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ORIGINAL ARTICLE

Prognostic implications of EGFR and HER-2 alteration assessed by immunohistochemistry and silver in situ hybridization in gastric cancer patients following curative resection

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Abstract
Background The aim of this study was to use immunohistochemistry (IHC) and silver in situ hybridization (SISH) to evaluate alterations in EGFR and HER2 in gastric cancer in order to determine the relationship with prognosis in gastric cancer patients following curative resection.
Patients and methods In this study, we analyzed EGFR and HER-2 status by IHC and SISH in 254 stage I–III gastric cancer patients who underwent curative surgery.
Results Thirteen cases (2.48 %) showed EGFR alteration by IHC or SISH. EGFR alteration was associated with older age ($P = 0.021$), intestinal type ($P = 0.040$) and higher stage disease ($P < 0.001$). The patients with operable state gastric cancer who had EGFR alteration had an unfavorable prognosis, and multivariate analysis confirmed that EGFR alteration was an independent unfavorable prognostic factor. Twenty-seven cases (10.6 %) showed HER-2 alteration by IHC or SISH. HER-2 alteration was associated with older age ($P = 0.006$), well or moderately differentiated histology ($P < 0.001$) and intestinal type ($P = 0.002$).
Conclusion HER-2 alteration is not an independent prognostic factor for curatively resectable gastric cancer. We observed EGFR alteration in a subset of cases with operable state gastric cancer and determined that it was associated with an unfavorable prognosis.

Keywords Gastric cancer · EGFR · HER-2 · Prognosis · SISH · IHC

Background

Gastric cancer is one of the most common epithelial malignancies worldwide [1]. Although our understanding of this disease has improved during the past decade, the prognosis for patients with advanced gastric cancer remains poor. Gastric cancer is associated with poor prognosis, and early diagnosis is challenging because most patients do not show symptoms until the late stages. However, current cancer screening programs include gastrofiberscopy, enabling earlier diagnosis and opportunities for curative intent surgery. At present, a cure can often be achieved primarily in localized disease with the appropriate local treatment [2–5]. Most patients with stage I–III gastric adenocarcinoma have local disease and can be cured with adequate D2 dissection. Nonetheless, the recurrence rate is high even after curative surgery. Therefore, the importance of adjuvant treatment is emphasized in these early stage cancers as well as in metastatic or recurrent disease.

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Furthermore, it is important to identify independent prognostic factors to select high-risk patients for treatment with tailored therapies.

EGFR and HER-2 are located at chromosome bands 7p12 and 17q12–q21, respectively. Gene amplification and/or protein overexpression of EGFR and HER2 have been observed in a variety of solid tumors. Increased EGFR expression is associated with an advanced stage and an unfavorable prognosis in many human tumors, such as non-small cell lung carcinoma, colorectal carcinoma, and breast cancer. In gastric cancers, the efficacy of these targeted therapies has not been clearly evaluated except for that of trastuzumab treatment in a metastatic or recurrent setting [6]. EGFR and HER-2 have been extensively studied in gastric cancer, but the results are highly variable. The rates of HER-2 overexpression in patients with gastric cancer vary, and the variability of this incidence rate may also result from differences in the detection methods and interpretation criteria [7–9].

EGFR and HER2 status is usually determined by immunohistochemistry (IHC) and/or in situ hybridization (ISH). Fluorescence in situ hybridization (FISH) requires a fluorescence microscope, and assessment of biopsies with heterogeneous staining patterns can be extremely laborious. Silver in situ hybridization (SISH) methods with traditional transmitted light microscopy have been introduced recently. There has been little published on the use of SISH in gastric cancer. In this study, we intended to validate the use of the SISH technique for assessing EGFR and HER2 gene amplification in gastric cancer.

In this study, we assessed the prognostic role of EGFR and HER-2 in a consecutive series of 254 gastric cancer patients who underwent curative surgery. The EGFR and HER-2 status of these patients was analyzed together with clinicopathological features and disease recurrence and survival, with the aim of identifying gastric cancer patients who could benefit from tailored and targeted treatments.

Patients and methods

Gastric cancer tissue specimens were obtained from 254 patients who underwent curative D2 dissection at Ulsan University Medical College, GangNeung Asan Hospital between March 1999 and December 2009. We reviewed the medical charts and pathological records for clinicopathological parameters such as age, gender, histological subtype, presence of lymphatic invasion, invasion depth, presence of lymph node and pathological stage. The mean patient age was 64.5 years, and all of the patients had undergone a curative resection (R0 according to the International Union Against Cancer guidelines). The clinical outcome was determined from the date of surgery until death or 31 December 2011, resulting in a follow-up period of from 1 to 125 months (mean 34.0 months). The cases that were lost to follow-up and deaths caused by problems other than gastric cancer were censored in the survival analysis. This study was approved by the Institutional Review Board of Gangneung Asan Hospital of the University of Ulsan College of Medicine.

Tissue microarray construction

Formalin-fixed using 10 % neutralized buffered formalin for 24 h, paraffin-embedded tissue samples of gastric cancer from patients who underwent curative surgery ($n = 254$) were obtained and arrayed using a tissue arraying instrument (Quick-Ray, Unitma Co., Ltd., Seoul, South Korea). Representative areas of each tumor were selected and marked on the H&E-stained slides, and the corresponding tissue block was sampled. The designated area of each donor block was punched with a 2-mm diameter tissue cylinder, and the sample was transferred to a recipient block. Each sample was arrayed to the duplicated blocks to minimize tissue loss.

Immunohistochemistry of EGFR and HER-2

EGFR and HER-2 protein expressions were evaluated by IHC using the EGFR pharmDx^{TM} kits (DAKO, Carpentaria, CA, USA) and the HercepTest (polyclonal antibody; DAKO, Glostrup, Denmark), respectively, according to the manufacturer's recommended protocols, as summarized below.

First, 4-μm-thick sections were transferred onto poly-L-lysine-coated adhesive slides and dried at 62 °C for 30 min. After epitope retrieval, the samples were incubated with primary antibodies against HER2 (HercepTest's polyclonal). The sections were subsequently incubated with a biotinylated antimouse immunoglobulin, peroxidase-labeled streptavidin (LSAB kit; DAKO), and 3,3′-diaminobenzidine. Slides were then counterstained with Harris hematoxylin. For EGFR assay, after deparaffinization, 4-μm-thick sections were treated with proteinase K solution for 5 min at room temperature. After peroxidase blocking for 5 min, the sections were incubated with primary antibody for 30 min at room temperature. They were then labeled with a polymer for 30 min at room temperature and reacted with diaminobenzidine tetrahydrochloride solution. Immunopositivity was scored using the instructions in the EGFR pharmDx and HercepTest kit. Reactivity was scored as zero when there was no membranous reactivity within the tumor and as positive when there was reactivity of

the tumor cell membrane that was detected to be greater than the background level. The positive samples were classified further into 1+, 2+, and 3+ reactivity levels according to the guidelines provided by the manufacturer. The highest intensity of reactivity of all tissue cores from the same tumor was used as the final immunohistochemical result for that tumor.

Silver in situ hybridization

Dual-color staining (silver) of HER2 and chromosome 17

For dual-color SISH, 4-μm-thick sections from each microarray block were prepared. The slides were processed using an automated system following the manufacturer's protocols for INFORM HER2 DNA and chromosome 17 (CEP17) probes (Ventana Medical System) [10]. Both probes were labeled with dinitrophenol (DNP) and were optimally formulated for use with the ultraView SISH Detection Kit and accessory reagents from the Ventana Benchmark series of automated slide stainers. The HER-2 DNA probe was denatured at 95 °C for 12 min and hybridized at 52 °C for 2 h. After hybridization, slides were washed three times at 72 °C. The CEP17 probe was denatured at 95 °C for 12 min and hybridized at 44 °C for 2 h. After hybridization, slides were washed three times at 59 °C. Hybridization and wash stringencies for HER-2 and CEP17 probes were determined empirically. HER-2 and CEP17 DNP-labeled probes were visualized using rabbit anti-DNP primary antibody and the ultraView SISH Detection Kit, which contains horseradish peroxidase- and alkaline phosphatase-conjugated goat antirabbit antibodies against HER2 and CEP17, respectively, as chromogenic enzymes. After sequential addition of silver acetate, hydroquinone, and H_2O_2, a silver precipitate was deposited in the nuclei. A single copy of the HER-2 gene was visualized as a black dot. A red dot for chromosome 17 appeared following the reaction with fast red and naphthol phosphate. The specimen was then counterstained with Harris hematoxylin.

Single staining (silver) of EGFR and chromosome 7

EGFR gene status is reported as an absolute copy number as well as a function of the ratio of the average number of copies of the EGFR gene to the average number of copies of chromosome 7 (Chr 7) per cell.

Visualization of the EGFR gene and Chr 7 was performed on one single slide using the SISH detection kit for the EGFR gene and the Ventana Alkaline Phosphatase (AP) Red ISH detection kit for Chr 7, with a dual color staining technique.

Interpretation of SISH and definitions of EGFR and HER-2 alterations

HER-2 gene amplification status was evaluated by counting signals in 20 non-overlapping tumor cells with the highest gene count. The interpretation followed the criteria of the ASCO/CAP guidelines [11]: negative for HER-2 gene amplification if the HER-2/CEP17 ratio was lower than 1.8, equivocal if the HER-2/CEP17 ratio was 1.8–2.2, and positive if the HER-2/CEP17 ratio was higher than 2.2. EGFR gene amplification status was interpreted using the same criteria as for EGFR/Chr 7.

Definitions of EGFR and HER-2 alteration

We defined EGFR and HER-2 alterations as scores of 1+, 2+ and 3+ for IHC or equivocal results for positive gene amplification.

Statistical analysis

Differences between and among groups were compared using Chi square test or Fisher's exact test for qualitative variables. Survival curves were estimated using the Kaplan–Meier method, and the significance of differences between survival curves was determined using the log rank test. Multivariate analysis was performed using Cox proportional hazards regression modeling. Null hypotheses of no difference were rejected if two-sided P values were <0.05. All analyses were performed using the statistical package SPSS version 17.0 (SPSS, Inc., an IBM Company, Chicago, IL, USA).

Results

Clinicopathologic characteristics of all patients

In this study, we enrolled 254 patients treated between March 1999 and December 2009. Of these patients, 26 % were clinical stage IA–IB, 29.5 % were clinical stage IIA–IIB, and 44.5 % were clinical stage IIIA–IIIC, all of which were restaged according to the AJCC 7. The age of patients ranged from 28 to 91 years (median age 64 years). At a mean follow-up of 34 months, 5-year recurrence-free survival (RFS) and overall survival were 65.4 and 61.8 %, respectively. In total, 148 (58.3 %) patients received adjuvant chemotherapy with a practice-based heterogeneous protocol according to the pathologic stage.

IHC and SISH analysis

EGFR protein expression status was determined by IHC for the 254 gastric cancer tissues (Fig. 1; Table 1). EGFR gene

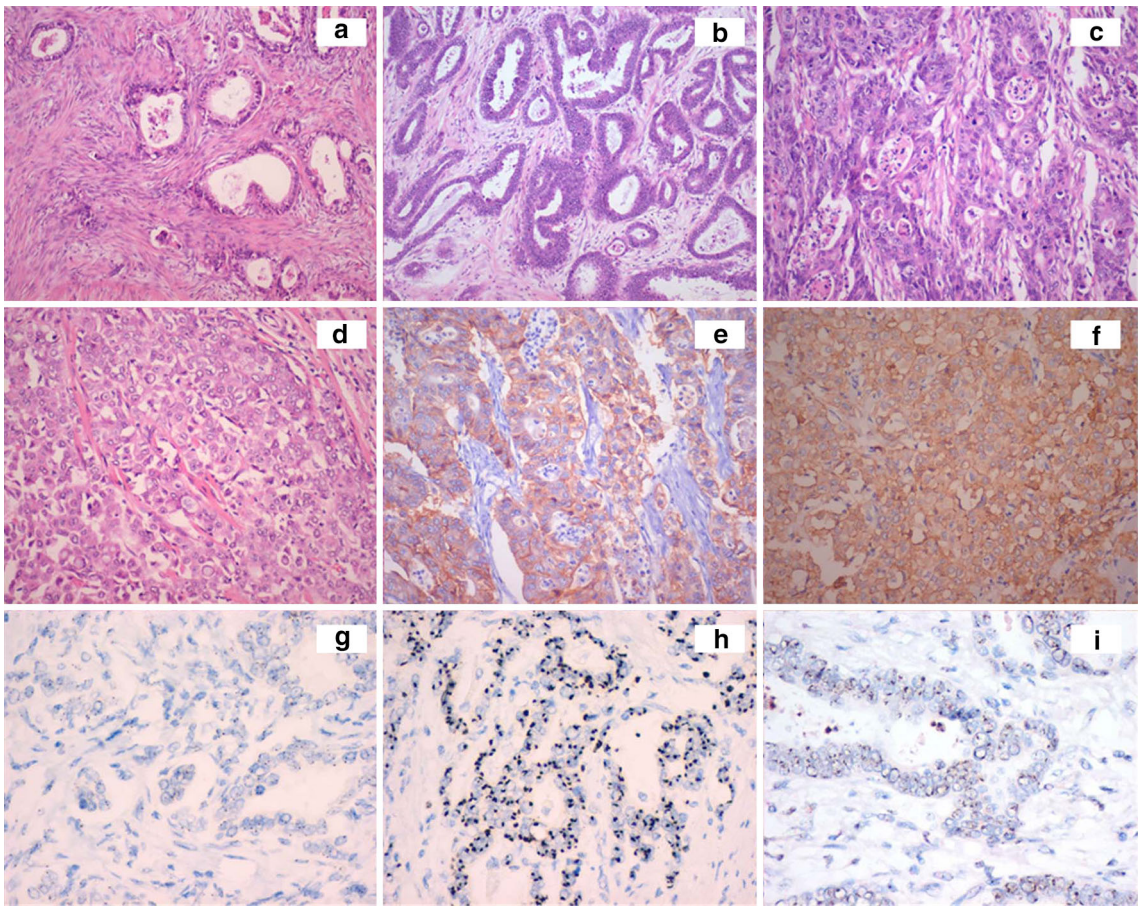

Fig. 1 The expression of EGFR and HER2 protein detected by IHC, and gene expression status evaluated by SISH. Representative H&E staining [×100 (**a**, **b**), ×200 (**c**, **d**)]. Representative EGFR IHC (×200, **e**) and HER-2/neu IHC (×200, **f**). Chr. 7 amplification (× 400, **g**) and EGFR gene amplification (× 400, **h**) detected by single color SISH. HER-2/neu *gene* amplification detected by dual-color SISH (× 400, **i**)

Table 1 Comparison between immunohistochemistry (IHC) and silver-enhanced in situ hybridization (SISH) results for EGFR

IHC	SISH			
	Negative	Equivocal	Positive	Total
0	241		2	243
1+	6			6
2+			1	1
3+			4	4
Total	247		7	254

Concordance rate 98.8 %

Table 2 Comparison between immunohistochemistry (IHC) and silver-enhanced in situ hybridization (SISH) results for HER-2/neu

IHC	SISH			
	Negative	Equivocal	Positive	Total
0	227	4	1	232
1+	5	1		6
2+	3		5	8
3+	2		6	8
Total	237	5	12	254

Concordance rate 93.7 %

amplification was determined by SISH in all cases and the results show in Table 1. Thirteen cases (5.1 %) showed EGFR alterations by IHC or SISH.

HER-2 protein expression status by IHC and HER2–neu gene amplification by SISH showed in Table 2. Twenty-seven cases (10.6 %) showed HER-2 alteration by IHC or SISH.

Alteration of EGFR and HER-2 with clinical features

The clinical features and pathologic data according to the alterations in EGFR and HER-2 are summarized in Table 3. EGFR alteration was associated with older age, intestinal type and higher stage disease.

Table 3 Correlations of EGFR, HER-2 alteration with clinicopathologic variables

Variable	Patient number (%)	IHC(+) or SISH(+) EGFR			IHC(+) or SISH(+) HER-2		
		Negative	Positive	*P* value	Negative	Positive	*P* value
Age							
<70	172 (67.7)	167 (97.1)	5 (2.9)	**0.021**	160 (93.0)	12 (7.0)	**0.006**
≥70	82 (32.3)	74 (90.2)	8 (9.8)		67 (81.7)	15 (18.3)	
Sex							
Male	193 (76.0)	182 (94.3)	11 (5.7)	0.455	169 (87.6)	24 (12.4)	0.097
Female	61 (24.0)	59 (96.7)	2 (3.3)		58 (95.1)	3 (4.9)	
Depth							
EGC	62 (24.4)	61 (98.4)	1 (1.6)	0.150	55 (88.7)	7 (11.3)	0.846
AGC	192 (75.6)	180 (93.8)	12 (6.3)		172 (89.6)	20 (10.4)	
pTNM							
Stage I–II	141 (55.5)	140 (99.3)	1 (0.7)	**<0.001**	126 (89.4)	15 (10.6)	0.996
Stage III	113 (44.5)	101 (89.4)	12 (10.6)		101 (89.4)	12 (10.6)	
LN meta							
(−)	77 (30.3)	76 (98.7)	1 (1.3)	0.068	71 (92.2)	6 (7.8)	0.333
(+)	177 (69.7)	165 (93.2)	12 (6.8)		156 (88.1)	21 (11.9)	
Size (cm)							
<5	143 (56.3)	138 (96.5)	5 (3.5)	0.183	132 (92.3)	11 (7.7)	0.085
≥5	111 (43.7)	103 (92.8)	8 (7.2)		95 (85.6)	16 (14.4)	
Diff							
Well-mod.	127 (50.0)	118 (92.9)	9 (7.1)	0.155	104 (81.9)	23 (18.1)	**<0.001**
Poor or others	127 (50.0)	123 (96.9)	4 (3.1)		123 (96.9)	4 (3.1)	
Lauren							
Intestinal	125 (49.2)	115 (92.0)	10 (8.0)	**0.040**	104 (83.2)	21 (16.8)	**0.002**
Diff. or mixed	129 (50.8)	126 (97.7)	3 (2.3)		123 (95.3)	6 (4.7)	
LVI							
(−)	93 (44.5)	90 (96.8)	3 (3.2)	0.109	82 (88.2)	11 (11.8)	0.733
(+)	116 (55.5)	106 (91.4)	10 (8.6)		104 (89.7)	12 (10.3)	
PNI							
(−)	62 (61.4)	58 (93.5)	4 (6.5)	0.491	54 (87.1)	8 (12.9)	0.413
(+)	39 (38.6)	35 (89.7)	4 (10.4)		36 (92.3)	3 (7.7)	

LVI lymphovascular invasion, *PNI* perineural invasion

HER-2 alteration was associated with older age, well to moderately differentiated histology and intestinal type (Table 3).

Prognostic implication of EGFR and HER-2/neu

The mean duration of follow-up was 34.0 months (range 1–125 months) after surgery. During the follow-up period, 85 patients relapsed. Mean recurrence time was 18.8 months (range 1–95 months; median 13 months). Three-year RFS was 70.3 %. Depth of invasion, stage, lymph node metastasis, tumor size, lymphovascular invasion, perineural invasion, and EGFR alteration were all significantly associated with a worse RFS rate in univariate analysis. However, on Cox's analysis, only EGFR alteration and stage were shown to be independent prognostic factors for RFS (Table 4).

During the follow-up period, 97 of the 254 patients (38.1 %) died.

In our univariate analysis, old age (≥70 years), tumor size (≥5 cm), depth of invasion, advanced stage, lymphovascular invasion, perineural invasion and EGFR alteration ($P = 0.028$) were all associated with poor survival (Table 4). On multivariate analysis as determined by the log rank test, EGFR alteration ($P = 0.001$) was an independent prognostic indicator, although pTNM stage and tumor size were stronger predictive factors (Table 4). The survival curves according to EGFR alteration and HER-2 alteration are shown in Figs. 2 and 3, respectively.

Recurrence free survival and overall survival according to HER-2/neu followed criteria of ToGA trial [12] also showed similar prognostic implication compare to alteration criteria in this study (Supplement Figure 1).

Table 4 Prognostic significance of clinicopathologic variables including EGFR and HER-2

Variable	Patient number (%)	Recurrence-free survival			Overall survival		
		5-year RFS rate	*P* value (univari)	*P* value (multi)	5-year OS rate	*P* value (univari)	*P* value (multi)
Age							
<70	172 (67.7)	66.9	0.665		68.7	0.001	
≥70	82 (32.3)	62.0			48.8		
Sex							
Male	193 (76.0)	63.9	0.191		60.5	0.158	
Female	61 (24.0)	70.0			72.2		
Depth							
EGC	62 (24.4)	95.7	<0.001		88.3	<0.001	
AGC	192 (75.6)	55.6			55.2		
pTNM							
Stage I–II	141 (55.5)	87.8	<0.001	**0.001**	86.0	<0.001	**<0.001**
Stage III	113 (44.5)	37.4			33.7		
LN meta							
Neg.	77 (30.3)	92.4	<0.001		89.4	<0.001	
Pos.	177 (69.7)	53.4			51.9		
Size (cm)							
<5	143 (56.3)	79.4	<0.001	0.077	77.9	<0.001	**<0.001**
≥5	111 (43.7)	47.6			44.7		
Diff							
Well-mod.	127 (50.0)	70.6	0.100		61.6	0.754	
Poor or others	127 (50.0)	60.4			64.3		
Lauren							
Intestinal	125 (49.2)	66.4	0.925		64.4	0.515	
Diff. or mixed	129 (50.8)	64.5			62.0		
LVI							
(−)	93 (44.5)	83.2	<0.001		83.1	<0.001	
(+)	116 (55.5)	54.6			53.5		
PNI							
(−)	62 (61.4)	81.6	0.001		79.2	0.008	
(+)	39 (38.6)	50.3			48.3		
EGFR							
(−)	241 (94.9)	67.3	<0.001	**0.023**	65.5	<0.001	**0.001**
(+)	13 (5.1)	27.7			18.5		
HER-2							
(−)	227 (89.4)	66.0	0.313	**N/S**	63.4	0.347	**N/S**
(+)	27 (10.6)	59.8			61.4		

LVI lymphovascular invasion, *PNI* perineural invasion

Subgroup analysis of intermediate stage (stage II–III) for excluding most favorable stage I group also showed similar results compare to all patients analysis (Supplement table; Supplement Figures 2, 3).

Discussion

The aim of this study was to evaluate potential prognostic factors, including clinical pathological characteristics and biomarkers, in gastric patients who had undergone curative resection. Gastric cancer is still one of the most common cancers and is also a leading cause of cancer mortality in Korea [13]. In operable gastric cancer, both the extent of surgery and the role of adjuvant treatment remain an international controversy. Many reports from Western countries have shown no survival benefit for D2 dissection because of high peri-operative morbidity and mortality [14, 15]. However, in Korea and Japan, a D2 dissection is the

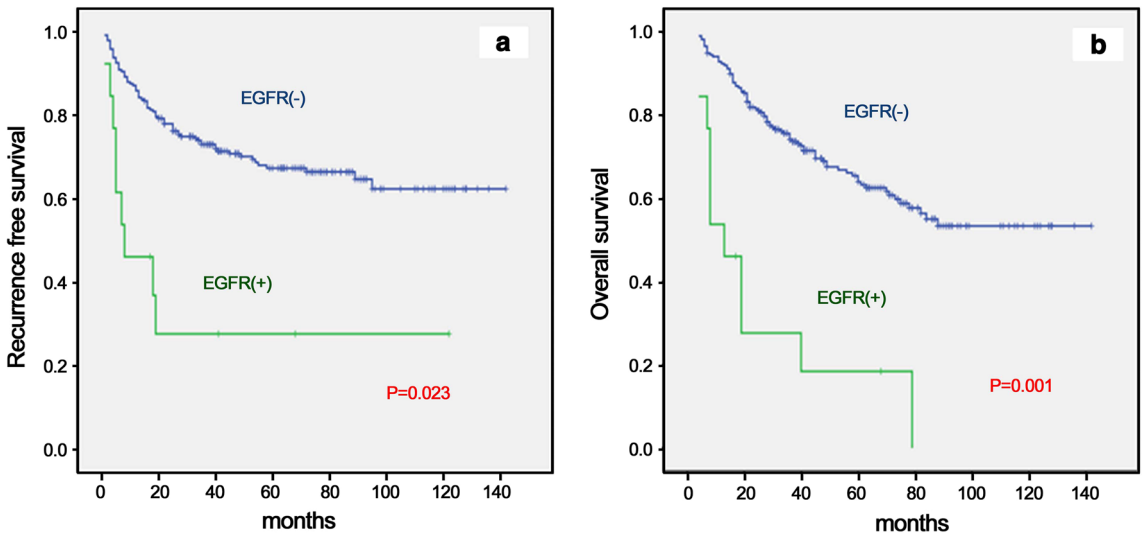

Fig. 2 Recurrence-free survival (**a**) and overall survival (**b**) according to EGFR

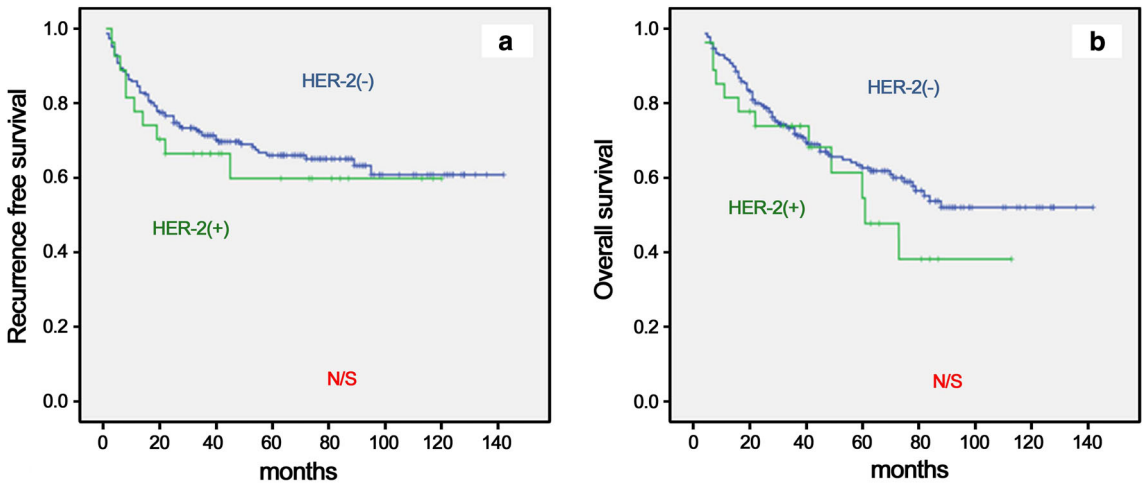

Fig. 3 Recurrence-free survival (**a**) and overall survival (**b**) according to HER-2/neu

standard surgical procedure because of its acceptable safety profile (about 1 % hospital mortality) and superior treatment outcome [16, 17]. Even after potentially curative D2 dissection, survival rates are still modest, and high rates of loco-regional recurrence and metastatic spread exist. The recurrence after surgical resection has provided a powerful rationale for use of integrated treatments basically represented by postoperative treatment for reducing the recurrence rate. Therefore, adjuvant treatment is important for metastatic or recurrent disease as well. Consequently, we restricted our study to D2-dissected gastric cancer patients.

In this study, we focused on the role of EGFR and HER-2 as prognostic markers for predicting cancer behavior and clinical outcome in gastric cancer patients undergoing potentially curative surgery.

We found that 5.1 % (13 cases) and 10.6 % (27 cases) of gastric cancers showed EGFR and HER-2 alteration, respectively. The percentage of EGFR positivity in gastric cancer patients has been reported with great variability [7, 18–22], and HER-2 positivity also varies extensively [7–9]. One possible explanation for this low rate of EGFR and HER2 alteration is the relatively high proportion of patients with diffuse-type histology in this analysis. For example, a comparable study [23] with locally advanced or metastatic disease showed diffuse type histology in only 20 % of patients compared with 56 % in this study. Furthermore, HER-2 expression is known to be more common in advanced-stage compared to early-stage gastric cancer [24, 25]. In this study, only subjects with operable stages were enrolled. EGFR alteration was more frequently seen in patients who were elderly and who had advanced stage disease and intestinal-type tumors. Several studies have shown that the advanced stage more frequently developed EGFR alteration but is not related to Lauren's classification [26].

Higher rates of HER2 alteration were seen in patients with intestinal-type tumors, older patients, and patients with well-differentiated tumors. In accord with earlier findings [27–29], these results show the HER-2 amplification is more common in tumors with intestinal type histology by the Lauren classification and with well to moderately differentiated histology. In this study, there was no difference in the rate of alteration between the early stage group and the advanced stage group. Most studies reported disease to be more frequently detected in advanced stages. We could not verify the reason for the relatively low alteration rate with advanced stage, but one possible explanation is the underestimation of advanced stage gastric cancer due to heterogeneity [30, 31]. This finding suggests the need for multi-foci IHC and SISH detection.

In 254 curatively treated patients, EGFR alteration correlated with disease recurrence and poorer survival in both univariate and multivariate analyses. In a multivariate model for predicting recurrence and survival, advanced stage, LN metastases, tumor size, and EGFR alteration were the only independent covariates. High levels of EGFR are reported to be a poor prognostic factor for overall survival in patients with resectable gastric cancer [32, 33]. Deregulation of EGFR, as a result of either over-expression or activating mutations, leads to the promotion of cell proliferation, inhibition of apoptosis, and induction of angiogenesis [34]. In contrast, Kim et al. [35] reported that patients with high EGFR expression levels who had chemotherapy following surgery for stage III and IV gastric cancer had better survival than patients with low EGFR expression levels. The majority (58.3 %) of patients in our study received adjuvant chemotherapy; however, when the analysis was restricted to patients receiving chemotherapy, the prognostic value was lost (data not shown). In the current study, EGFR alteration was a strong independent prognostic factor in the relatively early stages (stages I and III). EGFR could potentially be used for making decisions about adjuvant chemotherapy and anti-EGFR target therapy for gastric cancer as Galizia et al. [26] also reported that EGFR was a useful prognostic factor. In the case of HER-2, some previous studies have reported that amplification of the HER2 gene or overexpression of its encoded protein in gastric cancer is associated with significantly shortened disease-free survival and overall survival [9, 25, 27]. However, recent studies have shown no significant prognostic value of HER2 in gastric cancer [36, 37]. Recurrence pattern between loco-regional recurrence and distant recurrence was not related to alterations in EGFR and HER-2 (data not shown).

The SISH method is a novel technique that offers many advantages for detection of mutations. In the target agent era, high-throughput technologies now enable the identification of gene mutations and multiple oncogenic pathways, which are involved in various cancers including gastric cancer [38]. The SISH method is not generally used in gastric cancer until now but it has many advantages over other techniques. For example, SISH provides a permanent end result that can be visualized by an ordinary light microscope and is less labor- and time-intensive than FISH. FISH requires a fluorescence microscope, and assessment in biopsies with heterogeneous staining patterns can be extremely laborious. ISH methods allowing traditional transmitted light microscopy have been introduced recently. Excellent FISH/SISH correlation has been demonstrated [39, 40]. The SISH method can be a useful tool for detecting mutations in gastric cancer. The SISH method as applied to gastric cancer for detecting EGFR and HER-2 was an objective of this study. Especially in the case of EGFR, there was no equivocal positive result, so we could assume the distinct potential was higher than that with HER-2. To our knowledge, this is the first study using SISH to demonstrate that EGFR alteration is correlated with poor long-term prognosis in gastric cancer patients undergoing potentially curative surgery.

We defined the alteration in EGFR and HER2 based on the intensity of reactivity by IHC and SISH. Previous results analyzing the relationship between EGFR expression and other variables, as well as long-term outcome, have been controversial, thus raising doubts about the accuracy of techniques used to evaluate its expression and the prognostic significance of this molecular marker [19, 20, 41–44]. A discrepancy between HER2 gene amplification and protein expression has been reported in gastric cancer, and it has been suggested that HER2 protein overexpression may result from mechanisms other than gene amplification, including transcriptional activation by other genes or posttranscriptional modifications [45, 46].

Furthermore, there has not been a large-scale study to evaluate what factors have great impact on protein expression and gene amplification in EGFR and HER-2. Most studies have tried to define the positivity of EGFR and HER2 to predict the response of target agents for breast cancer, but gastric cancer has different characteristics compared with other cancers, notably the heterogeneity of stain in tumor cell membrane. Therefore, it is possible that we are underestimating the effect of mutation of EGFR and HER2 in gastric cancer. In the ToGA trial [12], the HER2 scoring criteria was focused on recurrent and metastatic settings. In addition, there are differences between the prognostic implication and predictive role of a certain agent. Therefore, we categorized alteration as including any positivity for mutation or protein expression and focused on the prognostic implication rather than predictive impact.

This study had some limitations. Even though we focused on gastric cancer treated by curative resection, we could not

evaluate the effect of adjuvant chemotherapy. Most early stage gastric cancer patients were not treated with adjuvant chemotherapy, and patients with more advanced stages were treated with heterogeneous adjuvant chemotherapy protocol and ineffective agents, including 5-fluorouracil (5-FU), mitomycin C, and doxorubicin cisplatin. Some agents had little effect as adjuvant chemotherapeutic agents for gastric cancer [47]. The influence of tumor location was not analyzed due to the small number of cardia origin patients (3.9 %) included in this study (data not shown). There is a relatively low incidence of proximal gastric cancer in Asian patients compare to the incidence in Western countries. Therefore, there were too few patients to evaluate differences in tumor location. In particular, for tumor collection, there was a difference in storage time after fixation, ranging from 1 to 120 months. We could not directly compare SISH with FISH. We did not study resection specimens but rather used the TMA technique. Most contemporary studies outside clinical trials use the very cost-effective TMA technique. However, given the high incidence of heterogeneous protein expression/amplification, we felt that using the TMA technique in this field could result in an underestimation of the incidence of alteration rates.

In conclusion, EGFR alteration was observed in a subset of cases with operable gastric cancer and was associated with an unfavorable prognosis. These findings warrant further investigation regarding EGFR-directed therapy in gastric cancer patients with curative resection as an adjuvant therapy. These findings suggest that EGFR alteration may be useful in identifying high-risk gastric cancer patients undergoing potentially curative surgery. Multimodal treatments should be considered in the adjuvant treatment of these patients.

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