

Prognostic impact of KRAS mutant type and MET amplification in metastatic and recurrent gastric cancer patients treated with first-line S-1 plus cisplatin chemotherapy

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ABSTRACT

Receptor tyrosine kinase (RTK)-related genes, including HER2, EGFR, MET, FGFR2 and KRAS, are target molecules that are clinically beneficial in gastric cancer (GC). We investigated the correlation between RTK-related genes and the curative effect of first-line S-1 plus cisplatin (SP) combination chemotherapy in metastatic and recurrent GC. We enrolled 150 patients with histopathologically confirmed metastatic and recurrent GC treated with SP. KRAS mutation was detected using direct sequencing. DNA copy number was measured by real-time PCR. Formalin-fixed paraffin-embedded specimens were examined immunohistochemically for HER2, EGFR, FGFR2 and MET. Among 144 patients, KRAS mutation was detected in five (3.5%) at codon 12 and one (0.7%) at codon 13. FGFR2, EGFR, HER2, MET and KRAS gene amplification was suggested in 4.4%, 5.9%, 9%, 3.7% and 10.3% of patients, respectively. KRAS mutation, but not KRAS amplification, was associated with significantly shorter overall and progression-free survival. MET membranous overexpression was associated with a significantly higher tumor response. MET amplification was associated with significantly shorter overall survival. We show for the first time that KRAS mutation and MET amplification are promising predictive markers in metastatic and recurrent GC patients treated with SP. KRAS status may be a useful prognostic marker in patients treated with SP.

INTRODUCTION

5-fluorouracil (including oral fluoropyrimidines S1 or capecitabine) with a platinum analog (cisplatin or oxaliplatin) is the most widely accepted first-line chemotherapy regimen for metastatic gastric cancer (GC) in Japan and other countries. S1 plus cisplatin (SP) combination therapy was significantly superior to S1 alone as first-line treatment for metastatic and recurrent GC in the phase III SPIRITS trial [1]. Current chemotherapy has improved survival in GC patients, although the survival

rate remains low [2]. New molecular-targeting agents are urgently needed to achieve clinical benefits for patients with GC and improve their survival.

Deng *et al.* [3] have reported that five genes, HER2, EGFR, MET, FGFR2 and KRAS, which are associated with the receptor tyrosine kinase (RTK)/RAS pathway, amplify the DNA copy number in GC. New drugs have been developed that target the RTK/RAS pathway. Trastuzumab (Herceptin) is a monoclonal antibody that specifically targets HER2 by directly binding with its extracellular domain [4]. Based on the results of the ToGA

study, trastuzumab has become the first antimolecular targeting drug for patients with HER2-positive advanced GC [5]. The epidermal growth factor receptor (EGFR) pathway plays a core role in regulating tumor cell growth and survival, and is associated with poor prognosis in GC [6]. The EXPAND [7] and REAL3 [8] trials are two concurrent randomized phase 3 trials assessing the addition of anti-EGFR monoclonal antibodies for first-line treatment of upper gastrointestinal cancer. However, these studies suggest that addition of EGFR antibodies does not convey additional benefit for patients with advanced gastric and gastroesophageal junction cancer. Hepatocyte growth factor (HGF) and its receptor MET promote the proliferation, migration and survival of tumors, and are associated with poor prognosis in GC [9-12]. Rilotumumab, which is a fully human IgG2 monoclonal antibody against HGF, showed anti-tumor activity in a phase Ib and II study [13]. A phase II study [RILOMET-1 (NCT01697072)] has confirmed these results in patients with MET-positive gastric and esophagogastric junction cancer [14]. Fibroblast growth factor receptor (FGFR)2 is an RTK that regulates cell growth and development. *FGFR2* amplification is associated with tumor cell proliferation, survival of GC cell lines [15], and indicates poor prognosis in patients with GC [16].

Few studies have examined the effect of RTK/RAS-related genes on clinical outcomes in GC. The aim of this study was to investigate the correlation between RTK/RAS-related genes and the curative effect of SP combination chemotherapy in metastatic and recurrent GC.

RESULTS

Patients

We enrolled 150 patients with metastatic and recurrent GC treated with first-line SP combination chemotherapy. Their baseline characteristics are summarized in Table 1.

Detection of KRAS mutations using direct sequencing

KRAS mutation was detected in six (4.2%) of 144 patients: at codon 12 in five (3.5%) and codon 13 in one (0.7%). *KRAS* codon 12 mutation consisted of G12D and codon 13 mutation consisted of G13D.

Detection of gene amplification using PCR

FGFR2, *EGFR*, *HER2*, *MET* and *KRAS* gene amplification was suggested in 4.4%, 5.9%, 9%, 3.7%

and 10.3% of patients, respectively. In diffuse-type GC, *FGFR2*, *EGFR*, *HER2*, *MET* and *KRAS* gene amplification was suggested in 5.9%, 5.9%, 5.9%, 3.0% and 9.8% of patients, respectively. In intestinal-type GC, *FGFR2*, *EGFR*, *HER2*, *MET* and *KRAS* gene amplification was suggested in 0%, 5.7%, 11.4%, 8.6% and 8.6% of patients, respectively. The frequency of overlapped amplifications among *FGFR2*, *KRAS*, *EGFR*, *HER2* and *MET* was observed in eight cases. Three (2.1%) of 144 tumors exhibited high level amplification of one component, with low level amplification of another (high *HER2*/low *MET* amplification, high *HER2*/low *KRAS* amplification, and high *EGFR*/low *KRAS* amplification in one case each). Four (2.8%) of 144 tumors exhibited a similar level of amplification between them (*EGFR*/amplification in three

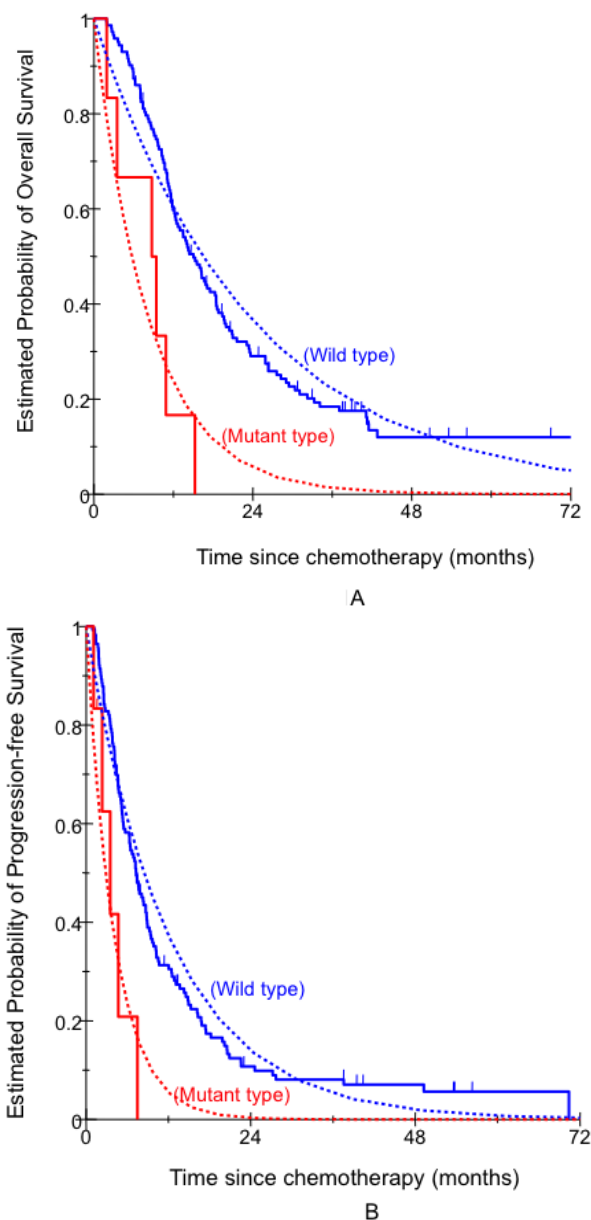


Figure 1: Comparison of clinical outcome by KRAS status. A. OS; B. PFS.

Table 1: Patient characteristics (n = 150)

		Number
Sex	Male/female	106/44
Age (y)	Median (range)	61 (16–78)
Tumor site ^a	U/M/L/other	47/61/34/8
ECOG PS	0/1/2	128/21/1
Stage	I/II/III/IV	1/6/12/131
Lauren classification ^b	intestinal/diffuse/mixed/unknown	37/100/12/1
Primary tumor resection	Yes/no	82/68
Liver metastasis	Yes/no	46/104
Lymph node metastasis	Yes/no	94/56
Peritoneal metastasis	Yes/no	70/80
Number of metastatic sites	≤ 2/> 2	127/23
Second-line chemotherapy	Yes/No	104/46

^aTumor site was classified based on the Japanese Classification of Gastric Carcinoma (3rd English edition): U, upper third; M, middle third; L, lower third.

^bFor Lauren classification, pap, tub, and por1 with type 1 or 2 were defined as intestinal type, and others as diffuse type.

Table 2: Correlation coefficient of pairwise between each gene copy number and protein expression

DNA copy number	IHC-score	correlation coefficient (95% CI)	p value
HER2	HER2	0.751 (0.667-0.8162)	<0.001
FGFR2	FGFR2-m	0.725 (0.634-0.786)	<0.001
EGFR	EGFR-m	0.642 (0.531-0.731)	<0.001
HER2	HER2	0.488 (0.348-0.607)	<0.001
FGFR2	FGFR2-c	0.23 (0.064-0.383)	0.007
MET	cMET-c	0.157 (-0.012-0.317)	0.069
MET	cMET-m	0.118 (-0.052-0.281)	0.172

FGFR2-m; FGFR2 membranous overexpression, EGFR-m; EGFR membranous overexpression, FGFR2-c; FGFR2 cytoplasmic overexpression, MET-c; MET cytoplasmic overexpression, MET-m; MET membranous overexpression.

(Supplementary Figure 1C, D). EGFR overexpression was observed in 25% of tumors and EGFR membranous-positive staining (EGFR positive) in 10%.

Correlation between gene copy and protein expression

Table 2 shows the correlation coefficient of pair-wise between each gene copy number and protein expression. There was close correlation between gene copy number and protein expression in EGFR and HER2 but not MET.

Relationship with overall survival (OS)

In univariate analysis, *KRAS* status indicated that *KRAS* mutant type was associated with significantly shorter OS. Patients with *KRAS* mutant type had significantly shorter median OS compared with those with *KRAS* wild type (Figure 1A, Table 3). In univariate analysis, *MET* amplification was associated with significantly shorter OS. Patients with *MET* amplification had significantly shorter median OS compared with those with *MET* non-amplification (Figure 2, Table 2).

Table 3: Association between RTK/RAS-related genes and clinical outcome

	N	OS			PFS		
		Median, months (95%CI)	HR (95%CI) ^a	HR (95%CI) ^b	Median, months (95%CI)	HR (95%CI) ^a	HR (95%CI) ^b
KRAS							
Wild type	144	14.9 (12.3–18.3)	1 (Reference)	1 (Reference)	7.3 (5.5–8.8)	1 (Reference)	1 (Reference)
Mutant type	6	8.8 (2.0–10.9)	10.97 (2.5–48.26)	5.06 (1.96–13.08)	3.1 (1.1–7.5)	8.66 (1.81–41.4)	5.93 (1.9–18.51)
<i>p</i> value			0.005	< 0.001		0.021	0.002
MET							
Non-amp	138	14.9 (12.3–17.5)	1 (Reference)	1 (Reference)	7.3 (5.7–8.8)	1 (Reference)	1 (Reference)
Amp	6	7.0 (5.9–10.5)	5.2 (1.40–19.27)	4.81 (1.53–15.12)	4.7 (0–20.5)	1.55 (0.54–4.51)	1.83(0.57–5.81)
<i>p</i> value			0.033	0.007		0.59	0.3

Non-amp, non-amplification; Amp, amplification.

P value was based on log-rank test for PFS and OS in the univariate analysis (^a) and Wald test for PFS and OS in the multivariable Cox regression model adjusting for age, Eastern Cooperative Oncology Group performance status, primary tumor site, number of metastatic sites, and liver involvement (^b).

In multivariate analysis, *KRAS* mutant type and *MET* amplification remained significantly associated with OS (Table 3). No significant associations were seen for the other factors (Supplementary Table 1).

Relationship with progression-free survival (PFS)

In univariate analysis, patients with *KRAS* mutant type had a significantly shorter median PFS compared with those with *KRAS* wild type (Figure 1B, Table 3). In multivariate analysis, *KRAS* mutant type remained significantly associated with PFS (Table 3). No significant associations were seen for the other factors (Supplementary Table 1).

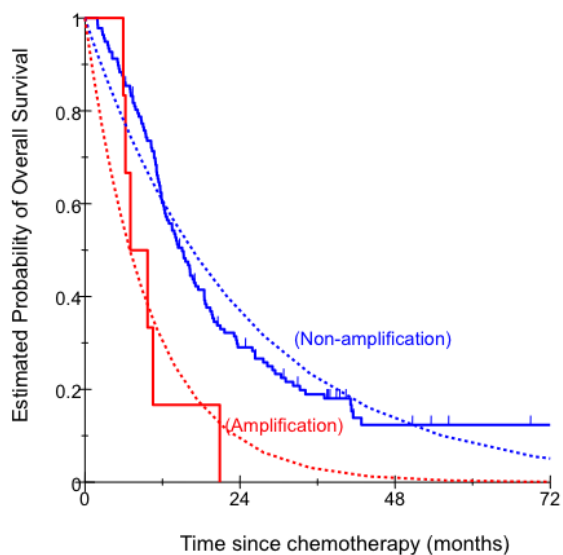


Figure 2: Comparison of OS by *MET* copy number.

DISCUSSION

To the best of our knowledge, we are the first to demonstrate that *KRAS* mutation in tumors independently predicts worse PFS and OS in patients with metastatic and recurrent GC treated with SP. Yang *et al.* [17] reported that *KRAS* mutation activates *NF-κB* to promote cisplatin-resistant cancer cells. No targeted therapies for *KRAS* mutant cancers are approved, because *KRAS* itself has proven difficult to target directly with small molecules. MEK inhibitors are the most effective agents in *KRAS* mutant cancer cell lines [18, 19]. It is reported that GC cell lines, which have *MEK1* mutation or *MEK1* and *KRAS* mutations, are hypersensitive to MEK1 inhibitors [20]. However, MEK inhibitors have failed to induce clinical responses in *KRAS* mutant advanced non-small cell lung cancer [21, 22]. That is because *KRAS* activates multiple critical effectors, such as the MEK–extracellular signal-regulated kinase, phosphoinositide 3-kinase (PI3K)–AKT, and nuclear factor-κB pathways. Preclinical studies have demonstrated that combining MEK inhibitors with PI3K inhibitors or BCL-XL may be an effective therapeutic strategy for *KRAS* mutant lung cancer [23, 24]. Monotherapy with MEK1 inhibitor and combination therapy with MEK1 and PI3K inhibitors or BCL-XL might be new effective strategies for *KRAS* mutant GC.

Our study indicated that *KRAS* mutation, but not *KRAS* amplification, was associated with PFS and OS in patients with metastatic and recurrent GC treated with SP without anti-EGFR antibody. *KRAS* mutations are known to be infrequent molecular events in esophagogastric adenocarcinoma [25]. The 4.1% frequency of *KRAS* mutation in our population is in keeping with the 3–10% reported in other studies [26, 27]. *KRAS* mutants were

not detected in five RTK-related gene amplifications in our population. Anti-EGFR antibody was expected to be effective against GC, because *KRAS* mutations are infrequent molecular events [28,29], and *KRAS*-mutated GC was not affected by capecitabine+cisplatin with or without cetuximab in our study. However, current clinical studies have reported that addition of EGFR antibodies to chemotherapy did not yield additional benefit for patients with advanced gastric and gastroesophageal junction cancer. An increased *EGFR* copy number is associated with a favorable response to anti-EGFR therapy in patients with *KRAS* wild-type colorectal cancer [30-32]. We found amplification of the *EGFR* gene in nine (6.3%) of the 144 samples analyzed. *EGFR* amplification overlapped *KRAS* amplification in five (56%) of nine patients in our study. Amplification of the *KRAS* gene was identified in 13 (9%) of the 144 samples analyzed. Mita *et al.* reported that *KRAS* amplification resulted in *KRAS* activation in the absence of mutation [33]. Mekenkamp *et al.* reported that copy number gains in wild-type *KRAS* were associated with poor PFS in patients with metastatic colorectal cancer treated with cetuximab [34]. High-frequency amplification overlapped *EGFR* and *KRAS* and may explain why addition of anti-EGFR antibodies to chemotherapy is not beneficial in patients with GC.

Our study showed that overlaps were observed in amplifications of *FGFR2*, *KRAS*, *EGFR*, *HER2* and *MET* in eight cases. Two of the eight cases had *FGFR2* amplification. One tumor exhibited a high level of *FGFR2* amplification with a low level of *EGFR* and *KRAS* amplification. The other tumor exhibited a similar level of amplification of *FGFR2* and *HER2*. Das *et al.* reported that correlation between *FGFR2* and *ERBB2* expression is exclusive using multicolor FISH [35]. One limitation of our study was that we did not confirm whether overlapped *FGFR2* and *HER2* amplification in the tumor was exclusive, because we did not perform multicolor FISH. We merged between *FGFR2* and *HER2* overexpression, because there was close correlation between *FGFR2* copy number and membranous expression, such as *HER2*. Each positive tumor cells between *FGFR2*-overexpression and *HER2*-overexpression were mutually exclusive (Supplementary Figure 2).

Our study has the limitation because the real-time PCR in this study cannot be said that the heterogeneity of RTK-amplification in an equivalence cell. We need to perform highly sensitive Real-time DNA Clamp PCR on extracted genomic DNA in LCM or multicolor FISH.

FISH has been widely used for detecting DNA copy number. However, small samples are limited for measurement of multiple genes using FISH. We used real-time PCR to detect DNA copy number. We identified 5 as the threshold copy number after considering that identified by FISH analysis, ≥ 4 , with 1 as the margin value. Frequency of *FGFR2* amplification at this threshold was similar to that in other studies. We performed dual color

in situ hybridization (DISH) in some samples to confirm whether the results of PCR were similar to those of DISH. DNA copy number analysis by PCR yielded results that were highly consistent with DISH (Supplementary Table 2). The different results for PCR and DISH may have been due to a genome domain counting the copy number of each other. *HER2* positivity (immunohistochemistry score 3+ and/or DNA amplification) was 18%, which was identical to previous studies that reported that *HER2* positivity (immunohistochemistry score 3+ and/or FISH-positive) in Japanese patients with GC was 10–20%.

We searched the frequency of *HER2* mutations in GC using the Cosmic database (Sanger Institute, <http://cancer.sanger.ac.uk/cosmic>) on November 12, 2015, and the frequency was 2.83% (25 of 884 cases). This was similar to the frequency of *HER2* mutations in colon cancer. In addition, the frequency became lower when we narrowed it down to the kinase domain of *HER2*. Therefore, it was difficult to do the analysis with a sufficient number of cases because there was a lower frequency of *HER2* than *KRAS* (codon12, 13) mutations.

Patients with *MET* amplification had significantly worse survival in our study. Some reports show that *MET* overexpression and higher *MET* copy number are associated with poor prognosis in patients with GC [36,37]. Fuse *et al.* reported that patients with *MET* overexpression had significantly worse OS than those without overexpression [38]. However, in view of the EXPAND study [7] and recent data on *MET* inhibitors presented at the ASCO 2015 Meeting, these results should be toned down.

In conclusion, our data show for the first time that *KRAS* mutation and *MET* amplification are promising prognostic markers in patients with metastatic and recurrent GC. Furthermore, *KRAS* mutation may be useful as a predictive marker in patients treated with SP combination chemotherapy. As this was a retrospective analysis with limited sample size, larger prospective studies are needed to validate our findings.

MATERIALS AND METHODS

Patients and study design

We enrolled 150 patients who had a histologically confirmed diagnosis of primary gastric adenocarcinoma and were treated with first-line SP from the Cancer Institute Hospital of the Japanese Foundation for Cancer Research (Tokyo, Japan).

Concurrent chemotherapy consisted of infusion of cisplatin (60 mg/m²) on days 1 and 8 plus oral S-1 (80 mg/m²/day) on days 1–21. This regimen was given unless there was evidence of progression, intolerable toxicity, or early withdrawal. Response was measured by computed tomography every 8 weeks, according to the Response

Evaluation Criteria in Solid Tumors (RECIST) 1.0. This study was approved by the Central Ethics Committee of the Japanese Foundation for Cancer Research and the Institutional Review Boards of the Cancer Institute Hospital, and all patients gave signed informed consent for the analysis of molecular correlates.

Detection of KRAS mutation

For *KRAS* mutation detection in formalin-fixed paraffin-embedded (FFPE) samples, we used a PCR followed by direct sequencing. Genomic DNA was extracted from FFPE samples using the QIAamp DNA FFPE Tissue Kit (Qiagen, Tokyo, Japan). RNase A (Qiagen) was used to digest single-strand RNA for the isolation of RNA-free DNA. DNA was quantified first by UV absorption (NanoDrop 2000 UV-Vis Spectrophotometer; Thermo Fisher Scientific, Kanagawa, Japan). The amplification products were analyzed in an ABI 3130xl Genetic Analyzer (Life Technologies Japan; Applied Biosystems, Tokyo, Japan).

Detection of gene amplification using real-time PCR

Starting DNA material was the same as that for *KRAS* mutation detection. Real-time PCR was performed using the TaqMan probes in an ABI 7900HT Real-Time PCR instrument (ABI, Foster City, CA, USA). Commercially available FAM-dye-labeled probes were designed to amplify *HER2*, *EGFR*, *FGFR2*, *MET* and *KRAS*. VIC-dye-labeled ribonuclease P RNA component H1 (*RPPH1*), telomerase reverse transcriptase (*TERT*) and human genomic DNA (Promega, Madison, WI, USA) were used as the endogenous control probes and control sample because *RPPH1* and *TERT* have exactly two copies per diploid human genome in healthy tissue, which are located on chromosome 14q11.2 and 5p15.33, respectively. The TaqMan copy number assay contained 1 μ l *HER2*, *EGFR*, *FGFR2*, *MET* and *KRAS* probe (20 \times , FAM labeled), 1 μ l *RPPH1* or *TERT* probe mix (20 \times , VIC labeled), 10 μ l TaqMan Universal PCR Master Mix (2 \times), 1.5 μ l genomic DNA and 6.5 μ l water. PCR cycling conditions were 95 $^{\circ}$ C for 10 min, followed by two-step cycling: 40 cycles of 95 $^{\circ}$ C for 15 s and 60 $^{\circ}$ C for 1 min. A manual threshold cycle threshold (Ct) of 0.2 and an automatic baseline were used to detect the template quantity of target genes and *RPPH1* or *TERT* using ABI sequence detection software version 2.4. The target probes and internal control were loaded in the same well and each reaction was performed in quadruplicate. CopyCaller version 2.0 (ABI) was used to calculate the copy number of each probe based on the real-time PCR data. Copy number of each target gene was calculated as the average of those based on two reference genes. The threshold of each gene amplification was

identified as ≥ 5 .

Immunohistochemistry

FFPE blocks were cut into 4- μ m serial sections. Immunohistochemical staining for *HER2*, *EGFR*, *FGFR2* and *MET* was performed with Histofine Histostainer 36A (Nichirei Biosciences, Tokyo, Japan) using primary antibodies against *HER2* (I-View Pathway anti-*HER2*/neu 4B5; Ventana), *EGFR* (Confirm anti-*EGFR* 3C6; Ventana), *FGFR2* (K-sam rabbit IgG; IBL, Gunma, Japan; 1: 20) and *MET* (anti-c-Met rabbit polyclonal antibody; Invitrogen, Carlsbad, CA, USA; 1: 200). The sections were deparaffinized in xylene and dehydrated with graded ethanol. After being washed in tap water, the sections were placed in retrieval buffer (Cell Conditioning 1; Ventana). For antigen retrieval, the sections were heated in a water bath at 90 $^{\circ}$ C for 30 min for *HER2* and *EGFR* staining, and heated in a Pascal Pressure Chamber (Dako, Tokyo, Japan) at 125 $^{\circ}$ C for 30 s and 90 $^{\circ}$ C for 10 s for *FGFR2* and *MET* staining. After washing with PBS, the sections were immersed in peroxidase blocking solution (Dako) to block endogenous peroxidase activity at room temperature for 10 min. Individual section slides were then incubated with primary antibodies at room temperature for 1 h. After further washing with PBS, the sections were incubated with the following secondary antibodies: NIEW Biotinylated Ig Secondary Antibody (Ventana) for *HER2* and *EGFR*, and N-Histofine Simple Stain MAX PO (R) for *FGFR2* and *MET*. After incubating with streptavidin horseradish peroxidase (NIEW SA-HRP; Ventana) at room temperature for 30 min, except for *FGFR2* and *MET*, the sections were washed with PBS, and were subsequently incubated with 3,3'-diaminobenzidine tetrahydrochloride (Dako) at room temperature for 3 min. The sections were then counterstained with Dako REAL Hematoxylin.

Immunohistochemical analysis

Immunostaining was assessed and scored subjectively by two pathologists blinded to clinical characteristics and outcomes. For *HER2*, *EGFR*, *MET* and *FGFR2*, immunostaining of the tumor cell membrane was scored using a four-grade scale (0, 1+, 2+ and 3+) and area percentage of positive cells (0–100%) in each grade according to scoring in GC. Immunostaining of the tumor cell cytoplasm was scored using a four-grade scale (0, 1+, 2+ and 3+) and area percentage of positive cells in each grade. *MET* and *FGFR2* reactivity was scored as 0 if there was no cytoplasmic reactivity within the tumor, or as 1+, 2+ or 3+ depending on the intensity above the background level. For *HER2*, *EGFR*, *FGFR2* and *MET*, we calculated H score by multiplying the staining intensity [0 (grade 0), 1 (grade 1+), 2 (grade 2+), 3 (grade 3+)] with the percentage of positive cells at each grade, and all values

were added to obtain the final immunohistochemistry score, ranging from 0 to 300. HER2, EGFR and MET were scored using a four-grade scale (0, 1+, 2+ and 3+) according to Hofmann's criteria [38]. FGFR2 was scored using a four-grade scale (grade 0, staining reactivity in <50% of cancer cells; grade 1+, cytoplasm and/or nuclear reactivity with an intensity score of 1+ in >50% of cancer cells, grade 2+, cytoplasm and/or nuclear reactivity with an intensity score of 2+ in >50% of cancer cells; grade 3+, cytoplasm and/or nuclear reactivity with an intensity score of 3+ in >50% of cancer cells) according to the criteria by Nagatsuma *et al.* [39]. The other evaluation of HER2, FGFR2 and MET was performed according to the defined negativity (grade 0 and 1+) and positivity (grade 2+ and 3+). EGFR was evaluated according to the defined negativity (grade 0, 1+ and 2+) and positivity (grade 3+).

Statistical analysis

The primary endpoint of this retrospective study was OS, and the secondary endpoint was PFS. OS was defined as the period from starting therapy to the date of death or censoring of the last contact if alive. PFS was defined as the interval from the first day of starting first-line SP therapy to the first day of documented disease progression or death. If progression or death was not observed, PFS was censored on the day of the last computed tomography scan. All factors were tested for deviation from Hardy–Weinberg equilibrium using the χ^2 test with 1 degree of freedom.

The associations between all factors and OS and PFS were examined in univariate analysis using Kaplan–Meier estimation and Cox–Mantel tests, and in multivariate analysis using a Cox proportional hazards regression model. The following baseline characteristics were adjusted in the multivariate analysis: sex, age, Eastern Cooperative Oncology Group performance status, Lauren classification, tumor location, primary tumor resection, lung metastases, liver metastases, peritoneal metastases, lymph node metastases, and ≥ 2 metastases. The relationships between all factors and response were assessed in the univariate analysis using χ^2 tests and in the multivariate analysis using a logistic regression model controlling for potential predictive variables. *P* values for all factors were adjusted for multiple testing using a modified test of Conneely and Boehnke that was applied for the correlated tests owing to linkage disequilibrium and the different modes of inheritance considered.

R version 3.2.0 software (www.r-project.org) was used to conduct all the analyses by Sugimoto Data Analysis Service (Aichi, Japan). All tests were two-sided at a significance level of 0.05.

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CONFLICTS OF INTEREST

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Ethical Standards

The protocol used for this study was approved by the ethics committee of the institutional review board of the hospital. Patients provided written informed consent for the collection of specimens, and the local ethics committee approved the study protocol.

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