorer prognosis of HER2-positive cases [13, 27]. In our study, patient survival was not significantly different between HER2-positive and -negative cases, even when the analysis included only intestinal type gastric cancer. Although the ratio of HER2-positive case was nearly half of our hypothesis, this study still maintained the enough statistical power to detect the general impact of HER2 expression on survival. Thus, in contrast to breast cancer in which HER2 overexpression is an established strong prognostic factor, HER2 status may not be a distinct prognostic factor in resectable gastric cancer. However, it is possible that HER2 is a

prognostic factor only for the advanced disease, because in the TNM stage III/IV subgroup, survival of HER2-positive patients was significantly shorter than that of HER2-negative patients. By applying the standardized scoring criteria on HER2 assessment in gastric cancer, further studies will provide distinct outcome around this issue.

Among 25 HER2-positive tumors in this study, 21 were intestinal type and four were mixed type according to Lauren's classification. This data is consistent with previous reports that the intestinal type showed a higher rate of HER2 positivity than the diffuse/mixed type [13, 14, 16, 27-31]. Strong correlation between HER2 positivity and intestinal histologic type is also supported by the finding that even in the four HER2-positive, mixed type cases, IHC staining of HER2 was positive only in the intestinal component. In the ToGA study, the HER2-positive ratio was higher in tumors at the gastroesophageal junction than in gastric cancer (33.2% vs. 20.9%). Similarly, the HER2-positive ratio was almost three times as much in the tumors invading to the esophagus compared to the other lesions in our study (33.3% vs 10.4%).

No correlation was found between HER2 positivity and T- or N-factor, as well as TNM stage in this study. HER2-positive tumor was found even in one of four T1a (tumor invades as far as lamina propria or muscularis mucosae) cases. Previous studies including all pathologic stages have also reported no correlation between pathologic stage and HER2-overexpression [13, 14, 27, 31]. Taken together, these suggest that HER2 overexpression occurs in early phase of gastric carcinogenesis. However, because numbers of patients in early stage were small in these reports, further studies are needed to determine the association between HER2 expression and development of gastric cancer.

This is the first study to examine the association between mucin phenotype and HER2 status of gastric cancer. Although we did not find significant correlation between HER2 status and mucin phenotype, HER2 expression was rarely detected in tumors without expression of any type of mucins (unclassified or null type). This observation is consistent with previous reports that the HER2 positive ratio is lower in diffuse or undifferentiated type

gastric cancer. Among four mucin markers, expression of CD10 (the marker for cells with small intestinal brush border differentiation) was significantly correlated with HER2 positivity. Because CD10 was strongly correlated with the intestinal histologic type in this study (P=0.0002), correlation between CD10 and HER2 expression may reflect the linkage between intestinal differentiation of cancer cells and HER2 expression. Correlation between intestinal phenotype and either better or poorer prognosis has been reported by some studies [20, 32]. However, no significant difference in patient survival was observed among the four different mucin phenotypes in this study.

Accumulation of p53 protein in the nuclei of carcinoma cells is known to correlate well with the presence of mutations in the p53 gene [33, 34]. Our study demonstrated the strong correlation between p53 overexpression and HER2 positivity, suggesting a possible role of p53 abnormality in the development of HER2-positive gastric cancer. These findings are consistent with previous studies, which have reported the correlation between p53 nuclear staining and HER2 positivity [17, 22]. Intriguingly, some studies also reported the linkage between alterations of p53 and the intestinal histologic type. Consistently, our study confirmed that p53 overexpression is more often found in the intestinal type of gastric cancer [17, 25]. These results suggest that the intestinal differentiation of cancer cells may also link to the expression of p53, as well as HER2 and CD10. Significance of expression of these molecules on the tumor biology or prognosis needs to be determined by further studies.

The concordance rate between IHC and DISH in the current study was as high as 96.7%, which is similar to previously published studies [3, 13, 14, 16, 27-31, 35]. Especially in IHC3+ cases, amplification of the HER2 gene was confirmed by DISH in all cases. While HER2 is usually homogenously expressed in HER2-positive breast cancer, HER2 expression in gastric cancer is often known to be heterogeneous [36]. The majority of HER2-positive tumors in our study also exhibited heterogeneous expression of HER2, and the ratio of HER2-positive cells varied **(**Table 5). In cases with smaller areas of HER2

expression, accurate diagnosis of HER2 using biopsy samples would be difficult, as biopsy taken from a negative area would return a false negative result. Therefore, to improve reliability of diagnosis in biopsy specimens, taking several samples from different parts of the tumor is recommended—the appropriate quantity or location for biopsy remains to be determined.

In IHC3+ cases, amplification of the HER2 gene was always confirmed by DISH, when the IHC stained area was evaluated. Even in a case with HER2 expression in less than 5% of tumor cells, HER2 amplification was confirmed in the small IHC stained area (Figure 3). Because HER2 amplification is exclusively detected in the IHC stained area, to properly examine amplification, it is crucial to examine HER2 expression first. In IHC2+ cases, however, even when the HER2 expression area is properly assessed, amplification was detected in only half of the patients. Thus, to determine the HER2 status of IHC2+ cases, HER2 amplification should also be evaluated. In comparison of DISH and FISH, FISH failed to detect HER2 amplification in four IHC3+/2+ cases, which were correctly diagnosed by DISH. Two of these cases expressed HER2 in less than 20% of tumor cells. In cases with a limited HER2 expression area, DISH may be easier for examination of the proper area, because comparison with IHC by conventional microscopy is possible.

In conclusion, our study indicated that HER2 expression in resectable gastric cancer is less frequent than metastatic or recurrent gastric cancer. The impact of HER2 expression on patient survival is limited, especially in earlier stages. When the variety of heterogeneity of HER2 expression is taken into consideration, assessing whole tissue sections or at least multiple biopsy samples is necessary to make proper diagnosis of HER2 status, and DISH proves to be superior for evaluating cases with limited HER2 expression. Further research is still need to clarify the relevance of HER2 heterogeneity for the clinical response to HER2 target therapy.

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Table 1 Comparison of IHC [a] and DISH test results for HER2 status

	IHC 3+	IHC 2+	IHC 1+	IHC 0	Total
DISH+	18	7	0	1	26 (12.2%)
DISH-	0	6	18	163	187 (87.8%)
Total	18 (8.5%)	13 (6.1%)	18 (8.5%)	164 (77.0%)	213

[a] IHC scores are based on the ToGA HER2 scoring scheme.

IHC, immunohistochemistry; DISH, dual color *in situ* hybridization; HER2, human epidermal growth factor receptor 2; ToGA, Trastuzumab for Gastric Cancer.

Table 2 Comparison of clinicopathologic factors between HER2-negative and HER2-positive gastric cancer

Variable	HER2 status		P value [a]
	Positive (%) [b] (n = 25)	Negative (%) [b] (n = 188)	
Age (years) [c]	71.2 ± 9.5	66.2 ± 11.2	0.0309
Gender			0.0048
Male	23(16.1)	120(83.9)	
Female	2(2.86)	68(97.1)	
Tumor location			0.8387
Upper	8(13.8)	50(86.2)	
Middle	11(12.2)	79(87.9)	
Lower	5(8.6)	53(91.4)	
Other	1(14.3)	6(85.7)	
WHO classification			<0.0001
Tubular	20(21.7)	72(78.3)	
Papillary	1(50.0)	1(50.0)	
Mucinous	0(0)	5(100)	
Poorly cohesive	0(0)	88(100)	
Mixed	4(15.4)	22(84.6)	
Lauren's classification			<0.0001
Intestinal	21(22.3)	73(77.7)	
Mixed	4(15.4)	22(84.6)	
Diffuse	0(0)	93(100)	
Depth of tumor (T)			0.8666
1a	1(25.0)	3(75.0)	
1b	3(13.6)	19(86.4)	
2	9(13.2)	59(86.8)	
3	8(11.4)	62(88.6)	
4a	4(9.1)	40(90.9)	
4b	0(0)	5(100)	
Nodal stage (N)			0.9052
0	6(8.8)	62(91.2)	
1	7(14.6)	41(85.4)	
2	7(13.0)	47(87.0)	
3a	3(11.1)	24(88.9)	
3b	2(12.5)	14(87.5)	
TNM stage			0.7816
IB	5(10.0)	45(90.0)	
II A	8(16.7)	40(83.3)	
II B	3(10.3)	26(89.7)	
III A	3(10.3)	36(89.7)	
III B	5(15.2)	28(84.9)	
III C	1(5.6)	17(94.4)	

IV	0(0)	6(100)	
Lymphatic invasion			0.2754
Absent	11(15.1)	62(84.9)	
Present	14(10.0)	126(90.0)	
Venous invasion			0.2150
Absent	10(9.1)	100(90.9)	
Present	15(14.6)	88(85.4)	
Recurrence			0.9071
Absent	17(11.6)	130(88.4)	
Present	8(12.1)	58(87.9)	

[a] P values were calculated using chi-square tests for categorical variables and Wilcoxon tests for continuous variables.

[b] Percentages show the ratio of HER2-positive or -negative patients for each item.

[c] Age was reported as the mean ± SD.

EGJ, esophagogastric junction; HER2, human epidermal growth factor receptor 2; WHO, World Health Organization.

Table 3 Comparison of HER2 status and mucin expression or phenotypic classification

	HER2 status		P value [a]
	Positive (%) (n = 25)	Negative (%) (n = 188)	
Mucin expression			
CD10			0.0079
Negative	14 (8.5)	150 (91.5)	
Positive	11 (22.5)	38 (77.6)	
MUC2			0.8870
Negative	15 (12.0)	110 (88.0)	
Positive	10 (11.4)	78 (88.6)	
MUC5AC			0.9711
Negative	11 (11.8)	82 (88.2)	
Positive	14 (11.7)	106 (88.3)	
MUC6			0.5207
Negative	15 (10.7)	125 (89.3)	
Positive	10 (13.7)	63 (86.3)	
Phenotypic classification			0.4564
Intestinal (I)	6 (12.2)	43 (87.8)	
Gastric (G)	9 (13.9)	56 (86.2)	
Gastrointestinal (GI)	9 (13.2)	59 (86.8)	
Unclassified (U)	1 (3.2)	30 (96.8)	

[a] P values were calculated using chi-square tests.

HER2, human epidermal growth factor receptor 2.

Table 4 Concordance between DISH and FISH results

	DISH		FISH			
IHC score	Positive	Negative	Positive	Negative	Not available	Total
3+	18	0	15	3	0	18
2+	7	6	5	7	1	13
1+	0	6	0	6	0	6
0	1	37	0	35	3	38
Total	27	57	20	51	4	75[a]

[a] Four specimens were not available for FISH.

DISH indicates dual color *in situ* hybridization; FISH, fluorescence *in situ* hybridization; IHC, immunohistochemistry.

Table 5 Ratio of HER2-stained cells in HER2-positive cases

Percentage [a]	n	(%)
≥90%	7	(28.0)
50% to <90%	9	(36.0)
30% to <50%	2	(8.0)
10% to <30%	7	(28.0)

[a] Percentage of HER2-stained cells with IHC2+ and 3+.

HER2, human epidermal growth factor receptor 2; IHC, immunohistochemistry.

Figure Legends

Figure 1

A representative case of HER2-positive gastric cancer with CD10 expression. **a** Specimen showing intestinal type adenocarcinoma (H&E, ×100). **b** Specimen showing strong basolateral membranous HER2 immunoreactivity which is scored as 3+ (HER2-IHC, ×100). **c** Specimen showing HER2 gene amplification with a HER2/Chr17 ratio of 5.62 (HER2-DISH, ×1000). Black and red signals represent HER2 and Chr17, respectively. **d** Specimen showing CD10 immunoreactivity along the luminal surface of the carcinoma glands (CD10, ×100; inset original magnification ×400).

Figure 2

Kaplan–Meier curves of HER2-positive and -negative patients for overall survival (**a**), recurrence-free survival (**b**), overall survival in TNM stage IB/II (**c**), and overall survival in TNM stage III/IV (**d**).

Figure 3

HER2-negative case with IHC2+-stained cells in less than 5% of tumor cells and HER2 amplification by DISH in the IHC2+-stained area. **a** Specimen showing IHC2+ in less than 5% of tumor cells (HER2-IHC, ×40; inset original magnification ×400). **b** Specimen showing high amplification in the IHC2+-stained area (HER2-DISH, ×1000)

Fig. 1

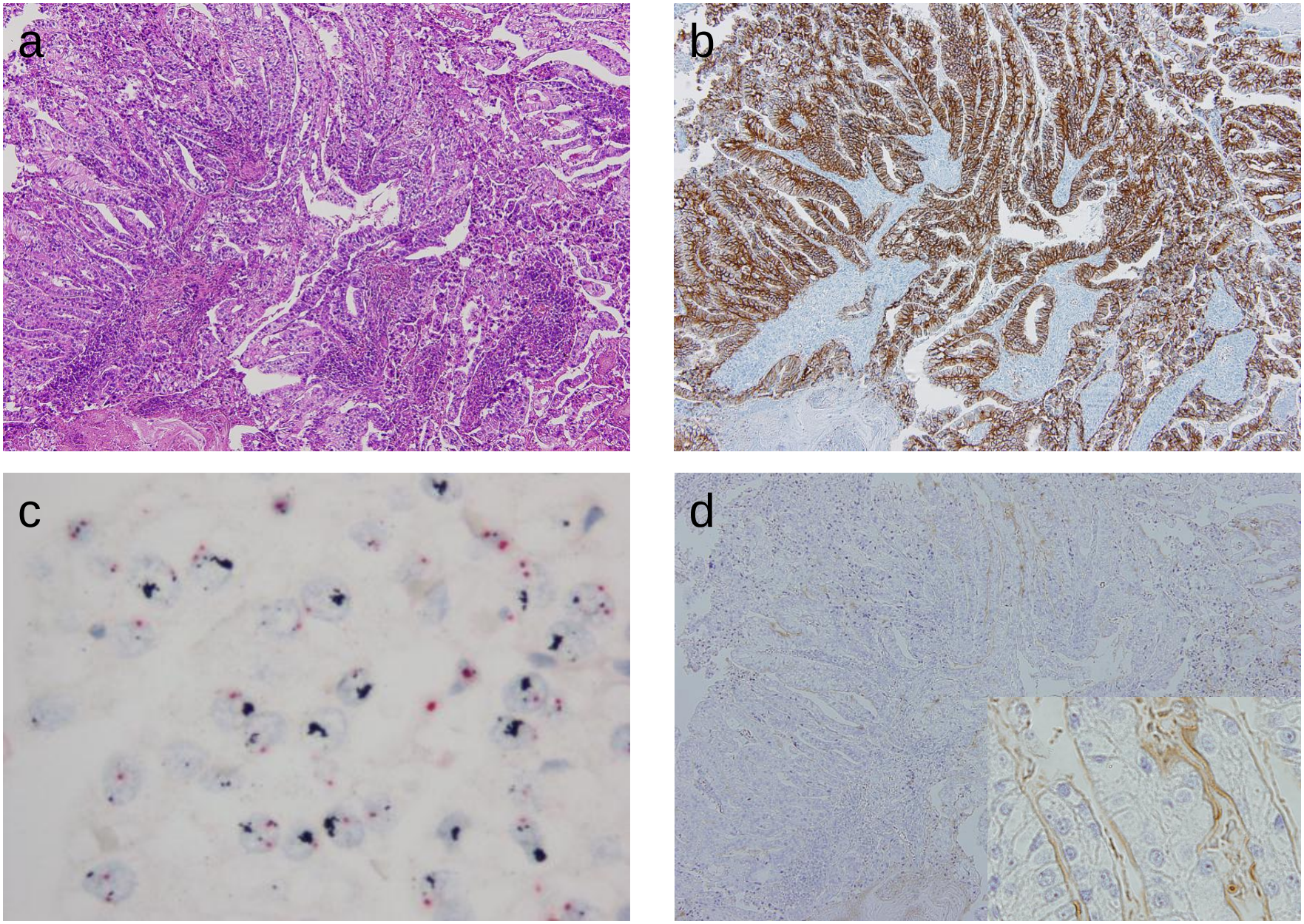

Fig. 2

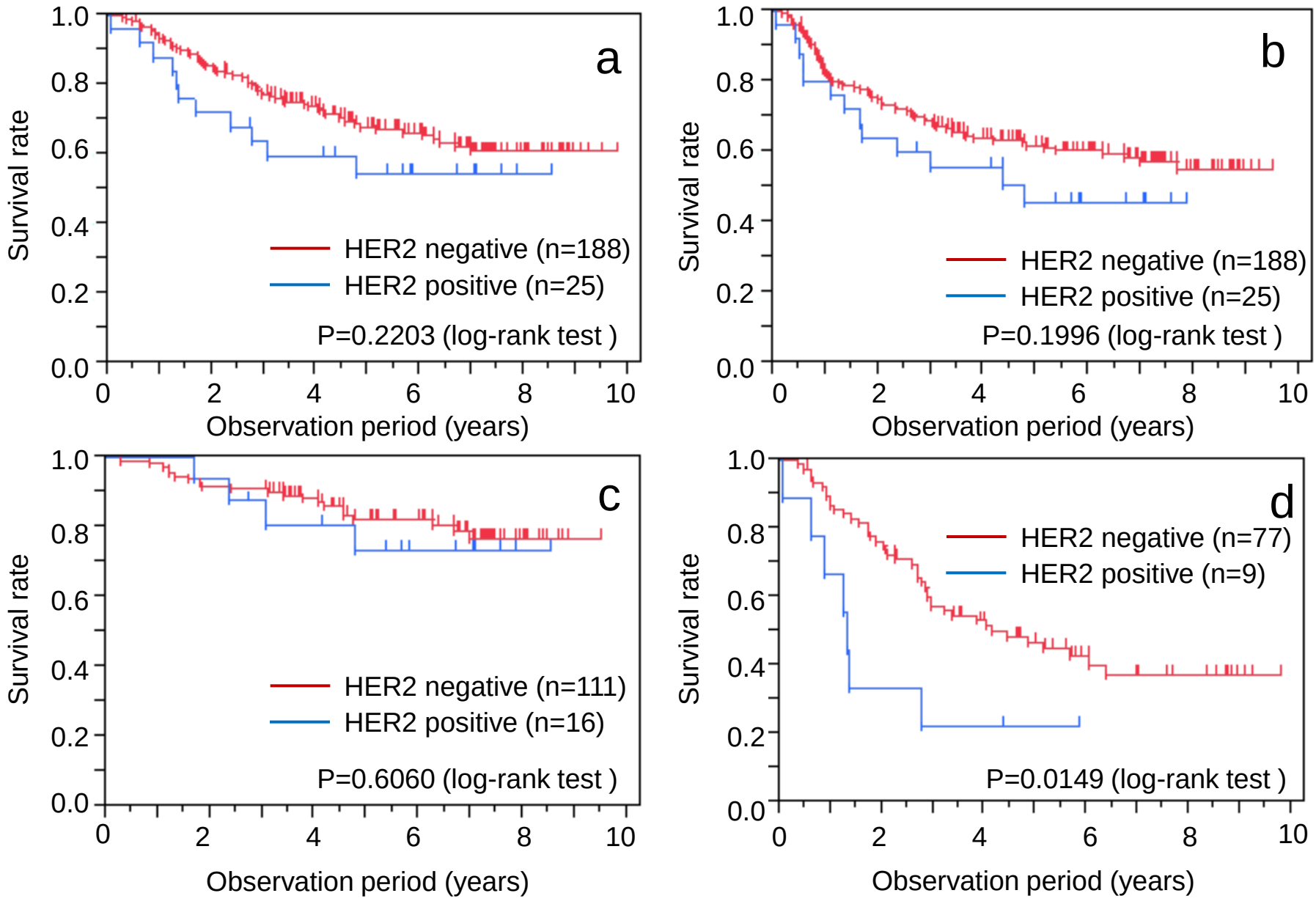
a
Survival rate
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
8
10
Observation period (years)
HER2 negative (n=188)
HER2 positive (n=25)
P=0.2203 (log-rank test)
b
Survival rate
Observation period (years)
HER2 negative (n=188)
HER2 positive (n=25)
P=0.1996 (log-rank test)
c
Survival rate
Observation period (years)
HER2 negative (n=111)
HER2 positive (n=16)
P=0.6060 (log-rank test)
d
Survival rate
Observation period (years)
HER2 negative (n=77)
HER2 positive (n=9)
P=0.0149 (log-rank test)

Fig. 3