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In summary, LINE-1–hypomethylated ESCC tumors presented highly frequent genomic gains at various loci containing *CDK6*. In addition, we found that LINE-1 methylation levels were associated with CDK6 expression in ESCCs and that the prognostic impact of LINE-1 hypomethylation in patients with ESCC might be attenuated by CDK6 expression. Collectively, these findings may suggest that global DNA hypomethylation in ESCC might contribute to the acquisition of aggressive tumor behavior through genomic gains of oncogenes such as *CDK6*. Future studies are needed to confirm our findings, and also to examine other potential mechanism(s) by which genome-wide DNA hypomethylation affects tumor behavior.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Significance of Accurate Human Epidermal Growth Factor Receptor-2 (HER2) Evaluation as a New Biomarker in Gastric Cancer

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Abstract. *Background: HER2 testing in gastric cancer differs from testing in breast cancer because of inherent differences in tumor biology; gastric cancer more frequently shows HER2 heterogeneity and incomplete membrane staining. The aim of the present study was to evaluate the frequency and accuracy of detection of HER2 expression by application of standard criteria in Japanese patients with gastric cancer. Material and Methods: A total of 198 tumor specimens were assessed for HER2 expression by immunohistochemistry (IHC) using the antibodies HercepTest™ and 4B5. Both hand-operated and automated IHC were performed. Results: HER2 expression differed according to the IHC method and antibodies used. HER2 IHC3+ tumors were identified in 21 (10%) and 7 (3.5%) cases by hand-operated and automated IHC, respectively. Conclusion: Among patients with gastric cancer, FISH may be performed in cases of IHC1+ by automated IHC. Further research is required to clarify the relevance of HER2 staining and scoring for the clinical response to HER2-targeted therapy.*

The clinical benefit of trastuzumab in patients with inoperable or metastatic human epidermal growth factor receptor-2 (HER2)-positive advanced gastric or gastroesophageal junction cancer was shown in the Trastuzumab for Gastric Cancer (ToGA) study, an international phase III randomized controlled trial (1). Although many studies have previously evaluated the HER2 status in gastric cancer, the patient cohorts and scoring criteria have varied, resulting in discrepancies in HER2 positivity ranging from 8.2 to 53.4% (2). Frequent heterogeneity of the HER2 status in gastric adenocarcinoma has also made the diagnosis of HER2 overexpression difficult. To solve these problems, the ToGA study employed a new set of immunohistochemistry (IHC) scoring criteria, based on the study by Hofmann *et al.* (3), which consider the biological features of gastric cancer. Using these new criteria, the ToGA study found HER2-positive tumors in 22.1% of metastatic gastric cancer cases. The efficacy of trastuzumab for treating metastatic gastric cancer was clearly demonstrated in the ToGA study, suggesting that anti-HER2 therapy is promising for advanced and metastatic HER2-positive gastric and gastroesophageal cancer. However, the frequency of HER2-positive tumors in patients with resectable gastric cancer, as determined by the new criteria, has not been examined. IHC3+ and fluorescence *in situ* hybridization (FISH)-positive cases were diagnosed as HER2-positive in the ToGA study. FISH-positive cases with an IHC score of 0 were included in the trastuzumab-administered group in the ToGA study, and a difference in overall survival was demonstrated; the FISH-positive group showed a better prognosis. In Japan, the criteria for HER2 positivity in gastric cancer is IHC3+ and FISH-positive IHC2+ cases. Therefore, if patients with an IHC score of 0 or 1 are administered trastuzumab, they may have a better prognosis; however, these patients are not administered trastuzumab in Japan.

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

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resectable gastric cancer needs to be determined. Some studies have reported that HER2 expression is associated with a poorer prognosis in gastric cancer (4-6), although a direct correlation has not been proven (7-10). Interpretation of these controversial results is difficult because each study used a different definition of HER2 overexpression or amplification. Regarding the clinicopathological features of HER2-positive gastric cancer, HER2 expression and intestinal histological type have shown a strong correlation (7, 10, 11). When focusing on the IHC staining of the antibody or differentiation of gastric adenocarcinoma, the development of HER2-positive tumors could be linked to the particular type of differentiation. The purpose of this study was to evaluate the frequency of HER2-positive gastric cancer by applying the standard scoring criteria in patients with resected gastric cancer. The relationships between HER2 expression and clinicopathological features and expression rates using several methods were also examined. We, herein, discuss the heterogeneity of HER2 overexpression in gastric cancer, carefully review cases of discordance of HER2 overexpression and gene amplification, and comment on the role of FISH.

Table I. *The relationships between HER2 expression and clinicopathological factors.*

HER2 status Variables	0 (n=101)	1+ (n=48)	2+ (n=28)	3+ (n=21)	*p*-Value
Age	62.4±11.9	62.4±13.3	65.7±13.7	70.8±7.6	N.S.
Gender					
Male	65	28	18	17	
Female	36	20	10	4	N.S.
Lauren's classification					
Intestinal	42	18	15	17	
Diffuse	59	30	13	4	*p*=0.0014
Depth of tumor					
T1,2	68	26	12	10	
T3,4	33	22	16	11	N.S.
pStage					
I	71	29	13	10	
II	7	7	4	4	
III	8	8	5	3	
IV	15	4	6	4	N.S.

IHC: Immunohistochemistry; *HER2*: human epidermal growth factor receptor-2.

Patients and Methods

Patients. Among patients who underwent curative resection for primary gastric cancer at Kyushu University Hospital between January 2003 and December 2007, 198 patients diagnosed with pathological TNM stage I to IV were included in this retrospective study. Clinicopathological parameters, including age, gender, histological classification, and pathological TNM stage, were retrieved from the medical charts or pathology reports. Histological classification was determined according to the Lauren's classification.

Methods. All tissues were fixed with 10% buffered formalin and then paraffin-embedded. Sections (4-μm thick) were de-paraffinized in xylene and hydrated through a graded ethanol series. First, IHC staining of HER2 was manually performed with the HercepTest II™ (DAKO, Glostrup, Denmark). Second, IHC staining of HER2 was performed with the HercepTest II™ and PATHWAY® HER2/neu (4B5) antibodies (Ventana Medical Systems, Tucson, AZ, USA) using an automated slide stainer (BenchMark XT; Ventana Medical Systems). The scoring scheme of the ToGA study was employed for IHC scoring (1) and the results were evaluated by two pathologists (S.A. and Y.Z.).

Out of the 198 specimens, 49 with HER2 defined as either IHC3+ or IHC2+ were evaluated by FISH. FISH analysis was carried-out using the PathVysion HER-2 DNA Probe Kit (Abbott, Des Plaines, IL, USA) after pre-treatment with the Paraffin Pre-treatment Kit (Abbott). Nuclei of invasive tumor cells were scored using a Biozero 8000 microscope (Keyence, Osaka, Japan) equipped with 4',6-diamidino-2-phenylindole (DAPI)/Green/Orange triple bandpass filters. In 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 >2.2. Negativity for *HER2* amplification was defined as a *HER2*/Chr17 ratio of <1.8. When the ratio was 1.8-2.2, signals in another 20 nuclei were counted, and the *HER2*/Chr17 ratio in a total of 40 nuclei was determined. When the ratio was ≥2.0, amplification was defined as positive; it was otherwise defined as negative.

To compare the stainings, the serial sections of the same block were examined and DNA was gathered from the same part on FISH.

Statistical analysis. Pearson's χ^2 test and Wilcoxon's test were performed to assess the correlation of clinicopathological parameters with HER2 positivity. All *p*-values were two-sided, and $p<0.05$ was considered to be statistically significant.

Results

HER2 positivity and clinicopathological factors. The association of HER2 status with the HercepTest II™ by hand-operated IHC in 198 specimens and the clinicopathological features are summarized in Table I. Out of the 198 patients, 21 (10.6%) and 28 (14%) were diagnosed with HER2 IHC3+ and IHC2+ cancer, respectively; the positive rate was similar to that of the ToGA study. According to Lauren's classification (12), HER2 overexpression was more often detected in the intestinal histological type than in the diffuse type. No correlation was found between HER2-positivity and tumor invasion (pT) or pTNM stage. Table II shows the relationships between HER2 status and several antibodies. HER2 positivity differed according to the IHC method, whether hand-operated or automated. A HER2 IHC3+ status with the HercepTest II™ was present in 21 (10%) specimens by hand-operated IHC. Conversely, a HER2 IHC3+ status was present in 7 (3.5%) and 12 (6%) specimens with the HercepTest II™ and 4B5, respectively, using the automated stainer.

Table II. *Comparison of HER2 expression among several antibodies.*

HER2 status	Hercep test (DAKO)	Automatic	
		Hercep test (DAKO)	4B5 (Ventana)
3+	21 (10)	7 (3.5)	12 (6.0)
2+	28 (14)	9 (4.0)	5 (2.5)
1+	48 (25)	6 (3.0)	7 (3.5)
0	101 (51)	176 (89)	174 (88)

(%)

Table III. *Relationships between HER2 status and FISH results with Hercep test.*

Hercep test (hand-operated)	FISH-positive (n=12)	FISH-negative (n=27)	N.D.
3+ (n=21)	12 (57.1)	6 (28.8)	3 (14.1)
2+ (n=28)	0	21 (66.7)	7 (33.3)

(%)
$p<0.0001$

Diagnosis of HER2 positivity and FISH. The 49 cases shown to be HER2 IHC2+/3+ with the HercepTest II™ were assessed by *HER2* amplification using FISH, and the results are shown in Table III. Of the 21 IHC3+ cases, 12 were FISH-positive, 6 were negative, and 3 were not detected. Of the 28 IHC2+ cases, 21 were FISH-negative and 7 were not detected.

We also examined the relationships between the HER2 status of each antibody and FISH in 39 cases of these, excluding the 10 cases that were not detected. Table IV shows the relationship between the HER2 status and FISH for each antibody. Out of these 49 IHC2+/3+ cases by hand-operated IHC, all 7 cases that were IHC3+ by automated IHC with the HercepTest II™ were FISH-positive (100%). Eleven of the 12 cases that were IHC3+ by automated IHC with 4B5 were FISH-positive (92%). However, some cases became FISH-positive despite the fact that they were negative by automated IHC (cases 30 and 32).

Figures 1 and 2 show the HER2 expression in two cases. Case 26 was HER2-positive by hand-operated IHC and negative by automated IHC. Case 39 was HER2-positive by both IHC methods.

Discussion

The present study included 198 patients with primary gastric cancer, and HER2 expression was assessed using the scoring scheme employed in the ToGA study (1).

Table IV. *Relationships between HER2 status and FISH results with each antibody.*

Case no.	DAKO	DAKO	4B5	FISH
No.1	2	0	0	negative
No.2	2	0	0	negative
No.3	2	0	0	negative
No.4	2	1	1	negative
No.5	2	0	0	negative
No.6	2	0	0	negative
No.7	2	0	0	negative
No.8	2	0	1	negative
No.9	2	0	0	negative
No.10	2	0	0	negative
No.11	2	0	1	negative
No.12	2	0	0	negative
No.13	2	0	0	negative
No.14	2	2	2	negative
No.15	2	1	1	negative
No.16	2	0	0	negative
No.17	2	1	1	negative
No.18	2	0	0	negative
No.19	2	2	2	negative
No.20	2	0	0	negative
No.21	2	0	0	negative
No.22	3	2	2	negative
No.23	3	0	0	negative
No.24	3	2	2	negative
No.25	3	2	3	negative
No.26	**3**	**0**	**0**	**negative**
No.27	3	2	2	negative
No.28	3	3	3	positive
No.29	3	3	3	positive
No.30	3	1	1	positive
No.31	3	3	3	positive
No.32	3	1	3	positive
No.33	3	3	3	positive
No.34	3	3	3	positive
No.35	3	2	3	positive
No.36	3	2	3	positive
No.37	3	2	3	positive
No.38	3	3	3	positive
No.39	**3**	**3**	**3**	**positive**

We performed IHC and FISH to confirm the difference and accuracy of HER2 staining. First, we evaluated the staining difference between the hand-operated and automated stainer. Second, we confirmed whether FISH performed only for HER2 IHC2+/3+ gastric cancer cases is acceptable.

We used the automated stainer BenchMark XT for IHC in the present study; this stainer is used worldwide. This autostaining system has been frequently compared with hand-operated methods. Bankfalvi *et al.* examined the difference between automated and manual determination of the HER2 status in breast cancer (13). Manual

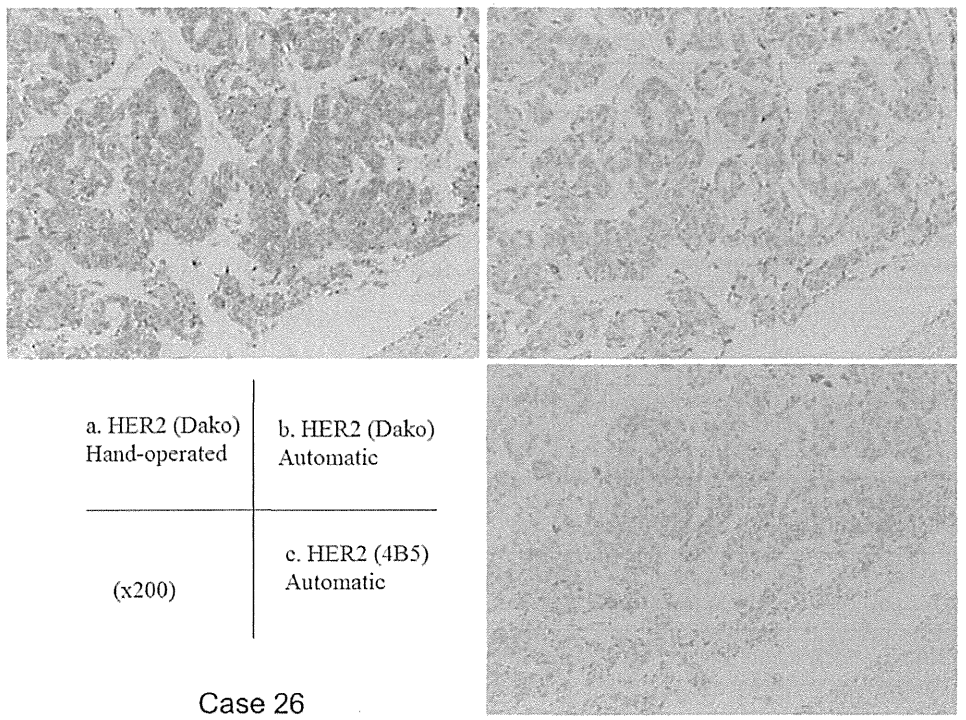

Figure 1. *HER2 IHC staining pattern of case 26. (a) HER2 IHC3+ stained with the HercepTest™ by hand-operated IHC. (b, c) HER2 score of 0 with the HercepTest™ and 4B5 using automated IHC, respectively. Magnification, 400×.*

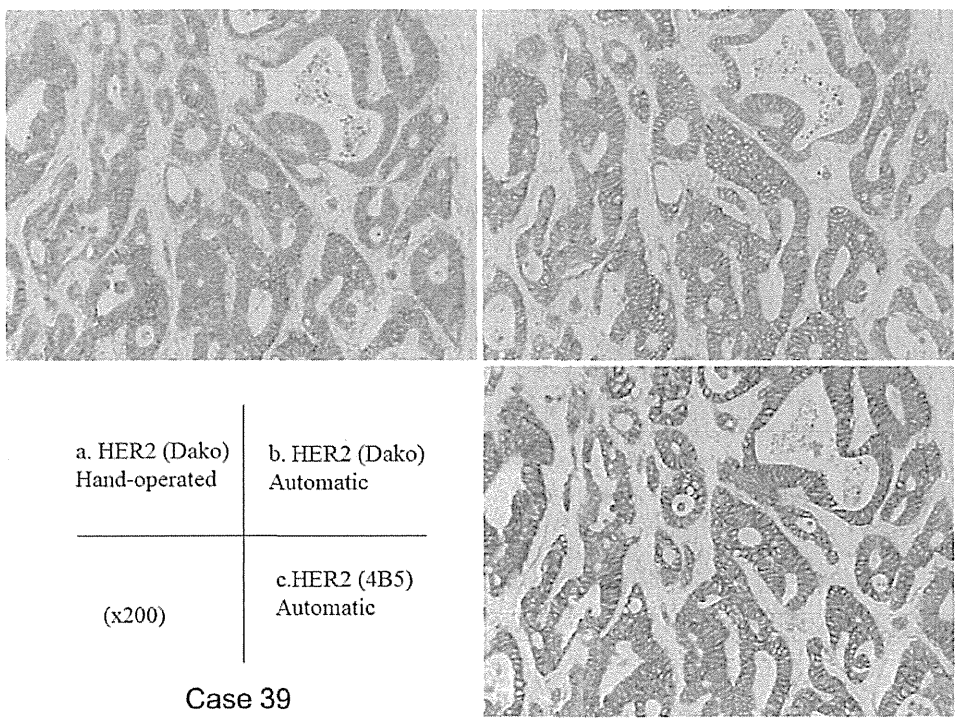

Figure 2. *HER2 IHC staining pattern in case 39. (a) HER2 stained with the HercepTest™ using hand-operated IHC and with (b, c) the HercepTest™ and 4B5 using automated IHC, respectively. All cases were diagnosed as HER2-positive with a score of 3+. Magnification, 400×.*

immunostaining was performed by the HercepTest II™. Automated IHC and FISH were carried-out in the Ventana BenchMark platform using the Pathway-CB11 antibody and the INFORM(R) HER2 probe, respectively. Positivity rates varied between the HercepTest II™ (26%), automated CB11 IHC (23%), and automated FISH (22%) (13). The overall concordance between positive (2+/3+) and negative (0/1+) results of manual and automated IHC was 97%, that between automated FISH and IHC was 92%, and that between automated FISH and the HercepTest II™ was 89%. They concluded that automation improves the accuracy of HER2 detection in diagnostic breast carcinoma tissues (13). By using different methods and different antibodies, the IHC result between antibodies was approximately the same, and it differed from our results.

In the ToGA study, IHC was performed with the HercepTest II™, and FISH was performed with pharmDx (DAKO). The staining method was not described in detail. The estimated HER2-positive ratio was 22.1%, which included IHC 3+ or FISH positivity. In FISH-positive cases, an HER2 IHC 0/1+ status was observed in 61 (10.4%) and 70 (12%) cases, respectively. The present study identified HER2 positivity in 24.7% of all included cases; however, 3-6% of HER2-positive cases were identified with automated IHC. The 49 HER2 IHC2+/3+ cases identified by hand-operated IHC using the HercepTest II™ underwent *HER2* amplification by FISH (Table III). Ten of these 49 cases were proven to be FISH-negative; furthermore, all IHC2+ cases were FISH-negative. The FISH-positive ratio of IHC3+ tumors using automated IHC was higher than that using hand-operated IHC (100% and 92%, respectively). Case 26, whose HER2 status was a score of 3+ by the hand-operated HercepTest II™, scored 0 by automated staining of the two antibodies shown in the serial section in Figure 1. Case 39, whose HER2 status was a score of 3+ by both methods, is shown in Figure 2. Even the same antibody showed different ratios with different staining. The concordance rate between IHC and FISH in the present study was 57.1%, which differs from that of previously published studies (3, 7, 9-11, 14-17); the concordance rate using automated IHC was otherwise similar to that rate. In IHC2+/3+ cases in particular, amplification of the *HER2* gene was confirmed by FISH in all cases. This study also differs from those of breast cancer. HER2 is usually homogeneously expressed in breast cancer; however, HER2 expression in gastric cancer is often heterogeneous (3).

The difference in HER2 expression between the antibodies was lower than that between the IHC methods (hand-operated or automated) in our study. One of the most important reasons for this is the heterogeneous nature of the gastric carcinoma cells in the postoperative specimens. The specimens after curative operation included early gastric cancer; therefore, the amount or areas of carcinoma differed from that of advanced cancer, and heterogeneity was more pronounced.

FISH-positive cases with an IHC score of 0 were included in the trastuzumab-administered group in the ToGA study, in which overall survival improved from 10 to 13 months. It is worth considering that patients with an IHC score of 0 or 1 may benefit from trastuzumab treatment. Based on our results, FISH should be performed for recurrent or unresectable HER2 IHC1+ cases because there were no FISH-negative cases with an IHC score of 0 by automated IHC; however, some IHC1+ cases were FISH-positive. This difference in the HER2-positive ratio between the ToGA study and our study may be attributed to the different backgrounds of patients. The ToGA study only included patients with metastatic or recurrent gastric cancer, while the present study population comprised of patients with resected gastric cancer. A recent study reported an HER2-positive ratio of 8.1% for curatively resected gastric cancer (9), similar to our finding. Taken together, these results suggest that the presence of HER2 positivity might be less frequent in resectable gastric cancer than in metastatic cases.

Of the 21 HER2-positive tumors in the present study, 17 were of the intestinal type according to Lauren's classification. These data are consistent with previous reports in which the intestinal type showed a higher rate of HER2 positivity than the diffuse type (7, 9-11, 14, 16, 17). No correlation was found between HER2 positivity and T- or TNM stage in the present study. HER2-positive tumors were found in 11 of 21 cases of T1/2 cancer (tumor invades as far as the lamina propria or muscularis mucosa). Previous studies that included all pathological stages also reported no correlation between pathological stage and HER2 overexpression (7, 10, 11, 17). Taken together, these findings suggest that HER2 overexpression occurs in the early phase of gastric carcinogenesis. Furthermore, the occurrence of HER2 expression in the early stage strongly suggests that there is no relationship between HER2 expression and prognosis. However, since only small numbers of patients in the early stage were included in these reports, further studies are needed to determine the association between HER2 expression and gastric cancer development. Because HER2 staining results are important in terms of whether patients receive trastuzumab, the accuracy of HER2 staining is important for patients with gastric cancer.

Conclusion

Our study indicated that the HER2-positive rates with hand-operated IHC in patients with resectable gastric cancer were almost identical to that of the ToGA study. A lower HER2 score in resectable gastric cancer has been previously reported; the positive rate of HER2 expression in gastric cancer after curative surgery remains unknown. HER2 expression in gastric cancer differs from that in breast cancer due to heterogeneity. FISH examination should be considered

in cases with an IHC1+ status by automated IHC. Accurate and reliable HER2 testing and scoring will allow for the appropriate selection of patients eligible for treatment with trastuzumab. Further research is required to clarify the relevance of HER2 staining and scoring for the clinical response to HER2-targeted therapy.

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ORIGINAL ARTICLE – GASTROINTESTINAL ONCOLOGY

Phase II Study of Docetaxel and S-1 (DS) as Neoadjuvant Chemotherapy for Clinical Stage III Resectable Gastric Cancer

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ABSTRACT

Background. We conducted a phase II trial to evaluate the efficacy and safety of preoperative chemotherapy with docetaxel (DTX) plus S-1 for resectable advanced gastric cancer.

Patients and Methods. A total of 47 patients from 14 centers were centrally registered. Patients received DTX (35 mg/m^2) on days 1 and 15, and daily oral administration of S-1 (80 mg/m^2/day) for days 1–14 every 4 weeks for two courses, followed by gastrectomy with D2 lymphadenectomy. The primary endpoint was pathological response rate (pRR). This study was registered in the UMIN clinical trial registry (UMIN000000875).

Results. The primary endpoint pRR was 47 % (90 % confidence interval (CI), 34–60 %; $p < 0.0001$). The response rate to preoperative chemotherapy using Response Evaluation Criteria in Solid Tumors (RECIST) was 34 %. Forty-six patients (98 %) underwent surgery, and curative resection was performed in 44 patients. Thirty-seven patients completed the protocol treatment. The most common toxicities of neoadjuvant chemotherapy were grade 3/4 neutropenia (42 %), febrile neutropenia (4 %), grade 2 anorexia (21 %), and fatigue (15 %). Treatment-related death and operative mortality was not observed in this study.

Conclusions. The combination of docetaxel and S-1 was well tolerated. This is promising as a preoperative chemotherapy regimen for patients with potentially resectable advanced gastric cancer.

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Gastric cancer, the most common malignant tumor arising in the gastrointestinal tract, is the second leading cause of cancer-related death in the world.[1] The standard approach for locally advanced gastric cancer is to achieve R0 resection and treat it with adjuvant chemotherapy.[2–5] Although gastrectomy with D2 lymph node dissection, the so-called extended dissection in Japan, Taiwan, Korea, and several other countries, has now been foregone, peritoneal metastases, lymph nodes metastases, and liver metastases are

frequently found in advanced stage gastric cancer. To decrease the incidence of relapse after R0 resection, postoperative adjuvant chemotherapy is the standard treatment in Japan and Korea. The study known as Adjuvant Chemotherapy Trial of S-1 for Gastric Cancer (ACTS-GC) demonstrated that S-1 is an effective adjuvant treatment for Japanese patients who have undergone D2 dissection for locally advanced gastric cancer.[6] In Korea, the capecitabine and oxaliplatin adjuvant study in stomach cancer (CLASSIC) trial showed the favorable result that capecitabine and oxaliplatin (XELOX) chemotherapy after curative resection of gastric cancer improves patient survival.[7]

S-1 is an oral fluoropyrimidine derivative developed in Japan, based on the concept of biochemical modulation. S-1 consists of tegafur, gimeracil, and oteracil potassium in a molar ratio of 1:0.4:1, respectively: tegafur, a prodrug that slowly metabolizes to 5-fluorouracil; gimeracil, which reversibly inhibits dihydropyrimidine dehydrogenase, the rate-limiting degrading enzyme of 5-fluorouracil, and thereby increases the plasma concentration of 5-fluorouracil; and oteracil potassium, which is distributed in high concentrations in gastrointestinal tissue and inhibits phosphorylation of 5-fluorouracil, thereby reducing gastrointestinal toxicity.[8] A more intensive regimen, such as S-1 plus cisplatin, has demonstrated a significantly higher response rate and longer survival than S-1 alone in the SPIRITS trial.[9]

Docetaxel (DTX) (Taxotere®, Sanofi-Aventis, Paris, France) is a semi-synthetic taxoid derived from the European yew tree, *Taxus baccata*. DTX also showed acceptable outcomes in patient trials both as a single agent and in combination with fluoropyrimidines or other agents. Enhanced antitumor activity was reported in a laboratory study of human gastric cancer xenografts treated with S-1 and DTX.[10,11] In previous phase II studies, favorable results were shown with the combination of S-1 and DTX for patients with advanced and recurrent gastric cancers.[12–14] In the present study, we hypothesized that preoperative chemotherapy combining DTX and S-1 (DS) in Stage III resectable advanced gastric cancer would induce a pathological response rate (pRR) of 40 %. The aim of this phase II study was to evaluate the feasibility and efficacy of this regimen and to select candidates for an experimental arm in the next phase III trial. Several phase II studies have demonstrated that a regimen of S-1 and cisplatin (SC) was safe and feasible in the neoadjuvant setting.[15–18] At present, the Japan Clinical Oncology Group (JCOG) is conducting a phase III trial of neoadjuvant chemotherapy using two courses of SC followed by surgery and postoperative S-1 as a test arm compared with surgery and postoperative S-1 as a control arm for scirrhous-type gastric cancer. Moreover, promising survival results were reported from a small phase II trial evaluating two courses of SC in the neoadjuvant setting for bulky nodal disease.[17] These phase II trials have shown favorable results for neoadjuvant chemotherapy for potentially unresectable gastric cancer; however, it has not been established whether neoadjuvant chemotherapy is necessary for the curative patient. This is the first phase II study of neoadjuvant chemotherapy with D2 gastrectomy for patients with clinical Stage III gastric cancer.

METHODS

Eligibility Criteria

Patients with histologically and cytologically confirmed adenocarcinoma of the stomach, diagnosed as Stage IIIa or Stage IIIb according to the Japanese classification of gastric carcinoma, 13th edition,[19,20] were included in this study if they met all of the following criteria: age >20 and ≤75 years; Eastern Cooperative Oncology Group performance status (ECOG PS) ≤1; able to take oral drug; and adequate hepatic, renal, respiratory, and bone marrow function. Staging laparoscopy was required to confirm no peritoneal dissemination. Written informed consent was obtained from all patients prior to enrollment in the study. Patient enrollment began in July 2007 and ended in November 2009. This study was approved by the Institutional Review Board of all institutions and was registered in the UMIN clinical trial registry (UMIN000000875).

Treatment Schedule

DTX (35 mg/m^2) was administered as a 1-h infusion on the morning of days 1 and 15 of each cycle (every 4 weeks). S-1 (40 mg/m^2) was given orally twice daily (within 30 mins after morning and evening meals) for 2 weeks, followed by a drug-free interval of 2 weeks. Patients received two cycles of DTX with S-1 (DS) therapy followed by surgery.

Surgery

A total or distal gastrectomy with D2 lymphadenectomy was performed. Involved adjacent organ(s), if any, were also removed to achieve R0 resection. A laparoscopic gastrectomy was not prescribed in the protocol. If resectable M1 disease (hepatic, peritoneal, and/or lymphatic metastases) was found during surgery, it was removed to achieve R0 resection. After the R0 resection, adjuvant chemotherapy with S-1 was initiated within 42 days after surgery. A 6-week course consisting of 4 weeks of daily oral S-1 administration at a dose of 80 mg/m^2/day followed by 2 weeks of rest was repeated during the first year after surgery.

Endpoints

The primary endpoint of the study was the pathological response rate (pRR). Assessment of pathological response

was determined centrally by two pathologists and graded according to the proportion of necrosis in the tumor: grade 0, no necrosis; grade 1a, <1/3 necrosis; grade 1b, >1/3 or <2/3 necrosis; grade 2, >2/3 or greater than all necrosis; and grade 3, all parts of the tumors affected by necrosis. The secondary endpoints were 3-year relapse-free survival (RFS), overall survival (OS) from the registration, and adverse effects. During the 4 weeks before chemotherapy was commenced, all patients underwent the following studies: physical examination, complete blood cell count, hepatic and renal function tests, and chest and abdominal computed tomography (CT) or magnetic resonance imaging (MRI). Physical examination, hepatorenal function tests, and blood counts were performed before every cycle. Patients were assessed before starting each 2-week cycle according to the National Cancer Institute Common Toxicity Criteria (CTC) Common Terminology Criteria for Adverse Events, version 3.0 (CTCAE vers. 3). Surgical completion was assessed according to the Clavien–Dindo classification. All enrolled patients were followed for 5 years. Physical and blood examinations were conducted every 3 months for the first 3 years and every 6 months for the last 2 years. An abdominal CT was performed at least every 6 months.

Statistical Considerations

A sample size of 45 was calculated according to a two-stage attained design of Green and Dahlberg, with one-sided significance level of 0.05 and 90 % power. By using a pRR of ≤20 % for the null hypothesis versus pRR of ≥40 % for an alternative hypothesis, 25 patients were recruited to the first stage. If five or more patients achieved pRR, the study would proceed to the second stage, with an additional 20 patients recruited. The null hypothesis would be rejected if 14 or more patients achieved pRR.

The confidence interval (CI) for the response rate was estimated by the Clopper-Pearson method. The duration of survival was measured from the day of gastrectomy, and the OS and RFS curves were calculated by the Kaplan–Meier method. All statistical analyses were performed using the SAS for Windows Release 9.3 (SAS Institute Inc., Cary, NC).

RESULTS

Patient Characteristics

A total of 47 patients from 14 centers were centrally registered between November 2007 and November 2009. All patients were eligible for analysis. The performance status (PS) was 0 in 41 patients, and one in six. Baseline patient characteristics are listed in Table 1. Forty-six patients (98 %) underwent surgery, and curative resection was performed in 44 patients. The treatment protocol was completed in 44 patients (93.6 %) of the total patient population, but only 37 (78.7 %) of those who underwent curative resection. All 47 patients underwent tumor resection (curative, 44; noncurative, three). The surgical methods were total gastrectomy ($n = 31$), distal gastrectomy ($n = 15$), and proximal gastrectomy ($n = 1$). Concomitant resection of the spleen was performed in 12 patients.

Clinical and Pathological Response

The response to preoperative chemotherapy using Response Evaluation Criteria in Solid Tumors (RECIST) was 34 %. Stable disease (SD), progressive disease (PD), not evaluable (NE) were 51, 4.3, and 10.6 %, respectively (Supplementary Table 1). Pathological responses are listed in Table 2. Pathological response, the primary endpoint (grade 1b to 3), was observed in 46.8 % of primary lesions. The overall response rate, as determined by the independent committee, was 46.8 %, with a 90 % CI of 34.2–59.7 %.

TABLE 1 Baseline patient characteristics ($N = 47$)

Factor		$N = 47$
Age (range)		63 (37–79)
Gender (male/female)	Male	36
	Female	11
Performance status	0	41
	1	6
Location of tumor	U	11
	M	19
	L	16
T stage	T2	4
	T3	38
	T4	5
Borrmann macroscopic type	2	13
	3	24
	4	9
	5	1
N stage	0	1
	1	30
	2	16
Clinical stage	IIIA	31
	IIIB	16
Histological type	Intestinal	13
	Diffuse	34

L lower, *M* middle, *N1* metastasis to D1 regional lymph nodes, *N2* metastasis to D2 regional lymph nodes, *T2* tumor invades the muscularis propria or subserosa, *T3* tumor invasion extends to or beyond the serosa, *T4* tumor invades adjacent structures (SI), *U* upper

TABLE 2 Pathological efficacy

Grade	Full analysis set	($N = 47$) (%)
0	0	0
1a	24	51.0
1b	7	14.9
2	13	27.2
3	2	4.3
Unresectable	1	2.1
Pathological response rate (grade 1b, 2, 3)	22	(47 [34.2–59.7])

Grade 0, no necrosis; grade 1a, <1/3 necrosis; grade 1b, >1/3 or <2/3 necrosis; grade 2 > 2/3 or greater than all necrosis; grade 3, all parts of the tumors affected by necrosis

Toxicity and Tolerability

The most common toxicities of neoadjuvant chemotherapy were grade 3/4 neutropenia (42.6 %), leukopenia (17.0 %), anorexia (8.5 %), febrile neutropenia (6.4 %), nausea (4.3 %), neuropathy (4.3 %), and allergic reaction (4.3 %) (Table 3). Seven patients did not complete the neoadjuvant therapy due to allergic reactions ($n = 2$), grade 3 anorexia ($n = 1$), grade 2 nausea and anorexia ($n = 2$), and PD ($n = 2$); all seven patients had gastrectomy). Among patients who received preoperative DTX with S-1 (DS) therapy, surgical complications developed in 19 patients (40.4 %) (Supplementary Table 2). The number of complications according to the Clavien–Dindo classification was 3 for grade I, 10 for grade II, and 9 for grade IIIa. The most frequent complication, pancreatic juice leakage, developed in eight patients (17.0 %), and the next most frequent complication, intra-abdominal abscess requiring drainage therapy, developed in six patients (12.8 %). No patient suffered from severe surgical complications of grade IIIb or higher. No patients died due to surgical complications.

Survival After Resection

After a median follow-up of 3 years, the median relapse-free survival (RFS) and overall survival (OS) was not reached (Fig. 1a, b). RFS was assessed for 44 patients who underwent gastrectomy, and OS was assessed for all 47 patients. The 3-year RFS rate was 53.2 % (95 % CI 19.4 % to not estimable) in 44 assessable patients, and 3-year OS was 60.9 % (95 % CI 28.1 % to not estimable) in 47 assessable patients. When the survival rate was separated by pRR, the 3-year RFS rate of grade 1b/2/3 cases was 62.9 %, whereas that of grade 0/1a cases was 42.9 % (Fig. 2a). The OS of grade 1b/2/3 cases and grade 0/1a cases were 72.7 and 52.2 %, respectively (Fig. 2b). Because of the small sample size, this difference in OS was not statistically significant (hazard ratio = 0.45).

DISCUSSION

Neoadjuvant chemotherapy is commonly administered for advanced, but resectable cancer to diminish the undetected cancer cells. In Europe, preoperative and postoperative (perioperative) chemotherapy is the standard treatment for advanced gastric cancer because the perioperative combination chemotherapy of epirubicin, cisplatin, and infused fluorouracil (ECF) improved survival in patients with locally advanced gastric cancer in a phase III trial.[21] Another phase III trial showed a survival advantage of 5-flurouracil and cisplatin (FP) perioperative

TABLE 3 Treatment-related adverse events

Adverse events	Grade 1*	Grade 2	Grade 3	Grade 4	Incidence (%)	Incidence of grade 3/4 (%)
Hematological toxicity						
Leukopenia	12	18	7	1	80.9	17.0
Neutropenia	1	8	16	4	61.7	42.6
Hemoglobin	27	14	1	0	68.1	2.1
Non-hematological toxicity						
Anorexia	19	6	4	0	61.7	8.5
Nausea	15	4	2	0	44.7	4.3
Vomiting	5	2	0	0	14.9	0
Allergic reaction	3	1	0	2	12.8	4.3
Diarrhea	5	1	0	0	12.8	0
Neuropathy	2	1	2	0	10.6	4.3
Febrile neutropenia	—	—	3	0	6.4	6.4

*Adverse effect was assessed according to the National Cancer Institute Common Toxicity Criteria (CTC) Common Terminology Criteria for Adverse Events version 3.0 (CTCAE vers. 3)

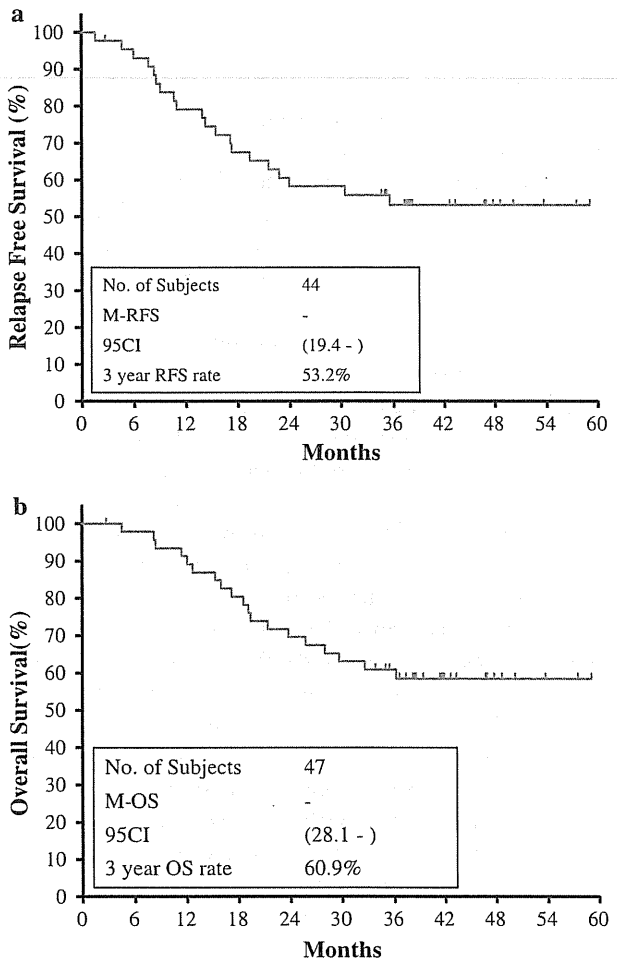

FIG. 1 After a median follow-up of 3 years, the median relapse-free survival (RFS) and overall survival (OS) was not reached. **a** RFS. **b** OS. *95 CI* 95 % confidence interval, *M-OS* median overall survival, *M-RFS* median relapse-free survival, *NR* not reached

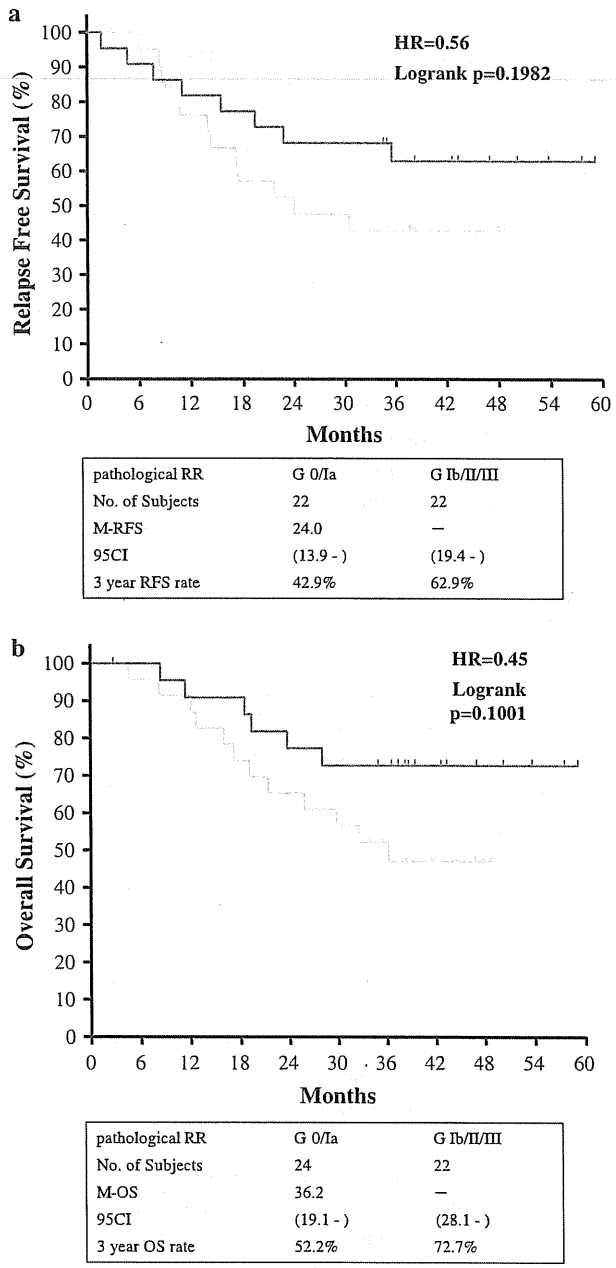

FIG. 2 Patients with high pathological response had improved relapse-free survival (RFS) and overall survival (OS) compared with those for patients with low pathological response. **a** The RFS of each subset of pathological response. The *solid line* represents the survival of the high pathological response group with grade 1b/2/3 (G 1b/2/3) pathological response. The *dotted line* represents the survival of the pathological response group with grade 0/1a (G 0/1a) pathological response. **b** The OS of each subset of pathological response. The *solid line* represents the survival of the high pathological response group with grade 1b/2/3 (G 1b/2/3) pathological response. The *dotted line* represents the survival of the pathological response group with grade 0/1a (G 0/1a) pathological response. One patient could not be assessed for pathological response. *95 CI* 95 % confidence interval, *HR* hazard ratio, *M-OS* median overall survival, *M-RFS* median relapse-free survival, *NR* not reached, *RR* response rate

chemotherapy for resectable gastric cancers.[22] The patient enrollment criteria of these European clinical trials included lower esophageal cancer, and D2 gastrectomy was not conducted in all patients. In Asian countries, D2 gastrectomy is the standard treatment for gastric cancer, and neoadjuvant chemotherapy has not been used for advanced, but resectable cancers. In Japan, it has been proven that overall survival and disease-free survival are improved by adjuvant S-1 monotherapy in advanced gastric cancer.[5] It has not been established that neoadjuvant chemotherapy is safe and effective for patients who have resectable advanced gastric cancers, who undergo D2 gastrectomy

We conducted this phase II trial of neoadjuvant chemotherapy for patients with advanced gastric cancer who could be resected with standard D2 gastrectomy. The objective of this clinical trial was to test the safety and effectiveness of neoadjuvant docetaxel and S-1

combination chemotherapy. Because exact evaluation of cancer staging before treatment was necessary to conduct a clinical trial of neoadjuvant chemotherapy for Stage IIIA or Stage IIIB patients, staging laparoscopy was required for all cases before enrollment. As a result, no patients were diagnosed with peritoneal dissemination at the planned operation.

In this study, operative complications were assessed in addition to chemotherapy-related adverse effects. The most common toxicities of neoadjuvant chemotherapy were grade 3/4 neutropenia (42.6 %) and leukopenia (17.0 %), similar to those in previous reports.[12,14] Seven patients did not complete the neoadjuvant therapy because of adverse effects. Abdominal abscess and pancreatic juice leakage were the most frequent surgical complications, but all cases recovered without reoperation. Anastomotic leakage was not experienced, but the incidence of abdominal abscesses was slightly higher than that expected with D2 operation.[4] Treatment-related death and operative mortality were not observed in this study. This differs from the JCOG0001 study, which reported 5 % treatment-related death and 2 % operative mortality. We conclude that neoadjuvant DTX with S-1 (DS) chemotherapy can be used safely. The DS therapy has several advantages for neoadjuvant chemotherapy. This therapy can be adopted at outpatient clinics. Inpatient care was not necessary for most patients in this trial. It is necessary to be in the hospital if cisplatin (CDDP) is used for neoadjuvant chemotherapy because hydration is usually needed. In this study, renal function was not damaged in any patient, despite outpatient clinic treatment. This is an important factor to consider for neoadjuvant chemotherapy.

To show the effectiveness of neoadjuvant chemotherapy for advanced gastric cancer patients, we chose pathological RR (pRR) as the primary endpoint. Phase II studies of docetaxel and S-1 combination therapy for unresectable and recurrent gastric cancer were previously conducted, and the RR was reported as 40–56 %.[12,14,23] However, there has been no such clinical study evaluating resectable gastric cancer. For resectable gastric cancer, the evaluation of clinical response using Response Evaluation Criteria in Solid Tumors (RECIST) was quite difficult. Therefore, pRR was chosen as the primary endpoint in this study. The pRR was one of best surrogated endpoints for neoadjuvant chemotherapy for gastric cancer.[24] The pathological response (grade 1b or greater) of JCOG 0001, a phase II trial of neoadjuvant chemotherapy for locally advanced gastric cancer, was 7 of 55 patients (12.7 %).[17] Tsuburaya et al.[15] reported that grade 1b or greater pRR was observed in 18 of 42 patients (42.8 %) on paclitaxel and cisplatin neoadjuvant therapy.[15] In a preoperative setting, a phase II study of S-1 plus cisplatin for patients with locally advanced gastric cancer (JCOG0210) showed 48 % pRR in the primary lesion.[25] In our study, grade 1b or greater was observed in 22 of 47 patients (46.8 %). The mean pRR was 46.8 % among the 47 patients, which was higher than the expected pRR of 40 %. The lower end of the 95 % CI for pRR was 34.2 %, which also exceeded the threshold pRR of 20 %. This response was similar to the previous neoadjuvant study for patients with far advanced gastric cancer. This study also showed that patients with high pathological response had improved relapse-free survival and overall survival compared with those for patients with low pathological response (Fig. 2). Although the sample size was too small to show statistical significance of pathological response on patient survival, this is a very promising result for the treatment of gastric cancer. In addition, it is possible that the assessment of pathological response showed the chemosensitivity of postoperative chemotherapy. The preoperative chemotherapy regimen included docetaxel and S-1. On the other hand, S-1 monotherapy was only used as postoperative chemotherapy in our study. Therefore, postoperative DS therapy may play a role in improving RFS and OS in patients who show high response to neoadjuvant therapy. Recently, the monoclonal antibody trastuzumab has become the standard treatment for human epidermal growth factor receptor 2 (HER2)-positive advanced gastric cancer. In the future, combination therapy with trastuzumab and DTX with S-1 (DS) should be considered for patients with HER2-positive tumors.

In conclusion, preoperative DS therapy was highly active against resectable clinical Stage III gastric cancer, and this treatment was well tolerated with few toxicities. The favorable results of our study have raised expectations that this therapy may improve survival outcomes for patients with advanced gastric cancer.

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Loss of Heterozygosity of PTEN (Encoding Phosphate and Tensin Homolog) Associated with Elevated HER2 Expression Is an Adverse Prognostic Indicator in Gastric Cancer

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Key Words
Loss of heterozygosity of PTEN · HER2 · Gastric cancer

Abstract
Objective: PTEN (the encoding phosphate and tensin homolog) is a well-known cancer suppressor gene and its mutation and loss of heterozygosity (LOH) occurs in various types of carcinomas. This study aimed to examine the association between LOH of PTEN and prognosis in HER2-expressing and nonexpressing gastric cancer patients. ***Methods:*** Fresh-frozen tumor samples of 221 gastric cancer patients with a primary diagnosis of gastric carcinoma were examined for LOH of PTEN. The results were compared with pathological parameters and the HER2 status. To elucidate the role of LOH of PTEN, the activation of the PI3K/AKT pathway was examined immunohistochemically using a phosphorylation-specific antibody. ***Results:*** LOH of PTEN was observed in 20% of the patients (39 of 195 cases). LOH of PTEN was associated with vascular involvement (25 of 39 cases; $p = 0.0083$), equivocal to positive staining for HER2 ($p = 0.0080$), and phospho-Akt expression ($p = 0.0067$). Patients with HER2-expressing gastric cancer with LOH of PTEN had a significantly worse prognosis ($p = 0.0050$). ***Conclusions:*** Although HER2 expression itself was not a prognostic factor, the combination of HER2 expression and LOH of PTEN exacerbates the malignant potential of gastric cancer through its proliferative function.

Introduction

PTEN (the encoding phosphate and tensin homolog) is a well-known tumor suppressor gene located on chromosome 10q23.3 and encodes a phosphatase that dephosphorylates phosphatidylinositol-(3,4,5)-triphosphate (PIP3) causing the downregulation of the PI3K/AKT signaling pathway [1, 2]. The PI3K/AKT signaling pathway transduces intracellular signals for growth, proliferation, and cell survival [3]. The mutation or the deletion of PTEN is reported in several types of human cancers such as glioblastoma, melanoma, prostate cancer, and endometrial neoplasms [3–5], and its germline mutation causes familial syndromes such as Cowden disease, characterized by multiple benign hematomas and an increased risk of thyroid and breast neoplasms [6]. PTEN heterozygous mutant mice have been shown to develop neoplasms

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in multiple organs, including the endometrium, liver, prostate, gastrointestinal tract, thyroid, and thymus [7], whereas mice overexpressing PTEN have been shown to have an increase in mitochondrial oxidative phosphorylation and resistance to oncogenic transformation [8], suggesting its crucial role in tumorigenesis.

HER2 (human epidermal growth factor receptor 2) is encoded by *ERBB2*, a well-known proto-oncogene located on chromosome 17 [9]. HER2 overexpression promotes cell proliferation and suppresses apoptosis through several signaling pathways, including the PI3K/AKT signaling pathway [10–13]. HER2 expression is reported to be involved in the pathogenesis and poor outcomes of several types of cancer such as lung, colon, and breast cancers [14, 15], and it is also well known for its expression in gastric cancer [16]. Although it still remains controversial whether HER2 expression is a prognostic factor or not, the ToGA study [17] concluded that trastuzumab in combination with chemotherapy can be considered as a new standard option for patients with HER2-positive advanced gastric or gastroesophageal junction cancer. Among several markers that are reported to predict sensitivity to trastuzumab, the loss of PTEN function is said to be one of the mechanisms of resistance to trastuzumab [18].

Gastric cancer remains a major player in cancer-related deaths, but its pathogenesis and progression are not clearly understood. Previously, we have reported that loss of heterozygosity (LOH) of PTEN is a prognostic factor of gastric cancer in a study of 76 gastric cancer patients [19, 20]. For this study, we increased the sample size to 221 patients in order to examine the association between LOH of PTEN and prognosis in HER2-expressing and nonexpressing gastric cancer patients. To elucidate the role of LOH of PTEN in gastric cancer, we also examined the activation of the PI3K/AKT pathway by immunohistochemical (IHC) analysis of a phosphorylation-specific antibody.

Materials and Methods

Tissue Samples

Pairs of primary gastric carcinoma tissue and corresponding normal mucosa were obtained from 221 consecutive patients who underwent surgery at the Department of Surgery and Science, Kyushu University Hospital, Fukuoka, Japan, between 1996 and 2006. The study was conducted according to the principles set forth in the Declaration of Helsinki. Informed consent was obtained from all patients prior to their inclusion.

In all cases, the histopathological type of the tumor was adenocarcinoma. For the pathological evaluation, the 13th edition of the Japanese Classification of Gastric Carcinoma was used. Cancer tissues and well-separated normal gastric mucosa obtained by gastrectomy were immediately snap-frozen and stored in liquid nitrogen. Genomic DNA was prepared by proteinase K digestion and phenol/chloroform extraction, which was followed by ethanol precipitation.

LOH analysis

LOH was analyzed using a DNA sequencer with microsatellite markers, using the methods previously described [19]. In details, the oligonucleotide primers for D10S796 and D10S1765 were synthesized and purified by high-performance liquid chromatography. The sequences of the primers used for polymerase chain reaction (PCR) analysis were the following: D10S796 forward – 5′-CAATGGAACCAAATGTGGTC, D10S796 reverse – 5′-AGTCCGATAATGCCAGGATG, D10S1765 forward – 5′-CATGCCAAGACTGAAACTCC and D10S1765 reverse – 5′-AAACCCCAATGCCATAATGG. PCR reactions using genomic DNA were performed using a TAKARA GeneAmp PCR Reagent Kit (Takara, Tokyo, Japan) and run on the Perkin-Elmer GeneAmp PCR system 9700 (Norwalk, Conn., USA). The conditions for the PCR cycle were as follows: 1 cycle at 95°C for 4 min; 35 cycles at 95°C for 0.5 min, 55°C for 0.5 min, and 72°C for 0.5 min; and 1 cycle at 72°C for 10 min. The DNA derived from cancer tissues was amplified using the 6-carboxy-X-rhodamine-labeled 5′ primer and cold 3′ primer, whereas the DNA from normal tissues was amplified using the 6-carboxy-24,44,74,4,7-hexachloro-fluorescein-labeled 5′ primer and cold 3′ primer. The PCR reactions and running conditions on the Perkin-Elmer Genetic Analyzer 310 were determined as described previously [21, 22]. A total of 221 cases were analyzed, and data were processed using GeneScan software (ABI; Applied Biosystems, Foster City, Calif., USA). Cases in which the peak value of one gene locus was diminished by >30% in the carcinoma tissue were judged as having LOH.

Immunohistochemistry

For the IHC analysis of phospho-Akt (p-AKT) and HER2, formalin-fixed paraffin-embedded specimens were dewaxed in xylene and rehydrated in ethanol. For the antigen retrieval of p-AKT, the specimens were heated with target retrieval solution (DAKO) in an autoclave. For HER2, the specimens were heated in a microwave in citrate buffer (pH 6). Endogenous peroxidase activity was blocked by incubation with 0.3% hydrogen peroxide in methanol for 30 min. The tissue sections were preblocked with 10% goat serum in phosphate-buffered saline for 10 min. The sections were incubated with an anti-p-AKT (Ser473) polyclonal antibody (Cell Signaling; dilution 1:50) and HER2 polyclonal antigen (DAKO; dilution 1:400) overnight at 4°C. The sections were incubated with the DAKO EnVision™+ System for 60 min. A diaminobenzidine tetrahydrochloride working solution was used for color development. Finally, the sections were counterstained with hematoxylin. For the evaluation of p-AKT activity in gastric cancer cells, specimens that showed >50% cytoplasmic expression were considered positive based on our previous report [20].

For the evaluation of HER2, scoring criteria based on the report by Hofmann et al. [23] were used. The specimens were divided into 3 groups according to membranous HER2 expression: negative staining (0, 1+), equivocal staining (2+), and positive staining (3+). Because of the difficulty of performing FISH in all HER2-equivocal cases, we had to decide whether to include or exclude the HER2-equivocal (HER2 2+)-expressing group in the HER2-positive (HER2 3+) group. On the basis of the biological difference, it has been pointed out that HER2 protein expression and gene amplifi-

Table 1. Clinicopathological features of patients with gastric cancer according to LOH of PTEN

Variable	PTEN		p value
	LOH (n = 39)	ROH (n = 156)	
Gender			n.s.
Male	28	100	
Female	11	56	
Age, years	66.5	62.6	n.s.
Histology			n.s.
Intestinal	18	61	
Diffuse	20	95	
Depth of invasion			n.s.
pS+pSM	5	20	
pMP+pSS+pSE+pSI	34	136	
Lymphatic invasion			n.s.
ply0	12	43	
ply1	12	34	
ply2	10	46	
ply3[1]	5	32	
Vascular invasion			0.0083
pv0	14	95	
pv1	10	34	
pv2	10	20	
pv3[1]	5	6	
Lymph node metastasis			n.s.
Negative	11	48	
Positive	28	107	
Peritoneal cytology			n.s.
Negative	38	135	
Positive	1	20	
Liver metastasis			n.s.
Negative	36	151	
Positive	3	4	
Metastasis			n.s.
Negative	38	153	
Positive	1	2	
Stage			n.s.
I+II	17	67	
III+IV	22	89	
HER2 status			0.0080
0, 1+	18	115	
2+, 3+	15	34	

n.s. = Not significant.

[1] Classified according to the 14th edition of the Japanese Classification of Gastric Carcinoma.

cation is heterogeneous in gastric cancer compared with breast cancer. Previous reports noted that the HER2-equivocal staining group showed more phenotypic heterogeneity than the HER2-positive staining group [24], and almost 50% showed gene amplification. On the basis of this information, we decided to include the HER2-equivocal group in the HER2-positive group and defined them as HER2-expressing gastric cancer.

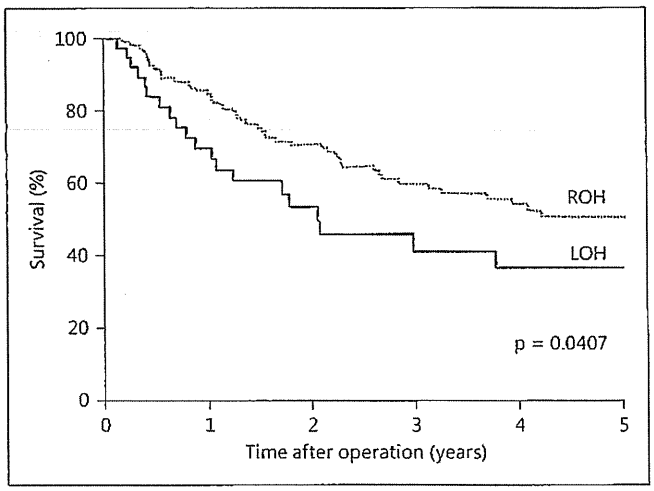

Fig. 1. Kaplan-Meier overall survival curves. Overall survival of patients with LOH of PTEN (solid line) and ROH of PTEN (dotted line).

Statistical Methods

Clinicopathological data were stored on an IBM 3090 mainframe computer (IBM, Armonk, N.Y., USA). Statistical analysis was performed using Microsoft Excel 2010 software and JMP software (version 9.0.2; SAS Institute, Cary, N.C., USA). Comparisons between LOH of PTEN and clinicopathological findings or IHC staining were evaluated using Pearson's χ^2 or Fisher's exact test. Patient survival was calculated using the Kaplan-Meier method. For multivariate analysis, a Cox regression analysis was performed. The results were considered significant at $p < 0.05$.

Results

LOH of PTEN and Clinicopathological Factors

In 26 cases, the lengths of the microsatellite sequences of the paternal and maternal alleles were almost the same, making it difficult to analyze LOH of PTEN. Thus, these 26 cases were excluded from the analysis. LOH of PTEN was observed in 39 of the remaining 195 cases (20%). On examining the HER2 status by IHC analysis, 38 cases were excluded because of a lack of samples. Among the remaining 183 cases, positive, equivocal, and negative staining rates for HER2 were 9.3% (17 cases), 17.5% (32 cases), and 73.2% (134 cases), respectively. HER2 positivity itself showed no correlation with clinicopathological features (data not shown). Among the clinicopathological parameters, LOH of PTEN was associated with vascular involvement (25 of 39 cases, p = 0.0083) and equivocal to positive IHC staining for HER2 (18 of 33 cases, p = 0.0080; table 1).

Table 2. Univariate and multivariate Cox proportional hazard analyses for overall survival in gastric cancer patients

Characteristics	Univariate p value	Multivariate HR (95% CI)	Multivariate p value
Lymphatic invasion	0.0049	2.91 (1.32–7.15)	0.0084
Vascular invasion	<0.0001	2.68 (1.45–4.91)	0.0032
Lymph node metastasis	<0.0001	3.56 (1.80–7.67)	<0.0001
LOH of PTEN	0.041	1.93 (1.00–3.56)	0.037
Liver metastasis	0.083	0.80 (0.280–2.71)	0.70
Remote metastasis	0.31	1.64 (0.257–5.78)	0.54
Cytodiagnosis of ascites	0.29	1.01 (0.419–2.19)	0.97
HER2 status (0, 1+ vs. 2+, 3+)	0.88	1.53 (0.826–3.00)	0.18

HR = Hazard ratio; CI = confidence interval.

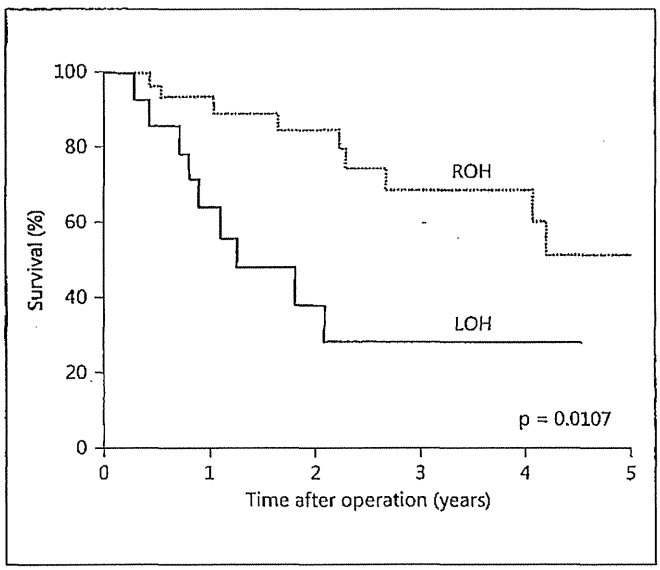

Fig. 2. Kaplan-Meier overall survival curves for HER2-expressing (equivocal or positive staining) gastric cancer. Overall survival of HER2-positive patients with LOH of PTEN (solid line) and ROH of PTEN (dotted line).

Survival Impact of LOH of PTEN

We also investigated the relationship between LOH of PTEN and overall survival using the Kaplan-Meier method. The overall survival rates after 5 years were 36.7% in patients with LOH of PTEN and 48.9% in those with biallelic PTEN (retention of heterozygosity, ROH); thus, patients with LOH of PTEN had a significantly lower survival rate (p = 0.0407) than those with ROH of PTEN (fig. 1). Univariate and multivariate Cox regression analyses revealed that lymphatic invasion, vascular invasion, lymph node metastasis, and LOH of PTEN were associated with patient survival (table 2).

Table 3. Relationship between p-AKT IHC staining and LOH of PTEN

	IHC staining of p-AKT	
	negative (n = 126)	positive (n = 61)
LOH of PTEN	18	19
ROH of PTEN	108	42

p = 0.0067.

Among all patients with equivocal or positive HER2 expression, the overall 5-year survival rates were lower in patients with LOH of PTEN than in patients with ROH of PTEN (28.9 vs. 52.2%, p = 0.0050; fig. 2). In cases with negative HER2 expression, the survival rates did not significantly differ with LOH of PTEN (for online suppl. fig. 1, see www.karger.com/doi/10.1159/000368984).

The Involvement of AKT Activation in Association with the PTEN Status

To investigate the function of LOH of PTEN, we examined the activation of the PI3K/AKT pathway by IHC analysis of p-AKT. Positive staining of p-AKT was observed in 32.6% patients (61 of 187 cases), and LOH of PTEN was more commonly seen in patients with positive staining for p-AKT (p = 0.0067; table 3). In HER2-expressing gastric cancer with LOH of PTEN, there was a tendency towards positive staining of p-AKT, but this was not statistically significant (data not shown).

Discussion

LOH of PTEN Activates the PI3K/AKT Signaling Pathway, Leading to Poor Prognosis in Gastric Cancer

PTEN is a well-known tumor suppressor, and in vivo studies on PTEN knockout mice have shown that hemizygous loss of PTEN with haploinsufficiency of the remaining allele leads to genomic instability and cancer development [25–27]. The relationship between the dysfunction of PTEN and tumorigenesis in gastric cancer is poorly understood. In our previous study investigating the PTEN heterozygosity status in 76 gastric cancer patients, we identified LOH of PTEN in 17.1% of all patients, but there was no correlation with clinicopathological parameters [19]. When we extended our investigation to 195 cases, LOH of PTEN was observed in 20% (39/195) of gastric cancer patients. With regard to clinicopathological features, vascular involvement and HER2 expression were associated with LOH of PTEN. The correlation between the loss of PTEN function and vascular invasion is reported in other malignancies such as breast cancer [28] and hepatocellular carcinoma [29]. Furthermore, specifically in gastric cancer, there are studies that have concluded that low PTEN expression causes lymph node metastasis, tumor infiltration, and remote metastasis [30, 31]. It has also been reported that LOH of PTEN is more frequently observed in cases of advanced gastric cancer [31]. In our study, gastric cancer patients with LOH of PTEN had a poor prognosis and showed a correlation with the IHC expression of p-AKT.

PTEN dephosphorylates PI3K, causing the downregulation of the PI3K/AKT signaling pathway, which plays an important role in cell proliferation [1, 2]. The activation of the PI3K/AKT signaling pathway has been reported in several malignancies such as breast [32], lung [33], and head and neck carcinomas [34]. In gastric cancer, a previous study showed that the IHC expression of p-AKT was correlated with clinicopathological features such as vessel infiltration and lymph node metastasis [35]. Our study revealed that LOH of PTEN causes an activation of the PI3K/AKT signaling pathway, leading to an adverse outcome in gastric cancer. We have previously shown, using MTT assay, that AKT phosphorylation is associated with LOH of PTEN, leading to chemoresistance in gastric cancer [20]. Further studies of the p-AKT status in gastric cancer tissue before and after chemotherapy should be conducted to elucidate the functional role of the activation of this pathway.

Poor Prognosis of HER2-Expressing Gastric Cancer with LOH of PTEN

HER2 positivity has been reported in 6.0–29.4% of all gastric cancer patients [16]. In our study, equivocal or positive HER2 staining was seen in 27% of all patients, and although HER2 expression itself was not correlated with other clinicopathological parameters and was not a prognostic factor, patients with LOH of PTEN had poor prognosis. HER2 overexpression is known to activate the PI3K/AKT pathway [13], and PTEN is its negative regulator [1, 2]. Interestingly, in gastric cancer cases with weakly or nonexpressing HER2, prognosis was not affected by LOH of PTEN. This suggests that, in gastric cancer, the combination of HER2 expression and LOH of PTEN accelerates the PI3K/AKT pathway and exacerbates the prognosis through its proliferative function. This could also be the reason why HER2 expression alone is not a definitive prognostic factor in gastric cancer.

The ToGA study [17] showed a benefit of trastuzumab combined with chemotherapy against advanced gastric cancer, and the oncologists' understanding of the importance of anti-HER2 targeting in gastric cancer is increasing. In a study of 39 HER2-positive breast cancer patients by Nagata et al. [18], those with PTEN-negative tumors had a significantly worse response to trastuzumab-based therapy than those with PTEN-positive tumors, and the continuous activation of the PI3K/AKT pathway due to PTEN dysfunction was hypothesized to be one of the causes. Although no patients in our study received trastuzumab treatment, there was a significant difference in the prognosis of HER2-expressing gastric cancer with LOH of PTEN. This result strongly suggests the importance of investigating the function of PTEN in gastric cancer patients who will receive trastuzumab treatment.

Conclusion

In conclusion, our investigation revealed that 20% of 195 gastric cancer patients had LOH of PTEN, which was associated with vascular invasion, equivocal or positive HER2 expression, and p-AKT expression. This study highlights the important role of PTEN in HER2-expressing gastric cancer by showing that the combination of HER2 expression and LOH of PTEN leads to poor prognosis. As trastuzumab treatment is used in HER2-positive advanced gastric cancer patients, we propose that the efficacy of trastuzumab in gastric cancer

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