

Immunohistochemical analysis of RTKs expression identified HER3 as a prognostic indicator of gastric cancer

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Standard treatment in Japan for the 13th Japanese Gastric Cancer Association stage II/III advanced gastric cancer is postoperative adjuvant S-1 administration after curative surgery. High expression of receptor type tyrosine kinases (RTKs) has repeatedly represented poor prognosis for cancers. However it has not been demonstrated whether RTKs have prognostic relevance for stage II/III gastric cancer with standard treatment. Tumor tissues were obtained from 167 stage II/III advanced gastric cancer patients who underwent curative surgery and received postoperative S-1 chemotherapy from 2000 to 2010. Expression of the RTKs including EGFR, HER2, HER3, IGF-1R, and EphA2 was analyzed using immunohistochemistry (IHC). Analysis using a multivariate proportional hazard model identified the most significant RTKs that represented independent prognostic relevance. When tumor HER3 expression was classified into IHC 1+/2+ ($n = 98$) and IHC 0 ($n = 69$), the cumulative 5-year Relapse Free Survival (5y-RFS) was 56.5 and 82.9%, respectively ($P = 0.0034$). Significant prognostic relevance was similarly confirmed for IGF-1R ($P = 0.014$), and EGFR ($P = 0.030$), but not for EphA2 or HER2 expression. Intriguingly, HER3 expression was closely correlated with IGF-1R ($P < 0.0001$, $R = 0.41$), and EphA2 ($P < 0.0001$, $R = 0.34$) expression. Multivariate proportional hazard model analysis identified HER3 (IHC 1+/2+) (HR; 1.53, 95% CI, 1.11–2.16, $P = 0.0078$) as the sole RTK that was a poor prognostic factor independent of stage. Of the 53 patients who recurred, 40 patients (75.5%) were HER3-positive. Thus, of the RTKs studied, HER3 was the only RTK identified as an independent prognostic indicator of stage II/III advanced gastric cancer with standard treatment.

Gastric cancer is the leading cause of cancer death in Japan as well as worldwide.⁽¹⁾ Advanced gastric cancer exhibits poor prognosis, and even optimal combination modalities of both surgery and chemotherapy cannot attain satisfactory survival outcomes.

S-1 is a fluoropyrimidine preparation consisting of a combination of tegafur, gimeracil, and oteracil potassium. A randomized phase III trial of S-1 (The Adjuvant Chemotherapy Trial of S-1 for Gastric Cancer (ACTS-GC)) was carried out in patients with the 13th Japanese Gastric Cancer Association (JGCA) stage II/III advanced gastric cancer. We participated in that trial and have contributed to standard treatment establishment in Japan.⁽²⁾ S-1 adjuvant chemotherapy was more effective than surgery alone for stage II/III gastric cancer.^(2,3) However, in S-1 subgroup analysis the 5-year overall survival (OS) rate of patients with stage III disease was 50.2%, leaving room for improvement with regard to prognosis.⁽³⁾

Molecular targeted therapy is an alternative therapeutic tool for advanced cancer, and the receptor tyrosine kinase (RTK) family is one such promising candidate target. The human epidermal growth factor receptor family including ErbB-1

(HER1; EGFR), ErbB-2 (HER2), and ErbB-3 (HER3) is a group of cell surface RTKs that plays an important role in the pathogenesis of solid tumors.^(4,5) In this study, we performed both clinicopathological and prognostic analysis of these RTKs in gastric cancer in order to identify the most promising target for therapy of stage II/III gastric cancer along with standard treatment.

Materials and Methods

Registration of patients. Between January 1, 2000, and December 31, 2010, 172 patients with the 13th JGCA stage II/III advanced gastric cancer underwent adjuvant S-1 chemotherapy after curative surgery in the gastrointestinal surgery division, Kitasato University Hospital. Among the 172 patients who underwent standard treatment, informed consent to use specimens was provided from 167 patients, for whom the median follow up term was 55 months (range 11–122 months).

Operation. Gastrectomy with D1 or D1+ lymph node dissection was performed for clinical early gastric cancer with preoperative diagnosis ($n = 25$) (D1; No. 7 lymph node dissection

performed regardless of the tumor location; D1+; No. 7, No. 8a and No. 9 lymph node dissections performed according to the guideline).⁽⁶⁾ Typical surgery with D2 lymph node dissection was performed for clinical advanced gastric cancer with preoperative diagnosis ($n = 142$).

Postoperative chemotherapy of S-1. The dose of S-1 was administered on the basis of body-surface area: $<1.25 \text{ m}^2$ (80 mg daily); $\geq 1.25 \text{ m}^2$ but $<1.50 \text{ m}^2$ (100 mg daily); $\geq 1.50 \text{ m}^2$ (120 mg daily). The adjuvant S-1 chemotherapy regimen was administered for 4 weeks followed by 2 weeks rest. This 6-week cycle was repeated principally during the first year after surgery. Details were described recently.⁽⁷⁾

Clinicopathologic factors. Clinicopathological factors included sex, age, tumor location, microscopic Lauren's histology, pathological T factor (the 13th JGCA), pathological N factor (the 13th JGCA), pathological stage factor (the 13th JGCA), infiltration pattern, lymphatic permeation, vascular permeation, operating method, expression of RTKs such as EGFR, HER2, HER3, Insulin-like growth factor receptor (IGF-1R) and erythropoietin-producing hepatocellular receptor (EphA2).

Immunohistochemical analysis. Tumor specimens used in this study were derived from routine formalin-fixed, paraffin-embedded tissue samples obtained from resected gastric cancer specimens. Sections (3- μm thick) were cut from the paraffin blocks and mounted on silanized slides. Staining for EGFR and HER2 was performed using the EGFR and HER2 antibodies that were included in the EGFR pharm Dx kit (Clone 2-18C9, mouse monoclonal antibodies; Dako) and the Hercep Test kit (rabbit polyclonal antibodies; Dako) respectively, according to the manufacturer's protocols.

For immunohistochemical staining of the other RTKs, tumor tissue sections were deparaffinized in xylene and dehydrated with graded ethanol. After washing with distilled water, tissue peroxidase was blocked with 3.0% hydrogen peroxide in methanol for 15 min at room temperature. For antigen retrieval using citric acid buffer, the slides were heated at 120°C for 20 min and then cooled for 20 min at room temperature. After washing, the slides were incubated with primary mouse monoclonal antibodies against HER3 (Ab-8, Clone SGP1, 1:35 dilution; Lab Vision Corporation, Fremont, USA) or against IGF-1R (α -Subunit Ab-1, Clone 24-31, 1:35 dilution; Lab Vision Corporation, Fremont, USA) or with rat monoclonal antibodies against EphA2 (RM-0051-8F21, 1:200 dilution; Abcam, Cambridge, UK) overnight at 4°C. The slides were washed with PBS. HER3 and IGF-1R stained slides were incubated with biotinylated anti-mouse IgG (Histofine Simplestain Max PO; Nichirei, Tokyo, Japan) as second antibody for 10 min at room temperature. The slides were washed with PBS again and then incubated with horseradish peroxidase-conjugated streptavidin (Histofine SAB-PO; Nichirei, Tokyo, Japan) for 5 min at room temperature. EphA2 stained slides were incubated with biotinylated anti-rat IgG (Vectastain Elite ABC Kit; Vector Laboratories, Cambridgeshire, UK) as second antibody for 30 min at room temperature. These slides were washed with PBS again and then incubated with third antibody (Vectastain Elite ABC Reagent) for 30 min at room temperature. For all slides, the immune reaction was demonstrated with DAB. The sections were then counterstained with Meyer's hematoxylin, dehydrated, and mounted. We used a thing dyed from a specimen well every time as positive control.

Scoring system of immunohistochemical staining. To assess RTK cell-membrane staining, all slides were observed under a microscope. Investigators were blinded to the prognostic analysis data. Immunohistochemical staining expression was graded

using a 4-point scale, where 0 = No staining is observed in invasive tumor cells, 1+ = Weak, incomplete membrane staining in any proportion of invasive tumor cells, or weak, complete membrane staining in less than 10% of cells, 2+ = Complete membrane staining that is non-uniform or weak but with obvious circumferential distribution in at least 10% of cells, or intense complete membrane staining in 30% or less of tumor cells, 3+ = Uniform intense membrane staining of more than 30% of invasive tumor cells. This grading was determined based on the diagnostic criteria of American Society of Clinical Oncology/College of American Pathology 2007 guidelines.⁽⁸⁾

Quantitative HER3 genomic amplification. We analyzed HER3 genomic status in 30 cases. Tissue sections from tumor and the corresponding normal mucosa, obtained at least 5 cm from the tumor edge, were sharply dissected on hematoxylin and eosin-stained slides, and genomic DNA was subsequently extracted using of a QIAamp DNA FFPE Kit (QIAGEN Sciences, Hilden, Germany). Quantitative genomic real-time polymerase chain reaction (PCR) was performed to quantify HER3 gene copy numbers using iQ™ Supermix (Bio-Rad Laboratories, Hercules, CA, USA) in triplicate on the iCycler iQ™ Real-Time PCR Detection system (Bio-Rad). We used DNA of MKN74 of gastric cancer cell line as positive control. ΔCt values were calculated as $\text{Ct}(\text{HER3}) - \text{Ct}(\text{beta-actin})$ for each sample. Relative copy number was determined as $2^{-\Delta\Delta\text{Ct}}$, where $\Delta\Delta\text{Ct} = \Delta\text{Ct}(\text{tumor}) - \Delta\text{Ct}(\text{corresponding normal})$.⁽⁹⁾ The increases of more than 2-fold relative to the corresponding normal were considered as genomic amplification.

Statistical analysis. Cumulative 5-year Relapse Free Survival (5y-RFS) was estimated by the Kaplan-Meier method, and statistical differences were tested by the log rank test. RFS was measured from the date of surgery to the date of recurrence or the last follow-up. Variables whose prognostic potential was suggested by univariate analysis ($P < 0.05$) were subjected to multivariate analysis using the Cox proportional hazards regression model. A value of $P < 0.05$ was considered statistically significant. All statistical analyses were performed with SAS software package JMP, version 10.0 (SAS Institute, Tokyo, Japan).

Results

Immunohistochemical staining of RTK family members in stage II/III advanced gastric cancer. Immunohistochemical staining of the RTK family members EGFR, HER2, HER3, IGF-1R, and EphA2 in stage II/III advanced gastric cancer was performed. This analysis indicated that these RTKs were predominantly expressed by cancer cells in the gastric cancer tissues. Very strong immunohistochemical staining (IHC 3+) of EGFR and HER2 was observed in some tissues, while 3+ staining of HER3, IGF-1R, or EphA2 was not detected. The breakdown of immunohistochemical grade of RTK expression in the total 167 patients was as follows: EGFR; IHC 1+ ($n = 62$; 37.1%), IHC 2+ ($n = 57$; 34.1%), and IHC 3+ ($n = 48$; 28.8%) (Fig. 1a). HER2; IHC 0 ($n = 96$; 57.5%), IHC 1+ ($n = 52$; 31.1%), IHC 2+ ($n = 8$; 4.8%), and IHC 3+ ($n = 11$; 6.6%) (Fig. 1b). HER3; IHC 0 ($n = 69$; 41.3%), IHC 1+ ($n = 68$; 40.7%), and IHC 2+ ($n = 30$; 18.0%) (Fig. 1c). IGF-1R; IHC 0 ($n = 79$; 47.3%), IHC 1+ ($n = 65$; 38.9%), and IHC 2+ ($n = 23$; 13.8%) (Fig. 1d). EphA2; IHC 0 ($n = 101$; 60.5%), IHC 1+ ($n = 54$; 32.3%), and IHC 2+ ($n = 12$; 7.2%) (Fig. 1e).

The clinical significance of immunohistochemical staining of RTK family members in stage II/III advanced gastric cancer. We then investigated the clinical significance of expression of each

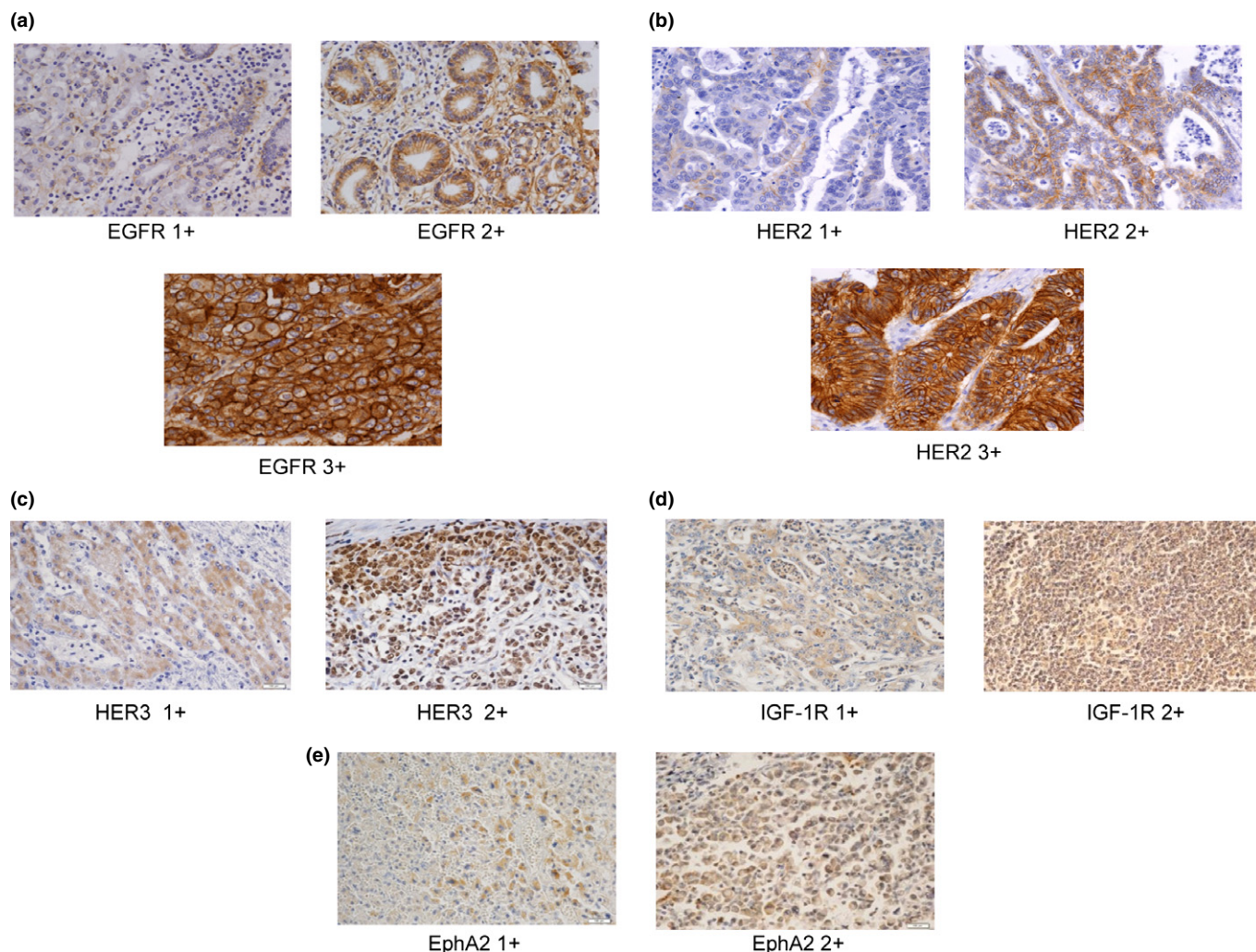


Fig. 1. Immunohistochemical staining of receptor tyrosine kinase (RTK) expression in the 13th JGCA stage II/III gastric cancer ($\times 400$). (a) EGFR immunohistochemical staining included very strong staining (IHC 3+). Other lower staining levels detected were IHC 2+ and IHC 1+. (b) HER2 immunohistochemical staining included very strong staining (IHC 3+). Other lower staining levels detected were IHC 2+, IHC 1+, and IHC 0. (c) HER3 immunohistochemical staining included strong staining (IHC 2+). Other lower staining levels detected were IHC 1+, and IHC 0. (d) IGF-1R immunohistochemical staining did not include strong staining (IHC 3+), but did include IHC 2+, IHC 1+, and IHC 0 staining levels. (e) EphA2 immunohistochemical staining did not include strong staining (IHC 3+), but did include IHC 2+, IHC 1+, and IHC 0 staining levels.

RTK by analysis of the correlation of RTK overexpression with each of 11 clinicopathological factors in stage II/III advanced gastric cancer. The clinical significance of HER3 is shown in Table 1, while the clinical significance of the other RTKs (EGFR, HER2, IGF-1R, EphA2) is shown in Table S1. Of the 11 clinicopathological factors, HER3 overexpression correlated only with the 13th pT factor ($P = 0.024$). Although EGFR overexpression correlated with the 13th pN factor ($P = 0.036$), a much higher correlation of EGFR overexpression was found with open surgery patients than with laparoscopic surgery patients ($P = 0.0063$), where laparoscopic surgery was indicative of patients with lower clinical T (definitely clinical T1 or T2). IGF-1R expression correlated with infiltration pattern ($P = 0.0004$), suggesting that IGF-1R overexpression (IHC 1+/2+) was correlated the invasive growth pattern of the gastric cancer nest. EphA2 overexpression, similar to HER3 overexpression, correlated with the 13th pT factor ($P = 0.0096$).

We also investigated the correlation of each RTK expression with the expression of other RTKs, as judged by

immunohistochemical staining (Fig. 2a). Intriguingly, HER3 overexpression was closely correlated with overexpressed IGF-1R ($P < 0.0001$, $R = 0.41$) and EphA2 ($P < 0.0001$, $R = 0.34$) (Table 1 and Fig. 2a). On the other hand, HER3 overexpression showed no correlation with EGFR and HER2 overexpression.

Univariate prognostic analysis of clinicopathological factors in stage II/III advanced gastric cancer treated with standard treatment. We next performed univariate prognostic analysis by log rank test for the 11 clinicopathological factors that are shown in Table 1. Significant prognostic factors representing poor survival were male sex ($P = 0.019$), age ≥ 67 years ($P = 0.0017$), the 13th JGCA pT factor ($P = 0.029$), and the 13th JGCA pN factor ($P = 0.0049$), as well as the 13th JGCA stage ($P < 0.0001$).

Univariate prognostic analysis by log rank test was also performed for the RTKs such as EGFR, HER2, HER3, IGF-1R, and EphA2 that are shown in Table 1. The significant prognostic RTKs representing poor survival were EGFR ($P = 0.030$), HER3 ($P = 0.0034$), and IGF-1R ($P = 0.014$).

Table 1. Distribution of clinical and pathological factors of correlation with HER3 and univariate prognostic analysis in 167 pStage II/III gastric cancer with gastrectomy and subsequent S-1 treatment

Variable	HER3 IHC 0	HER3 IHC 1+/2+	Chi-square test P value	5-year RFS	log rank test P value
Sex			0.36		0.019
Male	51	66		60.7	
Female	18	32		81.1	
Age (year)			0.46		0.0017
<67	42	54		78.0	
≥67	27	44		52.3	
Tumor location			0.37		0.16
Upper	19	34		57.2	
Middle	29	43		67.5	
Lower	21	21		79.4	
Lauren's histology			0.61		0.22
Diffuse type	47	63		70.5	
Intestinal type	22	35		60.3	
pT factor (13th JGCA)			0.024		0.029
T2	33	29		79.3	
T3	36	67		60.6	
T4	0	2		50.0	
pN factor (13th JGCA)			0.11		0.0049
N0	6	18		83.6	
N1	38	41		74.2	
N2	25	39		52.8	
pStage (13th JGCA)			0.46		<0.0001
II	26	30		88.5	
IIIA	31	44		63.1	
IIIB	12	24		43.0	
Operating method			0.63		0.17
Laparoscopic	11	13		76.1	
Open	58	85		65.5	
Infiltration pattern			0.45		0.88
α	7	5		75.0	
β	30	43		67.7	
γ	32	50		65.4	
Lymphatic permeation			0.61		0.059
Yes	66	92		65.2	
No	3	6		100.0	
Vascular permeation			0.91		0.088
Yes	63	89		65.1	
No	6	9		86.2	
EGFR expression			0.65		0.030
IHC 1+	27	35		78.7	
IHC 2+/3+	42	63		59.6	
HER2 expression			0.77		0.46
IHC 0/1+/2+	64	92		66.2	
IHC 3+	5	6		81.8	
HER3 expression					0.0034
IHC 0				82.9	
IHC 1+/2+				56.5	
IGF-1R expression			<0.0001		0.014
IHC 0	52	27		79.9	
IHC 1+/2+	17	71		56.6	
EphA2 expression			<0.0001		0.054
IHC 0	57	44		73.7	
IHC 1+/2+	12	54		57.7	

Multivariate prognostic analysis of RTK family members in stage II/III gastric cancer treated with standard treatment. The six significant variables including RTK family IHC expression (sex, age, the 13th JGCA stage, HER3, EGFR, and IGF-1R) that displayed prognostic potential in the above-described univariate analysis ($P < 0.05$) were next subjected to multivariate analysis. The 13th JGCA pT factor and the pN factor were not included, because they are thought to be critical confounding factors for 13th JGCA stage factor. This multivariate proportional hazard model identified HER3 IHC 1+/2+ (HR; 1.53, 95% CI; 1.11–2.16, $P = 0.0078$), male sex (HR; 1.51, 95% CI; 1.08–2.22, $P = 0.016$), and age (≥ 67) (HR; 1.39, 95% CI; 1.05–1.84, $P = 0.020$) as independent poor prognostic factors in addition to the 13th JGCA stage of gastric cancer (Table 2). EGFR and IGF-1R were eliminated as prognostic factors by this analysis.

Quantitative real-time PCR to explore genomic status of HER3 gene. The DNA increases of more than 2-fold relative to the corresponding normal were considered as genomic amplification by $\Delta\Delta C_t$ calculation methods. *HER3* gene amplification was detected 6.7% (2/30 cases). The relative copy number was 2.19 and 3.45 in the two cases.

Survival curves of stage II/III advanced gastric cancer treated with standard treatment according to RTK expression. Kaplan-Meier curves of the survival of patients with different levels of HER3 expression are shown in Fig. 3a. Significantly higher survival of patients with HER3 IHC 0 was found compared with patients with HER3 IHC 1+ ($P = 0.0083$), or HER3 IHC 2+ ($P = 0.0059$). The 5y-RFS was 82.9%, 58.0% and 52.3% respectively. When HER3 expression was classified into IHC 0 ($n = 69$; 41.3%) and IHC 1+/2+ ($n = 98$; 58.7%), the 5y-RFS was 82.9% and 56.5% respectively, and the difference was again statistically significant ($P = 0.0034$) (Fig. 3b). Thus, HER3 overexpression (IHC 1+/2+) exhibited poor prognosis.

We similarly constructed Kaplan Meier survival curves for analysis of the survival of patients according to the levels of expression of other RTKs. When EGFR expression was classified into IHC 1+ ($n = 62$; 37.1%) and IHC 2+/3+ ($n = 105$; 62.9%), the 5y-RFS was 78.7% and 59.6% respectively. This difference was statistically significant, and EGFR overexpression (IHC 2+/3+) also showed poor prognosis ($P = 0.030$) (Fig. 4a). HER2 expression was classified as follows; IHC 0 ($n = 96$; 57.5%), IHC 1+ ($n = 52$; 31.1%), IHC 2+ ($n = 8$; 4.8%) and IHC 3+ ($n = 11$; 6.6%). Unexpectedly, HER2 expression of IHC 3+ was not a prognostic factor. There was no significant difference between the survival of patients with IHC 3+ and that of patients with other HER2 IHC expression (Fig. 4b). When IGF-1R expression was classified into IHC 0 ($n = 79$; 47.3%) and IHC 1+/2+ ($n = 88$; 52.7%), the 5y-RFS was 79.9% and 56.6% respectively, and this difference was statistically significant ($P = 0.014$). IGF-1R overexpression (IHC 1+/2+) showed poor prognosis (Fig. 4c). EphA2 expression was also classified into IHC 0 ($n = 101$; 60.5%) and IHC 1+/2+ ($n = 66$; 39.5%), which showed a 5y-RFS of 73.7% and 57.7% respectively. This difference was not statistically significant ($P = 0.054$). Patients with EphA2 overexpression (IHC 1+/2+) tended to have a poor prognosis (Fig. 4d).

We also immunohistochemically analyzed RTK expression in patients who recurred. The most intriguing result was that, of the 53 patients who recurred, 40 of these patients (75.5%) were HER3-positive (Fig. 2b). We showed the initial recurrence patterns (including overlap cases) of the HER3 expression in the 53 patients in Table 3. As a result, the most frequent recurrent sites of HER3 overexpression (IHC 1+/2+)

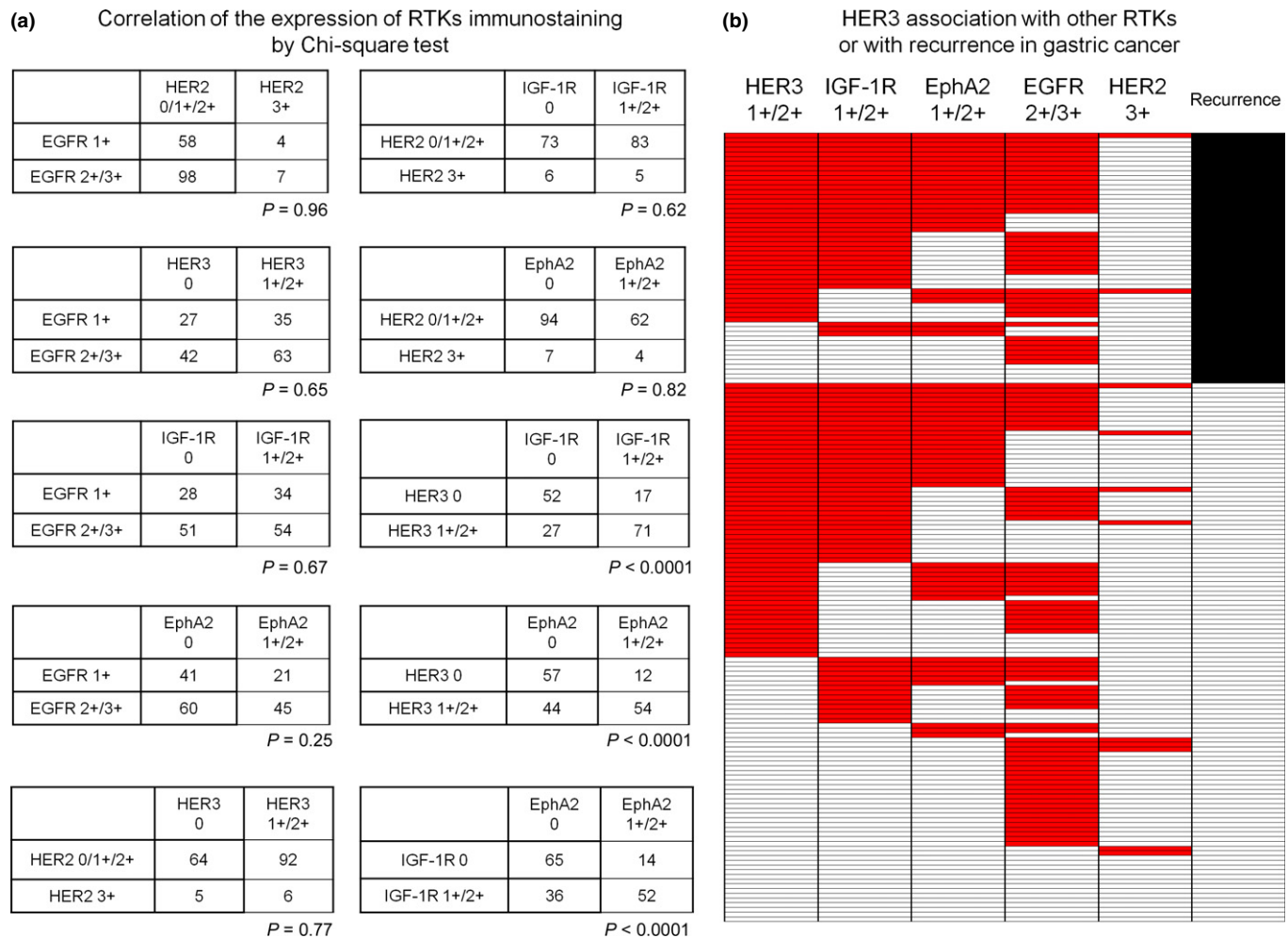


Fig. 2. Correlation of the immunostained expression of each RTK with other RTKs, and RTK expression pattern of recurrent tumors. (a) Individual RTKs were immunohistochemically stained and their staining level in the same tumor was correlated with that of the other RTKs. HER3 expression was closely correlated with that of IGF-1R ($P < 0.0001$, $R = 0.41$), and EphA2 ($P < 0.0001$, $R = 0.34$). (b) The tumors of the 53 patients who recurred were immunohistochemically analyzed for RTK expression; 40 patients (75.5%) were HER3-positive, 39 patients (73.6%) were EGFR-positive, two patients (3.8%) were HER2-positive, 36 patients (67.9%) were IGF-1R-positive, and 27 patients (50.9%) were EphA2-receptor positive. Of the RTKs expressed, HER3 overexpression showed the highest association with recurrence.

Table 2. Multivariate analysis of factors associated with relapse free survival of the 13th JGCA pStage II/III gastric cancer treated with standard treatment

Variable	HR	95% CI	<i>P</i> value
pStage (13th JGCA)			<0.0001
II	Reference		
IIIA	1.71	1.13–2.68	
IIIB	1.99	1.28–3.17	
HER3 (IHC 1+/2+)	1.53	1.11–2.16	0.0078
Sex (male)	1.51	1.08–2.22	0.016
Age (≥ 67)	1.39	1.05–1.84	0.020
EGFR (IHC 2+/3+)	1.32	0.96–1.86	0.085
IGF-1R (IHC 1+/2+)	1.16	0.86–1.59	0.35

cases were extra lymph node metastasis and/or peritoneal dissemination.

Recurrent cases included two cases in which the recurrence occurred over 5 years. The recurrent sites of the two cases were both peritoneal dissemination.

Survival curves of advanced stage gastric cancer according to RTK family expression. We analyzed the prognostic significance of expression of each RTK in advanced gastric cancer, focusing on the 13th JGCA pathological stage IIIB of gastric cancer, using the log-rank test (Fig. 5). When HER3 expression of these tumors was classified into IHC 0 ($n = 12$) and IHC 1+/2+ ($n = 24$), the 5y-RFS was 75.0% and 28.6% respectively, and this difference was statistically significant ($P = 0.019$). EphA2 expression was also classified into IHC 0 ($n = 17$) and IHC 1+/2+ ($n = 19$), the 5y-RFS was 54.8% and 31.6% respectively. This difference was also statistically significant ($P = 0.042$).

There were 36 stage IIIB cases, which was included 20 recurrences. In the 20 recurrent cases of stage IIIB, HER3 overexpression (IHC 1+/2+) and EphA2 overexpression (IHC 1+/2+) was recognized in 17 and 13 patients, respectively. Lymph node was the most dominant in the initial recurrent sites in stage IIIB gastric cancer with HER3 or EphA2 overexpression (IHC 1+/2+). On the other hand, HER3 expression (IHC 0) was recognized in three cases, and the recurrent sites were all peritoneal dissemination.

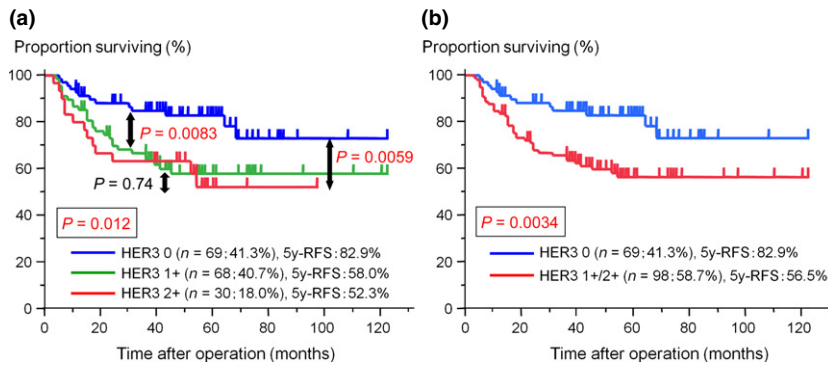


Fig. 3. The 5y-RFS of patients with different levels of HER3 expression, as detected using IHC, was analyzed using Kaplan Meier survival curves. (a) HER3 expression of IHC 1+ and IHC 2+ displayed a poor prognosis compared with HER3 IHC 0. (b) HER3 overexpression (IHC 1+/2+) displayed poor prognosis ($P = 0.0034$).

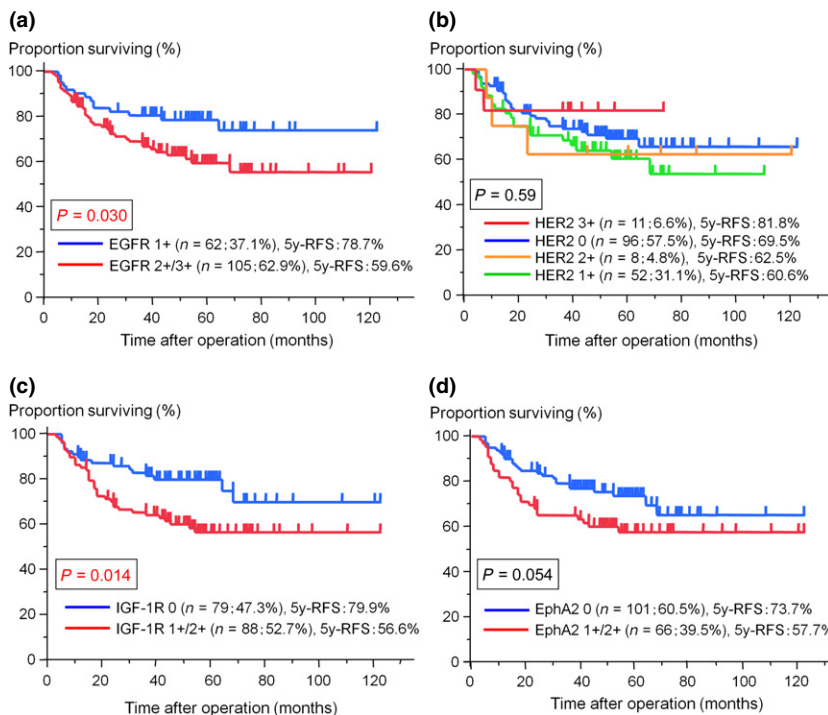


Fig. 4. The 5y-RFS of patients with different levels of the indicated receptor tyrosine kinase expression, as detected using IHC, was analyzed using Kaplan Meier survival curves. (a) EGFR overexpression (IHC 2+/3+) indicated poor prognosis ($P = 0.030$). (b) HER 2 overexpression (IHC 3+) did not indicate poor prognosis. (c) IGF-1R overexpression (IHC 1+/2+) indicated poor prognosis and difference was statistically significant ($P = 0.014$). (d) EphA2 overexpression (IHC 1+/2+) tend to indicate poor prognosis ($P = 0.054$).

Survival curves of combination both EGFR and HER3 according to individual stages of advanced gastric cancer. EGFR was not significant, but marginally significant independent prognostic factor instead of HER3 (Table 2). Combination of both EGFR and HER3 was significant poor prognostic factor in all the gastric cancer cases ($n = 167$) (Fig. 6a). Interestingly, 5-year RFS was excellent in stage II cases other than combination of both EGFR IHC 2+/3+ and HER3 1+/2+ (Fig. 6b). On the other hand, 5-years RFS was dismal in stage IIIA (34.5%) and stage IIIB (25.0%) in cases with combination of both EGFR IHC 2+/3+ and HER3 1+/2+ (Fig. 6c, and 6d).

Discussion

There have been many reports that RTKs are prognostic factors for gastric cancer. Overexpression of EGFR,^(10–19) HER2^(11,15,17,20,21) or HER3^(12,20–23) has been demonstrated to be a prognostic factor and these RTKs are novel molecular targets for gastric cancer. Other RTKs such as IGF-1R^(13,24,25) and EphA2^(26–29) have also been reported to be prognostic factors for gastric cancer.

In the present study, since we examined RTK expression in pathological stage II/III advanced gastric cancer treated with curative surgery and adjuvant S-1 chemotherapy (standard treatment), the background of the examined patients is unique compared to previous reports. In particular, there have been many reports regarding EGFR and HER2 as prognostic factors for gastric cancer.^(10–21) It was reported that the EGFR is more frequently over-expressed with advancing stage of gastric cancer.⁽¹⁷⁾ However multivariate prognostic analysis has shown that EGFR could be a prognostic factor at any stage of gastric cancer, because this analysis identified EGFR as a prognostic factor independent of stage. Moreover, in stage II/III gastric cancer patients of the ACTS-GC biomarker study, in which patient background was considered to be similar to that in our study, EGFR positive cases exhibited poor prognosis and it was thought that EGFR expression may explain one aspect of the molecular characteristics of high malignancy.⁽¹⁶⁾ In contrast, HER2 expression was also examined in the ACTS-GC biomarker study, and, as shown in the present study, it was concluded that HER2 overexpression is not a prognostic factor for gastric cancer with standard treatment.

Table 3. Initial recurrence patterns (including overlap cases) of the HER3 expression in 167 pStage II/III gastric cancer with gastrectomy and subsequent S-1 treatment

	Recurrence patterns				
	Local recurrence	Lymph node (regional)	Lymph node (extra)	Peritoneal dissemination	Distant metastasis
Recurrence cases with HER3 positive (IHC 1+/2+; n = 40)	2	8	13	16	Liver 7, Lung 1, Adrenal gland 1
Recurrence cases with HER3 negative (IHC 0; n = 13)	0	4	3	8	Liver 2, Lung 1, Bone 1, Adrenal gland 1
Total recurrence cases (n = 53)	2	12	16	24	Liver 9, Lung 2, Bone 1, Adrenal gland 2

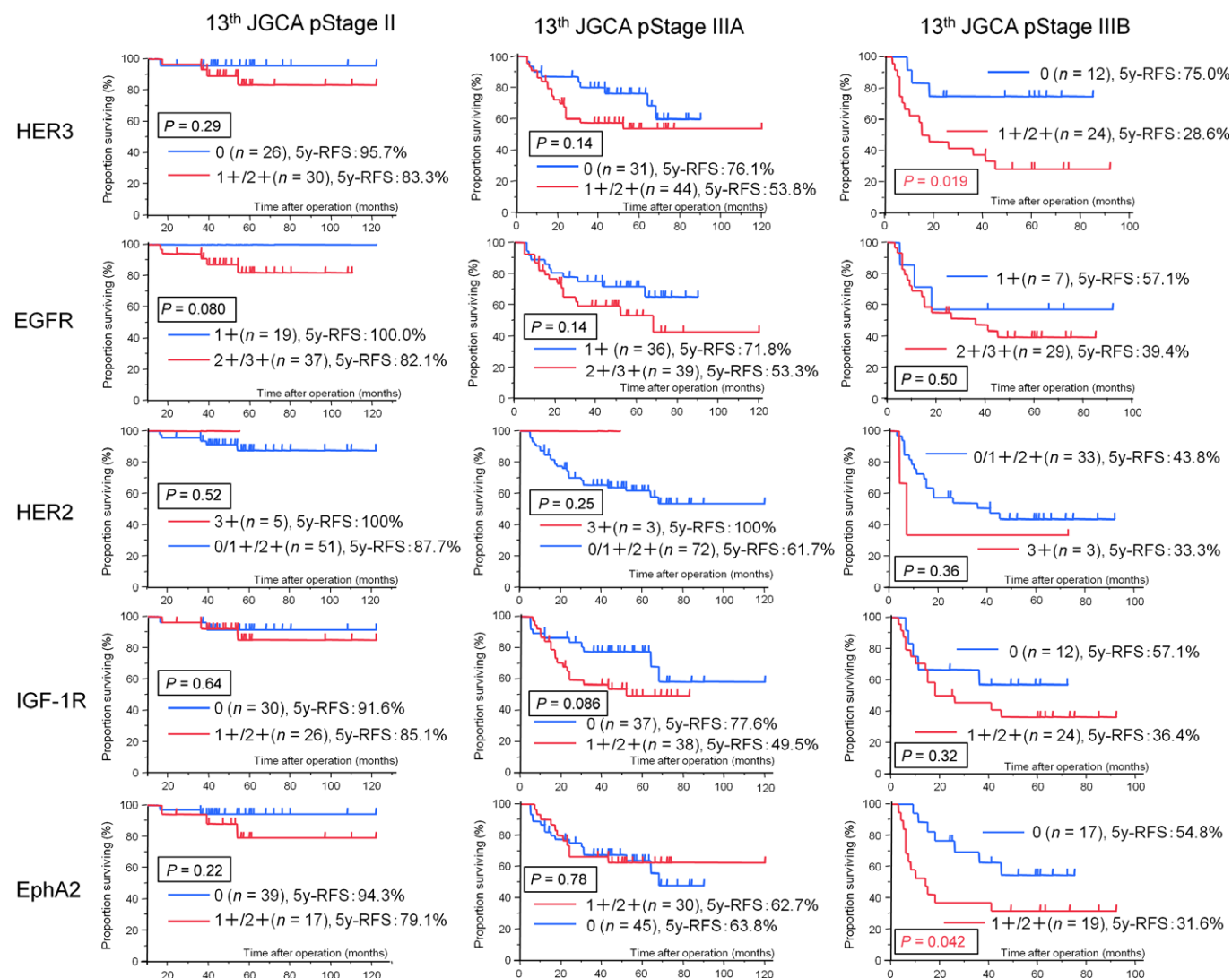


Fig. 5. The 5y-RFS of patients with 13th JGCA pathological stage IIIB tumors with the indicated RTK expression, as detected using IHC, was analyzed using Kaplan Meier survival curves. The 5y-RFS of patients with HER3 and EphA2 overexpression was statistically significantly different from that of patients with no HER3 or EphA2 expression respectively (HER3; P = 0.019, EphA2; P = 0.042). EGFR, HER2 and IGF-1R overexpression also tended to indicate poor prognosis in 13th JGCA stage IIIB tumors.

The rate of tumor EGFR positive expression varied in the previous reports. In our study, when an EGFR expression of IHC 3+ was judged as EGFR positive, 28.8% of the patients

were included. On the other hand, when EGFR expression of IHC 2+/3+ was judged as positive, 62.9% of the patients were included. According to the latest report of the National Cancer

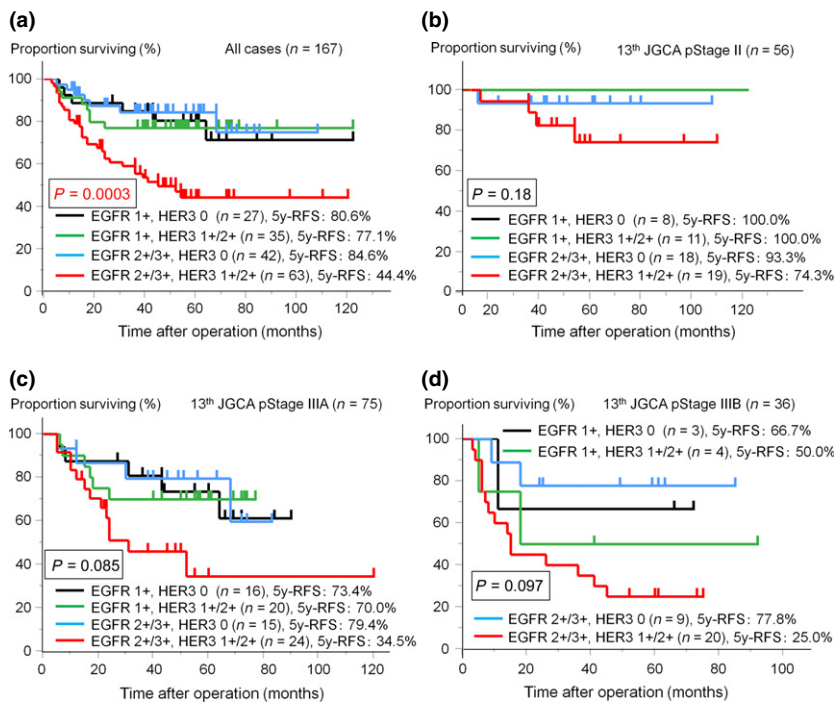


Fig. 6. The 5y-RFS of patients with combination both EGFR and HER3 expression, as detected using IHC, was analyzed using Kaplan Meier survival curves. (a) The 5y-RFS of combination both EGFR and HER3 overexpression was 44.4%, and indicated poor prognosis ($P = 0.0003$). (b) The 5y-RFS of combination both EGFR and HER3 overexpression was 74.3% in pStage II. (c) The 5y-RFS of combination both EGFR and HER3 overexpression was 34.5% and indicated poor prognosis comparing other combinations in pStage IIIA. (d) The 5y-RFS of combination both EGFR and HER3 overexpression was 25.0% and indicated poor prognosis comparing other combinations in pStage IIIB.

Center of Japan, the rate of positive EGFR expression was 35.3% in stage II/III gastric cancer and this was considered to be equivalent to the IHC 3+ stained cases in our study.⁽¹⁹⁾ In the present study, we analyzed prognosis using a cut-off line between EGFR 1+ and 2+/3+ staining. This cut-off line was used because staining of EGFR associated with massive lymph node metastasis was one of the strongest prognostic factors of gastric cancer that differed between 1+ and 2+/3+.⁽³⁰⁾ Even if we raised the cut-off line to EGFR 1+/2+ expression, and compared EGFR 1+/2+ with 3+, the EGFR positive cases showed significantly poorer prognosis as compared to the EGFR negative cases ($P = 0.042$) (data not shown). On the other hand, an EGFR expression of 3+ was only detected in 9% of patients in the ACTS-GC Biomarker Study,⁽¹⁶⁾ and the positive rate of EGFR expression was only 14.4% in the patients in the Osaka University.⁽¹⁸⁾ Thus, in those studies EGFR positive cases were infrequent in comparison with the cases of the present study (28.8%) or those reported by the National Cancer Institute (35.3%). The prognostic analysis of EGFR may thus be influenced by the cut-off line of EGFR staining level, and therefore further validation in terms of this point is required. At least in the present study, EGFR expression failed to be an independent prognostic factor for stage II/III gastric cancer, and only HER3 was finally remnant after multivariate analysis.

The HER3 receptor is a key member of the ErbB family and preferentially signals through the phosphatidylinositol 3-kinase pathway.^(31,32) Because of amino acid substitutions in the intracellular domain compared to other ErbB, the HER3 receptor lacks intrinsic tyrosine kinase activity. HER3 heterodimerizes with other HER family members to initiate signal transduction. Inappropriate signaling can occur as a result of receptor overexpression leading to cell proliferation, inhibition of apoptosis, cancer cell motility, angiogenesis and metastasis formation. Interdependence between the different members of the HER family including HER3, as well as complementary functions have been reported. The role of

HER3 in cancer has been a particular focus of attention in recent years. In our current study, combination of both EGFR 2+/3+ and HER3 1+/2+ exhibited the poorest prognosis in stage II/III advanced gastric cancer. Hence, concurrent expression of HER3 together with EGFR plays a critical role in gastric cancer progression.

Hayashi *et al.* were the first to report that HER3 is a prognostic factor for gastric cancer.⁽¹²⁾ They performed immunohistochemical staining of HER1-4, and proved that HER3 is an independent prognostic factor. HER3 was also reported to be a prognostic factor in a later analysis of a Brazilian case of gastric cancer.⁽²¹⁾ HER3 expression by solid tumors including gastric cancer was considered a strong prognostic factor in the latest meta-analysis.⁽²²⁾ Hence, there is strong interest in HER3 as a prognostic factor for gastric cancer. In the present study, if tumor HER3 expression was divided into 0 (negative) and 1+/2+ (positive), then the positive rate was 58.7%. This positive rate of tumor HER3 expression is similar to that of past reports.^(12,20,21) Furthermore and importantly, 75.5% of recurrent patients that were treated with standard therapy were HER3 positive. We therefore consider that clinical studies explore the therapeutic efficacy of HER3 antibody inhibition for recurrent cases should be a promising approach for improvement of prognosis of a large portion of stage II/III gastric cancer with recurrence.

In addition, high tumor HER3 expression was closely related to high expression of either IGF-1R or EphA2, both of which also showed poor prognosis. Inter-relationships between these three molecules have not been previously reported and therefore the underlying mechanism of these relationships is unclear. However, it will be necessary to perform basic research to determine which molecule is the most important target for gastric cancer therapy in the near future. Furthermore, since high expression of these molecules was largely detected in recurrent cases, combination therapy targeted towards these molecules might be promising for clinical application against gastric cancer.

HER3 activation of the phosphatidylinositol 3-kinase pathway is common in the diffuse type of gastric cancer, and it is known that downregulation of HER3 is promising as a targeted therapy for diffuse type gastric cancer.^(33–35) Moreover, HER3 is downstream of FGFR2 signaling. FGFR2 is a critical oncogene of diffuse type of gastric cancer, and has also been reported as an important molecular target.^(36,37) We therefore consider that inhibition of HER3 could be a promising molecular target for gastric cancer therapy in the future. Recently, gene mutation of HER3 has been reported in carcinoma including gastric cancer, and control of HER3 mutant activity by the HER2 inhibitor has also been proposed as a possible anti-cancer therapy.⁽³⁸⁾ On the other hand, *HER3* gene amplification has not been elucidated in gastric cancer. We for the first time reported HER3 genomic amplification in gastric cancer in the current study.

There have recently been reports of the relationship of the expression of other RTKs to the prognosis of gastric cancer. In a very recent report, c-kit expression was proved to be an important prognostic factor.⁽¹⁸⁾ Our study did not include

any information regarding FGFR2, which is an alternate, but well known, critical RTK in diffuse type of gastric cancer.^(19,39,40) So we are still investigating whether we can conclude that the prognostic significance of the specific RTKs we examined in the current study are sufficient, or whether other RTKs need to be examined. However, in the current study we were at least able to dissect the unique importance of HER3 expression for gastric cancer, and we plan to determine the most optimal molecular targets for gastric cancer therapy by comparing other RTKs with HER3 in the future.

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Disclosure Statement

The authors have no conflict of interest to declare.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Table S1. Distribution of clinical and pathological factors of correlation with EGFR, HER2, IGF-1R, and EphA2 in 167 pStage II/III gastric cancer with gastrectomy and subsequent S-1 treatment.