original reports abstract

Association of *ERBB2* Copy Number and Gene Coalterations With Trastuzumab Efficacy and Resistance in Human Epidermal Growth Factor Receptor 2–Positive Esophagogastric and Gastric Cancer

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PURPOSE *ERBB2* copy number (CN), measured using next-generation sequencing, is a predictive biomarker for trastuzumab efficacy in human epidermal growth factor receptor 2 (HER2)–positive advanced esophagogastric and gastric cancer (AGC). We aimed to investigate the association of *ERBB2* amplification and gene coalterations with response and resistance to trastuzumab-combined chemotherapy.

METHODS The SCRUM-Japan GI-SCREEN was a comprehensive genomic profiling project of GI cancer tissues using Oncomine Cancer Research Panel and Oncomine Comprehensive Assay. From 885 patients with AGC who successfully underwent gene profiling, 74 with *ERBB2* amplification (CN ≥ 4.0) and who received first-line trastuzumab-combined chemotherapy were selected, and *ERBB2* CN and gene coalterations were assessed.

RESULTS *ERBB2* CN did not differ in tumor response to trastuzumab-combined chemotherapy (one-way analysis of variance test, $P = .37$). Multivariate analysis using the Cox proportional hazard model revealed that *ERBB2* CN (continuous $\log_2$-converted CN, hazard ratio, 0.76; 95% CI, 0.62 to 0.93; $P < .01$) and receptor/oncogene amplifications in the HER2 signaling pathway (hazard ratio, 2.5; 95% CI, 1.2 to 5.3; $P = .01$) were significant predictors for progression-free survival (PFS). *ERBB2* variants coexisted in five patients (7%) and were missense mutations. Two patients with low variant allele frequencies (VAFs; 8%, 12%) showed high *ERBB2* CN (55, 80) and durable response (≥ 20 months), whereas three patients with high VAFs (66%–90%) showed low *ERBB2* CN (8-11) and no response with short PFS (1-10 months).

CONCLUSION *ERBB2* CN and gene coamplification in the HER2 signaling pathway were positive and negative predictors of PFS in trastuzumab-treated HER2-positive AGC patients, respectively. HER2-positive AGC patients with a high VAF of *ERBB2* showed poor outcomes and may need HER2 tyrosine kinase inhibitors and trastuzumab deruxtecan.

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ASSOCIATED CONTENT

Data Supplement

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INTRODUCTION

ERBB2 is an oncogene encoding human epidermal growth factor receptor 2 (HER2), a receptor tyrosine kinase (RTK).[1,2] Approximately 15% of advanced esophagogastric and gastric cancer (AGC) harbor *ERBB2* amplification that causes overexpression of HER2 and subsequent oncogenesis and tumor progression. Trastuzumab, a humanized immunoglobulin G1 monoclonal antibody against HER2, inhibits ligand-independent activation of overexpressed HER2 RTK.[3] Trastuzumab combined with fluoropyrimidine plus platinum showed a survival benefit for HER2-positive AGC and has become the first-line standard of care.[4]

Many studies regarding the prediction of trastuzumab efficacy and intrinsic/acquired resistance have demonstrated that amplified *ERBB2* copy number (CN) is a predictor of prolonged progression-free survival (PFS) and overall survival (OS) in trastuzumab-combined chemotherapy and that gene coalterations such as oncogenes and tumor-suppressor genes in RTK/RAS/PI3K signaling pathways, cell cycle regulators including *cyclin E1* (*CCNE1*) and *cyclin-dependent*

CONTEXT

Key Objective

There have been a few reports comprehensively investigating *ERBB2* copy number (CN) and gene coalterations of the human epidermal growth factor receptor 2 (HER2) signaling pathway in HER2-positive advanced esophagogastric and gastric cancer (AGC). In the SCRUM-Japan GI-SCREEN, a large-scale genomic profiling project of GI cancer tissues using next-generation sequencing, we studied *ERBB2* amplification and gene coalterations in association with response and resistance to first-line trastuzumab-combined chemotherapy.

Knowledge Generated

ERBB2 CN and gene coamplification other than *ERBB2* in the HER2 signaling pathway were positively and negatively associated with progression-free survival, respectively. A small number of patients with HER2-positive AGC had the *ERBB2* sequence variant, and those with high variant allele frequency showed poor prognosis.

Relevance

ERBB2 CN and gene coamplification of the HER2 pathway are useful biomarkers for the prediction of trastuzumab-treatment outcomes, and the *ERBB2* covariant should be further studied in the treatment selection for HER2-positive AGC.

kinase inhibitor 2A (*CDKN2A*), *TP53*, and *MYC* are resistant to trastuzumab therapy.[5-12]

Clinically, HER2 positivity is judged on the basis of degree of HER2 protein expression using immunohistochemistry (IHC3+) or in situ hybridization (ISH+) when the expression is equivocal (IHC2+).[3] This approach is considered standard of care for the determination of patients with HER2-positive disease amenable to anti–HER2-directed therapy. In recent years, high *ERBB2* CN has been reported to be an independent predictor of longer real-world PFS in patients with AGC treated with trastuzumab using next-generation sequencing (NGS) technology, and a significant association of continuous *ERBB2* CN with survival was also demonstrated.[12-14] Currently, commercial comprehensive genomic profiling can be used to investigate the relevance of gene alterations to the clinical benefits of chemotherapies in clinical practice. Thus, there is a growing need to clarify how information on gene alterations obtained by NGS can be used to better identify biomarker-selected patients uniquely poised to benefit from precision-guided therapies.

This study was a retrospective cohort and analysis that aimed to investigate the *ERBB2* CN and gene coalterations in patients with HER2-positive AGC treated with trastuzumab-combined first-line chemotherapy, who were enrolled in a nationwide cancer genome screening project in Japan.

METHODS

Study Design and Patients

SCRUM-Japan GI-SCREEN (Cancer Genome Screening Project for Individualized Medicine in Japan) was a multicenter study of the comprehensive genomic profiling for GI cancer.[14] The target gene sequences of tumor tissues were determined using NGS. In the gastric cancer cohort, patients who met the following criteria were eligible for NGS analysis: those with pathologically confirmed esophagogastric and gastric adenocarcinoma, with an Eastern Cooperative Oncology Group performance status (ECOG PS) score of 0-2, whose major organ functions were preserved for systemic chemotherapy, and who provided written informed consent for this study. The ethical, medical, and scientific aspects of this study were reviewed and approved by the institutional review board of each institution, and this study was registered in the University Hospital Medical Information Network Clinical Trials Registry (UMIN000016343). This study was conducted in accordance with the Declaration of Helsinki, as revised in 2000.

In this study, the clinical and genomic data of patients with *ERBB2*-amplified AGC who received first-line trastuzumab-combined chemotherapy were extracted from those enrolled in the SCRUM-Japan GI-SCREEN. The patients were treated according to the clinical practice guidelines in Japan.[15] Trastuzumab was administered at a standard dose and schedule.

Data Collection and Assessments

The primary end point was to determine how *ERBB2* amplification and gene coalterations were associated with the response and resistance to trastuzumab-combined chemotherapy. Data on the following clinical characteristics of eligible patients were collected: age, sex, ECOG PS score, presence of primary tumor before chemotherapy, number and location of metastatic organs, presence of ascites, method of tissue collection (tumor biopsy or surgical resection), tumor sites, histologic classification–based Lauren's criteria, HER2 score (IHC3+ or IHC2+ISH+), and concomitant chemotherapy regimen. Response rate, PFS, and OS of trastuzumab-combined first-line chemotherapy were assessed. Tumor response assessment was performed on the basis of the RECIST v1.1, complete response (CR), partial response (PR), stable disease, and progressive disease, if measurable lesions were present. PFS was defined as the time from the initiation of trastuzumab-

TABLE 1. Baseline Patient Characteristics

Characteristics	No. of Patients (%)
Age	
Median years (range)	68 (27-84)
Sex	
Male	52 (70)
Female	22 (30)
ECOG PS	
0	50 (68)
1	23 (31)
2	1 (1)
Primary lesion	
Absent	15 (20)
Present	59 (80)
No. of metastatic organs	
1	34 (46)
≥ 2	40 (54)
Metastatic sites	
Liver	38 (51)
Peritoneum	26 (35)
Lymph nodes	57 (77)
Lung	13 (18)
Bone	3 (4)
Ascites	25 (33)
Obtained site of specimens for gene analysis	
Primary tumor	
Endoscopic biopsy	60 (81)
Surgical resection	13 (18)
Metastatic lesion	
Needle biopsy	1 (1)
Histology[a]	
Intestinal	58 (78)
Diffuse	13 (18)
Others	3 (4)
HER2	
IHC3+	58 (78)
IHC2+ and ISH+	16 (22)
ERBB2	
Median copies (first-third IQR)	20 (10-55)
Mutation	5 (7)
Gene coalteration in HER2 pathway	
Mutation	
ERBB2/PIK3CA/PTEN/KRAS/BRAF	13 (18)
Coamplification	
FGFR/EGFR/AKT1/IGF1R/MET/PIK3CA/KRAS/BRAF	14 (19)
Chemotherapy used with trastuzumab	
S-1 + oxaliplatin	33 (45)
S-1 + cisplatin	10 (14)
S-1 + oxaliplatin + docetaxel	1 (1)
Capecitabine + cisplatin	16 (22)
Capecitabine + oxaliplatin	8 (11)
5-FU + oxaliplatin	3 (4)
5-FU + cisplatin	1 (1)
Paclitaxel	2 (3)

Abbreviations: *AKT1*, protein kinase B; *BRAF*, v-raf murine sarcoma viral oncogene homolog B; ECOG PS, Eastern Cooperative Oncology Group performance status; *EGFR*, epidermal growth factor receptor; *ERBB2*, Erb-B2 receptor tyrosine kinase 2; *FGFR*, fibroblast growth factor receptor; FU, fluorouracil; HER2, human epidermal growth factor 2; *IGF1R*, insulin-like growth factor 1 receptor; IHC, immunohistochemistry; IQR, interquartile range; ISH, in situ hybridization; *KRAS*, Kirsten rat sarcoma virus; *MET*, MNNG HOS transforming gene; *PIK3CA*, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; *PTEN*, phosphatase and tensin homolog.

[a]Lauren classification.

combined chemotherapy to disease progression and/or death from any cause. OS was defined as the time from the initiation of trastuzumab-combined chemotherapy to death from any cause. Patients who were lost to follow-up were censored on the last response evaluation for PFS and on the last contact for OS.

Target Sequencing

Formalin-fixed and paraffin-embedded tumor tissues were sent to the Life Technologies Clinical Services Laboratory (910 Riverside Parkway, West Sacramento, CA). Tumor DNA and RNA were extracted and analyzed using the Ion Torrent Oncomine Cancer Research Panel (OCP) and Oncomine Comprehensive Assay v3 (OCA; Thermo Fisher Scientific, Waltham, MA). The target regions of 143 cancer-related genes were amplified by OCP analysis and 161 genes were amplified by OCA analysis (Data Supplement). Gene amplification was defined as a CN of ≥ 4.0. Gene mutations were called out if the allele frequency was ≥ 5% and alternate allele observation count of ≥ 10, excluding synonymous mutations. Gene mutations were solely oncogenic mutations determined using the Oncomine Knowledgebase and annotated using Iron Reporter software. The mutation loci were mapped using the MutationMapper of the cBioPortal.[16,17] The data files were stored in the SCRUM-Japan Data Center.

Statistical Analyses

The difference between *ERBB2* CN and the antitumor effect was evaluated using one-way analysis of variance. The optimal cutoff value of *ERBB2* CN was determined by calculating the receiver operating characteristic (ROC) curve on the basis of the PFS rates at 12 and 24 months. Using an *ERBB2* CN cutoff value, the background factors in the high and low groups were compared using the Fisher's exact test. Log_2-converted *ERBB2* CN was also used in every analysis. The patients were classified according to the log_2 values from class 2 (CN 2^2-$< 2^3$) to class 7 (CN $\geq 2^7$) for the Kaplan-Meier plots of PFS and OS. For PFS, the prognostic and predictive factors were examined by conducting a multivariate analysis using the Cox proportional hazards model. The log-rank test was used to compare the survival of the patient group. All tests were performed using the JMP version 16.0.0 software program (SAS International, Cary, NC) and EZR version 1.55.[18] All *P* values were calculated using a two-sided test, and a *P* value of $< .05$ was considered significant.

RESULTS

Patients

From April 2015 to March 2019, 1,132 patients with AGC were enrolled. Among the 885 patients whose gene sequencing of tumor tissues was completed, 125 (14%) had *ERBB2* amplification (Data Supplement). Thirty-four patients whose tissue samples were collected after chemotherapy, 14 who did not receive trastuzumab as first-line chemotherapy, and three with insufficient clinical data were excluded; overall, 74 patients who had *ERBB2* amplification and received trastuzumab-combined first-line chemotherapy were finally included in this study. Comprehensive cancer genome profiling was performed using OCP and OCA in 45 and 29 patients, respectively.

The patient's median age was 68 years (Table 1). Most patients had good general condition, with ECOG PS scores of 0 (68%) and 1 (31%). The tissue samples were mostly obtained from the primary tumor site. The histologic classifications were intestinal type in 58 patients (78%) and diffuse type in 13 patients (18%). HER2 IHC3+ tumors were observed in 58 patients (78%), whereas IHC2+ISH+ were observed in 16 patients (22%). The median *ERBB2* CN was 20. Gene comutations (*ERBB2*, *PIK3CA*, *PTEN*, *KRAS*, and *BRAF*) and coamplifications (*FGFR*, *EGFR*, *AKT1*, *IGF1R*, *MET*, *PIK3CA*, *KRAS*, and *BRAF*) involved in the HER2 pathway were detected in 13 (18%) and 14 (19%) patients, respectively. Other amplifications were observed in *CCNE1*/*CCND1*/*CDK6* (30%) and *MYC* (16%). Mutations were commonly observed in the *TP53* (72%), *PIK3CA*/*PTEN* (8%), *ERBB2* (7%), and *KRAS*/*BRAF* (3%) genes. All detected gene alterations were common in the OCP and OCA except for *BRAF* amplification (n = 1), which was not included in the OCP. As for the HER2 scores, none of the patients' characteristics including the proportion of gene coalterations in the HER2 pathway were significantly different between the IHC3+ and IHC2+/ISH+ groups, except for *ERBB2* CN (median CN: 29 and 9, respectively; Wilcoxon test, $P < .01$).

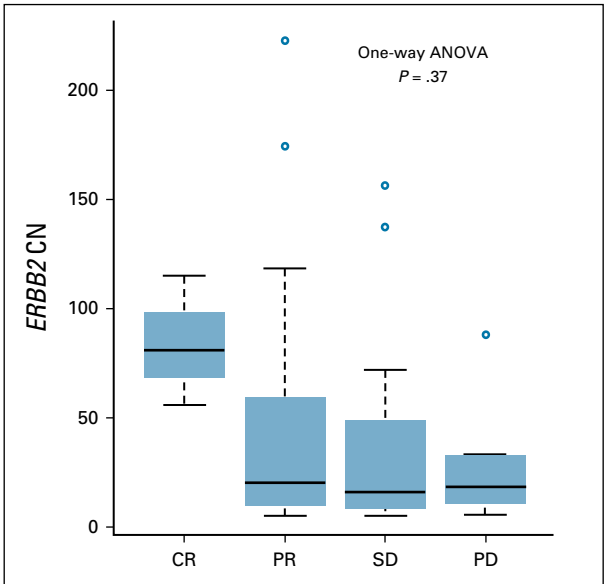
Tumor Response

In 68 patients with measurable lesions, three achieved CR (4%), and 43 achieved PR (63%), resulting in a 67% response rate (Fig 1). Neither nonconverted nor log_2-converted *ERBB2* CN showed a significant difference in tumor response (one-way analysis of variance, $P = .37$ and $P = .46$, respectively).

PFS and OS

The median PFS and OS were 8.8 months (95% CI, 7.4 to 11) and 29 months (95% CI, 20 to 38), respectively. The PFS and OS events occurred in 64 (86%) and 54 (73%) patients, respectively, until the data cutoff date of October 15, 2021.

In Figure 2, all 74 patients are sorted by PFS lengths, and their respective gene coalterations and *ERBB2* CN are depicted. Majority of patients with a PFS of > 12 months



Tumor Response		*ERBB2* CN	
	No. (%)	Median	25%, 75%
CR	3 (4)	81	56, 115
PR	43 (63)	20	10, 65
SD	16 (24)	16	8, 49
PD	6 (9)	18	10, 47

FIG 1. Tumor responses and *ERBB2* gene CN of patients with measurable lesions (n = 68) receiving first-line chemotherapy containing trastuzumab for advanced HER2-positive esophagogastric and gastric cancer. ANOVA, analysis of variance; CN, copy number; CR, complete response; *ERBB2*, erb-b2 receptor tyrosine kinase 2; HER2, human epidermal growth factor receptor 2; PD, progressive disease; PR, partial response; SD, stable disease.

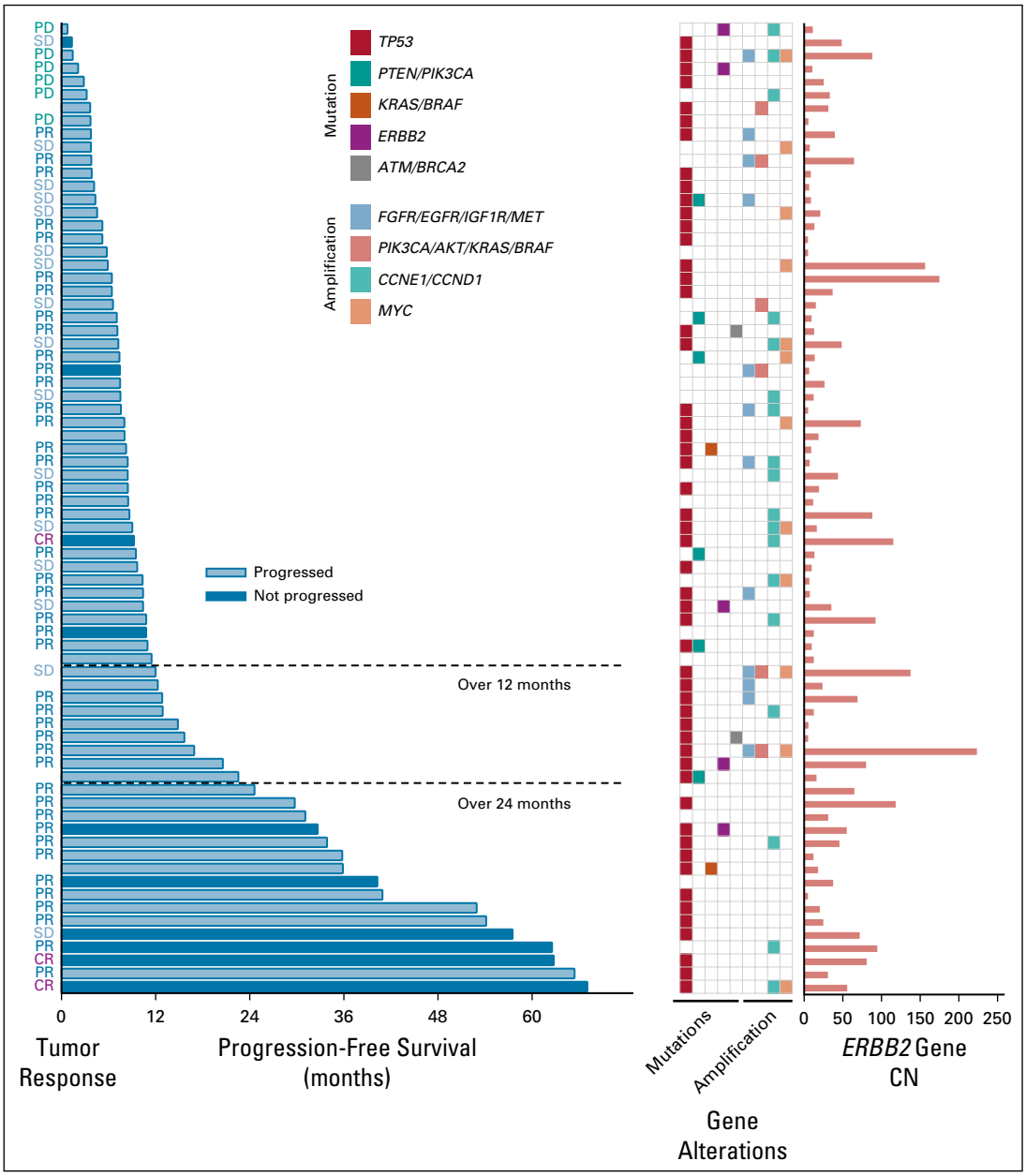


FIG 2. Efficacies and gene alterations in 74 patients treated with first-line chemotherapy containing trastuzumab for advanced HER2-positive esophagogastric and gastric cancer. *AKT*, protein kinase B; *ATM*, ataxia-telangiectasia mutated; *BRAF*, v-raf murine sarcoma viral oncogene homolog B; *BRCA2*, breast cancer susceptibility gene 2; *CCND1*, cyclin D1; *CCNE1*, cyclin E1; CN, copy number; CR, complete response; *EGFR*, epidermal growth factor receptor; *ERBB2*, erb-b2 receptor tyrosine kinase 2; *FGFR*, fibroblast growth factor receptor; HER2, human epidermal growth factor receptor 2; *IGF1R*, insulin-like growth factor 1 receptor; *KRAS*, Kirsten rat sarcoma virus; *MET*, MNNG HOS transforming gene; *MYC*, myelocytomatosis proto-oncogene; *PIK3CA*, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; PD, progressive disease; PR, partial response; *PTEN*, phosphatase and tensin homolog; SD, stable disease; *TP53*, tumor protein 53.

achieved CR and PR after receiving a trastuzumab-combined chemotherapy. Patients who showed a long PFS of > 24 months had a high *ERBB2* CN (mean $\log_2$ CN 5.2 *v* 4.4, Student's *t* test $P = .04$) and no gene coamplifications in the HER2 signaling pathway (Fig 2). Mutation of *TP53*, *PTEN*/*PIK3CA*, and *ERBB2*, and amplification of *CCNE1*/*CCND1* and *MYC* distributed independently of PFS lengths, showing no association of these alterations with PFS lengths. There was a significant difference in the PFS between patients with and without gene coamplifications other than *ERBB2* of the HER2 pathway ($P = .038$; Data Supplement).

The Kaplan-Meier curves were created for each log class of *ERBB2* CN. Patients with log classes 2 and 3 appeared to

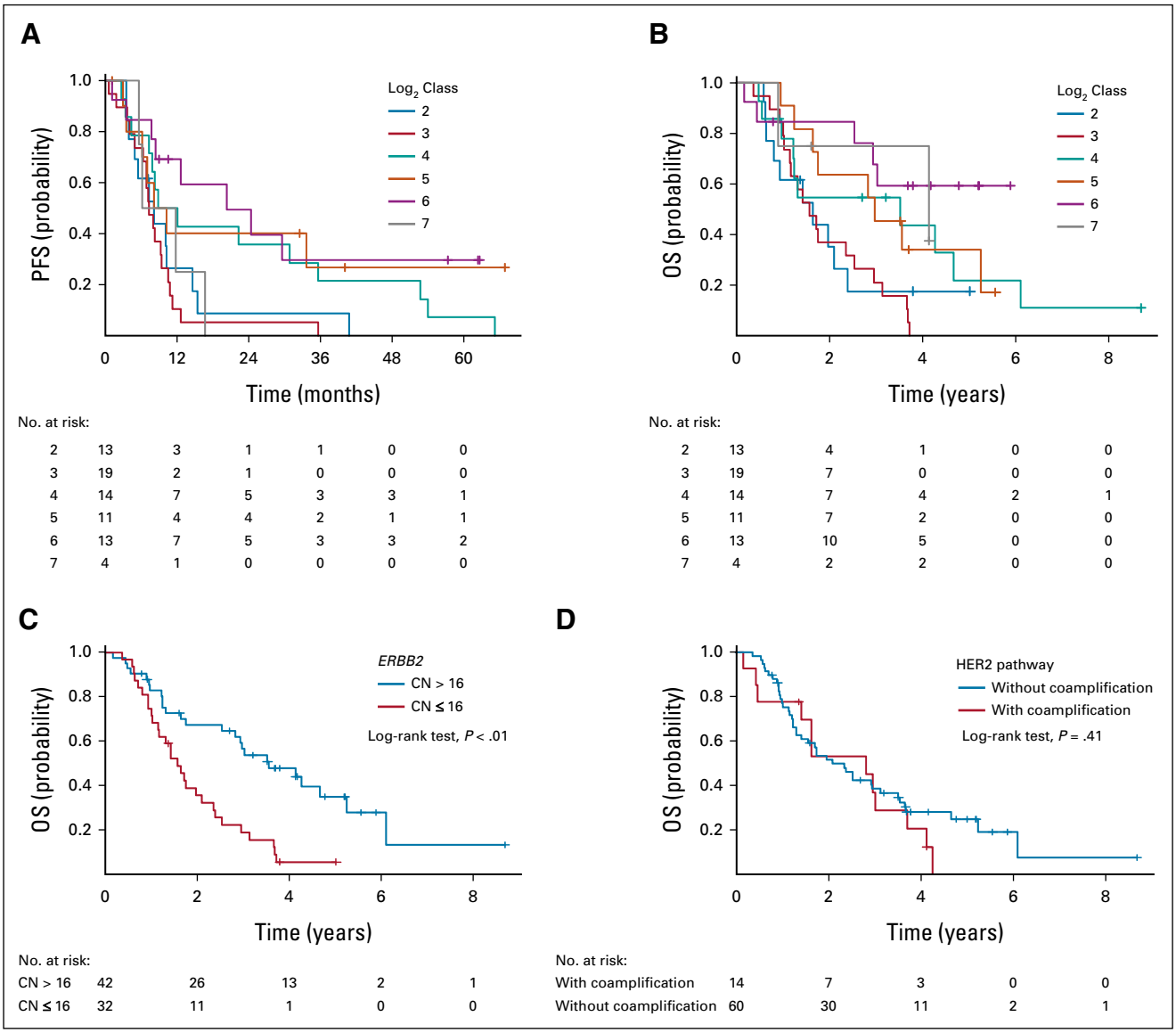


FIG 3. (A) PFS and (B) OS in patients with amplified *ERBB2* according to log_2-converted *ERBB2* CN class. Overall survival according to (C) high/low CN (cutoff 16) and (D) with/without coamplification genes other than *ERBB2* in the HER2 signaling pathway. CN, copy number; *ERBB2*, erb-b2 receptor tyrosine kinase 2; HER2, human epidermal growth factor receptor 2; OS, overall survival; PFS, progression-free survival.

have shorter PFS than those with log class 4 or more except for log class 7 (Fig 3A). Among four patients with log class 7 tumors, two had gene coamplifications of the HER2 signaling pathway. The optimal cutoff values of *ERBB2* CN for 12-month PFS and 24-month PFS on the basis of the ROC curve analyses were 15.2 copies (area under the curve: 0.64) and 16.6 copies (area under the curve: 0.68), respectively (Data Supplement). On the basis of these results, the cutoff value was determined to be CN 16 (log_2 value = 4.0). Multivariate analyses of HER2 markers associated with PFS were performed using a Cox proportional hazards model. As shown in Figures 4A and 4B, continuous log_2-converted *ERBB2* CN (hazard ratio [HR], 0.71; 95% CI, 0.57 to 0.88; $P < .01$) and dichotomized *ERBB2* CN at 16 (HR, 0.34; 95% CI, 0.19 to 0.63; $P < .01$) were significant predictive factors for PFS. In addition, receptor/oncogene amplification in the HER2 signaling pathway was a predictor of poor PFS in both settings (HR, 2.80; 95% CI, 1.38 to 5.69; $P < .01$, and HR, 2.73; 95% CI, 1.38 to 5.41; $P < .01$). Similar results were obtained when other clinical characteristics were included as variables (Data Supplement).

The Kaplan-Meier plots of log class *ERBB2* CN for OS resembled those for PFS (Fig 3B). OS was significantly longer in patients with high *ERBB2* CN (> 16) than in those with low CN (log-rank test, $P < .01$), whereas OS was not different between patients with and without amplified HER2 pathway genes (log-rank test, $P = .41$; Figs 3C and 3D).

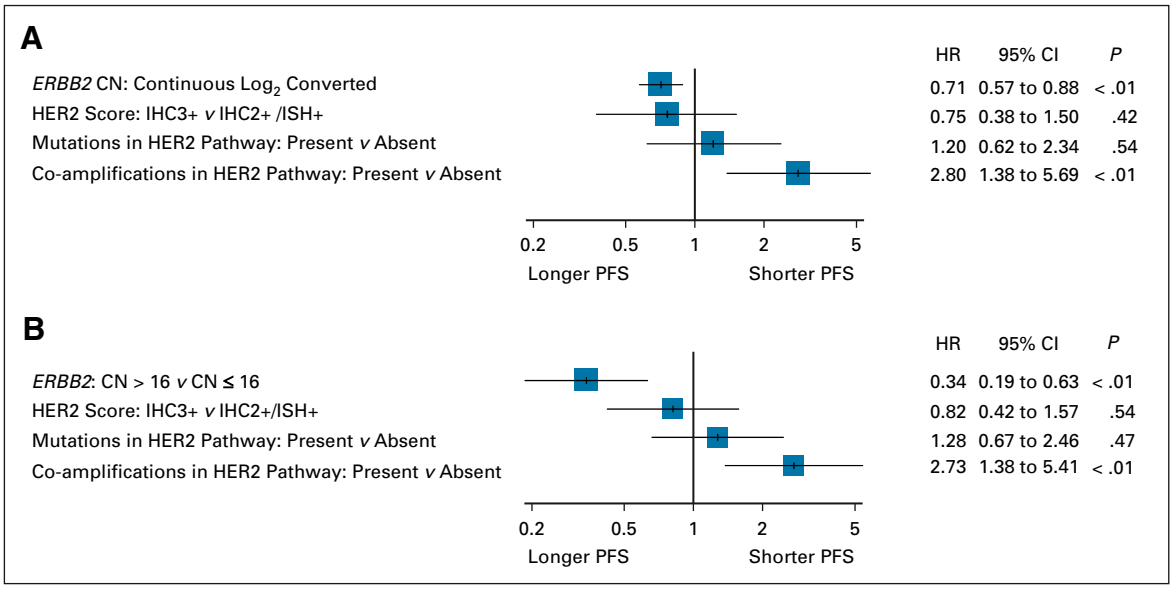


FIG 4. Multivariate analysis of HER2 biomarkers for PFS in 74 patients treated with first-line chemotherapy containing trastuzumab using (A) continuous log_2-converted *ERBB2* CN and (B) a cutoff value of *ERBB2* CN 16. CN, copy number; *ERBB2*, erb-b2 receptor tyrosine kinase 2; HER2, human epidermal growth factor receptor 2; HR, hazard ratio; IHC, immunohistochemistry; ISH, in situ hybridization; PFS, progression-free survival.

ERBB2 Covariants

Five patients (7%) had *ERBB2* covariants (all missense mutations); their details are presented in Table 2. Two patients with low variant allele frequencies (VAFs) showed high *ERBB2* CN, PR to trastuzumab-combined treatment, and long PFS and OS, whereas the remaining three patients with high VAFs showed low *ERBB2* CN, short PFS and OS, and no response to trastuzumab-combined treatment. Four *ERBB2* variants were located in the tyrosine kinase domain, whereas one variant was located in the furin-like cysteine-rich region (Data Supplement). In 42 cases of *ERBB2* mutations included in seven publicly available databases of esophagogastric and gastric cancer (1,012 cases; 1,079 samples) from cBioPortal, many missense mutations were found in the tyrosine kinase domain, furin-like cysteine-rich region, and transmembrane/juxtamembrane domain (Data Supplement).[12,19-24]

DISCUSSION

We demonstrated that *ERBB2* CN was a predictor of prolonged PFS, and coamplification of receptor/oncogene that activated the HER2 signaling pathway was an independent predictor of shorter PFS in patients with HER2-positive AGC treated with trastuzumab-combined first-line chemotherapy. In addition, a small subgroup of patients with *ERBB2* comutation was found, and those with a high VAF of *ERBB2* presented poor outcomes, although it should be noted that the number of patients was limited.

In the ToGA study, which showed prolonged survival in patients treated with trastuzumab-combined chemotherapy, the treatment response rate increased by 12.8% after the addition of trastuzumab (47.3% *v* 34.5%).[4] This finding indicated trastuzumab treatment shrunk tumor. Although this study included a small proportion of patients with HER2 IHC0-1+/fluorescence in-situ hybridization–positive, which was likely a contributor to the more mitigated difference in efficacy, the effect size of trastuzumab would be smaller, compared with the cytotoxic chemotherapy agents combined. This could be the reason why no significant difference in *ERBB2* CN was detected in the tumor responses. However, patients with CR tended to have a higher *ERBB2* CN (> 50). The remarkable response to trastuzumab may be influenced by the degree of tumor dependency on *ERBB2* CN as a driving force for tumor growth. As for the survival data, the median PFS in this study (8.8 months) was similar to that in the ToGA study (6.7 months), whereas the median OS was apparently longer in our study (29 months *v* 13.8 months). This may be attributed to the different criteria for HER2 positivity and the recent expansion of subsequent treatment options after trastuzumab discontinuation.

ERBB2 CN as a continuous value showed a significant association with PFS in this study, as well as in two recent studies using the nationwide (US-based) deidentified Flartiron Health-Foundation clinic genomic database and Foundation Medicine genomic database for gastroesophageal adenocarcinoma.[7,13] The former study demonstrated significant differences in the time to trastuzumab discontinuation and OS between patients with HER2 concordance (HER2+ and *ERBB2* amplification+) and those with discordance (HER2+ and *ERBB2* amplification–) and between patients with high *ERBB2* CN (≥ median value, 25) and those with low *ERBB2* CN.[7] The latter showed that the

TABLE 2. *ERBB2* Variants Through Next-Generation Sequencing Analysis of 74 *ERBB2*-Amplified Advanced Esophagogastric and Gastric Cancer

Age (years)/Sex	HER2 Expression	*ERBB2* CN	ERBB2 Amino Acid Variant	VAF (%)	Cellularity	Histology	First-Line Chemotherapy	Tumor Response	PFS (months)	OS (months)
71/male	IHC 3+	80	p.D769Y	12	0.5	tub2	CapeOX + Tmab	PR	20	50+
79/female	IHC 3+	55	p.D769Y	8	0.5	tub2	SOX + Tmab	PR	32+	42+
75/male	IHC 3+	11	p.G776V	75	0.7	pap	FLOX + Tmab	PD	1	4
82/female	IHC2+/FISH+	11	p.V777L	90	0.5	pap	SOX + Tmab	PD	2	12
64/male	IHC 3+	8	p.S310F	66	0.5	tub2	FP + Tmab	SD	10	17

NOTE: All patients were treated with fluoropyrimidine plus platinum with trastuzumab as the first-line treatment. + after the number means alive.

Abbreviations: CapeOX, capecitabine plus oxaliplatin; CN, copy number; FISH, fluorescence in-situ hybridization; FLOX, 5-FU/leucovorin plus oxaliplatin; FP, 5-FU plus cisplatin; HER2, human epidermal growth factor receptor 2; IHC, immunohistochemistry; OS, overall survival; pap, papillary adenocarcinoma; PD, progressive disease; PFS, progression-free survival; PR, partial response; SD, stable disease; SOX, S-1 plus oxaliplatin; Tmab, trastuzumab; tub2, moderately differentiated adenocarcinoma; VAF, variant allele frequency.

association of *ERBB2* CN with PFS was not observed in HER2-positive patients who were treated with chemotherapy without trastuzumab.[13] Taken together, *ERBB2* CN is a robust efficacy predictor of trastuzumab treatment in patients with HER2-positive AGC. Setting a common optimal cutoff value for *ERBB2* CN seemed to be difficult, because the trastuzumab effect continuously emerged as the *ERBB2* CN increased, and the NGS methods differed across studies. However, some reference values of *ERBB2* CN, such as the median, quartile, and ROC-derived optimal values, can be used to predict the efficacy of trastuzumab. In this study, it was 16 copies of *ERBB2*, and this should be validated in the future. As for poor PFS in patients with log class 7, it might be due to the small number of patients and gene coamplifications on the HER2 signaling pathway.

Gene coalterations in the RTK/RAS/PI3K pathway and cell cycle checkpoints were reported to contribute to the intrinsic resistance to trastuzumab.[9-12] Beside them, replacement of clones lacking *ERBB2* amplification and newly detected mutation of *ERBB2* were implicated in acquired resistance to trastuzumab.[12,25] Gene amplification of receptor/oncogene that activated the HER2 pathway was an independent negative predictor of PFS in this study; this finding is in agreement with that of previous studies.[9-12] The amplifications fortunately did not affect the OS; from this point of view, *ERBB2* CN is a more clinically meaningful biomarker than the *RTK*, *RAS,* and *PIK3CA* amplification. In addition, approximately 40% of patients who had log class 4 or more (*ERBB2* CN $>$ 16) showed a PFS of more than 24 months (Fig 3A), and *ERBB2* CN was a significant predictor for PFS, whereas IHC/ISH status was not in the multivariate analysis (Fig 4). These results suggested that *ERBB2* CN was more useful than the IHC/ISH status in predicting the PFS of patients who were selected by the standard HER2 IHC test combined with ISH.

ERBB2 mutations occur in $<$ 5% of patients with various types of carcinoma and 4.9%-7.7% in those with gastric cancer.[26,27] *ERBB2* mutation and amplification are mutually exclusive, and the co-occurrence is scarcely reported so far. Of note, co-occurrence was detected in 7% (5/74) of patients with HER2-positive AGC in this study, accounting for 1% of all patients with AGC if the HER2-positive rate was 15%. The mutation loci were distributed in the tyrosine kinase domain and furin-like cysteine-rich region. These were also the case in the cBioPortal database and all known as gain-of-function mutations.[12,19-24] This study suggested an inverse correlation between the VAF of comutant *ERBB2* and PFS/OS. Trastuzumab is considered ineffective in cells harboring high *ERBB2* VAF, and other drugs such as antibody-drug conjugates (trastuzumab deruxtecan)[28] and tyrosine kinase inhibitors (lapatinib, afatinib, neratinib, etc)[2,26] should be used instead of trastuzumab or in combination with trastuzumab in this case.

This study has some limitations. This was a retrospective study with a small sample size. Validation of the results and comparison of the study group with a control cohort consisting of patients with HER2-positive AGC who did not receive trastuzumab were not performed. Among the examined gene alterations, *BRAF* amplification was not included in the OCP. HER2-positive disease constitutes a relatively small fraction of AGC; hence, enrollment of patients in clinical studies is difficult. However, similar results using NGS obtained from multiple study groups have been piled up, including those reported in this study, and further collaborations are needed worldwide.

In conclusion, *ERBB2* CN and gene coamplification in the HER2 pathway were positive and negative predictors of PFS in trastuzumab-treated HER2-positive AGC patients. The orphan fraction of patients with a coalteration of *ERBB2* amplification and mutation was identified, and further investigation is warranted.

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All data relevant to the study are included in this article or uploaded in the Data Supplement. All raw data used in the current study are available from the corresponding author on reasonable request.

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