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ORIGINAL ARTICLE

Three-gene predictor of clinical outcome for gastric cancer patients treated with chemotherapy

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To identify transcriptional profiles predictive of the clinical benefit of cisplatin and fluorouracil (CF) chemotherapy to gastric cancer patients, endoscopic biopsy samples from 96 CF-treated metastatic gastric cancer patients were prospectively collected before therapy and analyzed using high-throughput transcriptional profiling and array comparative genomic hybridization. Transcriptional profiling identified 917 genes that are correlated with poor patient survival after CF at $P<0.05$ (poor prognosis signature), in which protein synthesis and DNA replication/recombination/repair functional categories are enriched. A survival risk predictor was then constructed using genes, which are included in the *poor prognosis signature* and are contained within identified genomic amplicons. The combined expression of three genes—*MYC*, *EGFR* and *FGFR2*—was an independent predictor for overall survival of 27 CF-treated patients in the validation set (adjusted $P=0.017$), and also for survival of 40 chemotherapy-treated gastric cancer patients in a published data set (adjusted $P=0.026$). Thus, combined expression of *MYC*, *EGFR* and *FGFR2* is predictive of poor survival in CF-treated metastatic gastric cancer patients.
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Keywords: gastric; cancer; chemotherapy; gene; expression

Introduction

Although the emerging area of targeted anticancer agents holds great promise, cytotoxic chemotherapy remains the primary treatment option for many cancer patients. Identifying patients who likely will or will not benefit from cytotoxic chemotherapy through the use of biomarkers could greatly improve clinical management by better defining appropriate treatment options for patients. None of the molecules experimentally identified to cause chemotherapy resistance *in vitro* was sufficiently validated in primary tumors and thus clinically applicable,[1] underscoring the importance of well-designed, clinical study to identify clinically relevant mechanisms for chemotherapy resistance. In fact, however, such predictors derived to date from high-throughput transcriptional profiling of primary tumors, especially gastrointestinal tract cancers, have not shown satisfactory performance.[2–5] It may be primarily owing to the high rate of false-positive discovery in high-throughput data, in addition to the high degree of genetic variation of individual tumor compared with limited number of samples available for the study.

To provide insight into clinically relevant mechanisms for chemotherapy resistance in gastric cancer, we prospectively collected and analyzed 123 endoscopic biopsy samples before cisplatin and fluorouracil (CF) chemotherapy from patients with extended follow-up, using high-throughput transcriptional profiling and comparative genomic hybridization (CGH) analyses. We could identify functional categories enriched in genes correlated with patient outcome, and develop a genomic predictor that was validated in two independent data sets.

Materials and methods

Patients

Sample collection, treatment and follow-up were performed according a protocol approved by the Institutional Review Board of the National Cancer Center Hospital in Goyang, Korea (NCCNHS01-003). All patients signed an Institutional Review Board-approved informed consent form. Eligibility for enrollment into the study included the following parameters: (1) age $\geqslant$18 years; (2) histologically confirmed gastric adenocarcinoma; (3) clinically documented distant metastasis; (4) no previous or concomitant malignancies other than the gastric cancer; (5) no previous history of chemotherapy, either adjuvant or palliative; and (6) adequate function of all major organs. Patients who were lost to follow-up before completing six cycles of chemotherapy, except for documented progressive disease, were excluded from this study.

Sample size calculation

Overall survival was the primary clinical end point of this study. As a minimum of 91 events were estimated to be required for the number of training set samples[6] at $\alpha = 0.001$, $\beta = 0.05$, τ (standard deviation of log intensity) $= 0.75$ and δ (hazard ratio (HR) associated with one-unit change of log intensity) $= 2$, we used the 96 samples collected until January 2005 as the training set for development of the predictor.

Ninety-six eligible patients who were treated with CF by one medical oncologist (HK) from August 2001 to January 2005 were used for the expression profiling training set. A second group of 27 eligible patients was used as the array validation cohort. Twenty-two patients in the validation cohort were treated with CF, and five patients were treated with cisplatin plus oral capecitabine (a fluorouracil pro-drug considered equivalent to fluorouracil; CX),[7] by another group of medical oncologists in the same institution between February 2005 and April 2006. Tissue procurement and processing were the same for the training and validation samples.

Treatment

Patients continued therapy indefinitely until they experienced unacceptable toxicities or progressive disease was documented. CF-treated patients received cisplatin 60 mg m^{-2} intravenously on day 1 and fluorouracil 1000 mg m^{-2} intravenously on days 1–5 of a 3-week schedule. The treatment schedule for fluorouracil could be shortened at the discretion of the oncologist to 3 instead of 5 days for elderly patients ($\geqslant$70 years) or patients with poor performance status (Eastern Cooperative Oncology Group performance status $\geqslant$2). Chemotherapy doses were reduced according to toxicities and the patient's performance status. Specific dose modification schemes for the subsequent cycle were left to the discretion of treating oncologist. Five patients (18.5%) in the validation group received oral capecitabine (Xeloda; Roche, Basel, Switzerland; 1250 mg m^{-2} twice a day for 2 weeks) instead of intravenous infusion of fluorouracil. Time to progression was measured from the initiation of chemotherapy to the progressive disease. In patients without any measurable lesions, time to progression was measured to the time when a change in therapy was required because unmeasurable lesions (such as ascites) unequivocally progressed.

Gene expression and CGH microarray analyses

Tissue samples were collected and processed for RNA and DNA extraction as described previously,[8] only if samples contained at least 50% tumor cells. Affymetrix (Santa Clara, CA, USA) HG-U133A gene expression microarray data were analyzed with survival analysis algorithms of BRB-ArrayTools (version 3.6, National Cancer Institute, http://linus.nci.nih.gov/BRB-ArrayTools.html).[9] The survival risk groups were constructed using a predictive index based on the supervised principal component method of Bair and Tibshirani.[10] A three-gene predictive index percentile was generated based on the weighted average of the log intensities of the three genes (*FGFR2* (211401_s_at), *EGFR* (210984_x_at) and *c-MYC* (202431_s_at)), using a proportional hazards regression on the first two principal components of the log intensities of those three genes, in which a high value of the predictive index corresponds to a high risk of death. If the predictive index of a sample in the validation set corresponded to the median predictive index of the training set, the sample was assigned a 50% predictive index. We specified the number of risk groups as 2 (high and low) and the predictive index percentile for defining the two risk groups as 67%, using a 67.1% rate of clinical benefit (partial response and stable disease) and 32.9% rate of progressive disease in the training set. We also performed Cox regression analyses using this three-gene predictive index percentile as a continuous variable, in which HRs for survival were calculated according to each percentile increase in three-gene predictive index percentile (from 0 to 100%). Array CGH data were generated using Agilent (Santa Clara, CA, USA) 4 $\times$ 44k HD-CGH Microarrays and analyzed using CGH Analytics software (version 3.5.14). Aberrations with average tumor/normal $\log_2$ ratio >2.0 were defined as amplifications. Experimental details are provided in Supplementary Materials and Methods.

Analyses of published DNA microarray data

The entire set of published Affymetrix U133 Plus 2.0 DNA microarray data[4] ($n = 40$) was combined with our training set data ($n = 96$), using common probe set IDs. MAS5 data of

the combined data set were $\log_2$ transformed, normalized using the median over the entire arrays and analyzed for survival risk prediction using BRB-ArrayTools 3.6, as described above.

Publicly accessible microarray data for surgically treated gastric cancer patients generated by the Stanford Functional Genomics Facility were obtained from the NCBI GEO database (GSE4007) and included about 30 300 genes common to these data sets. The microarray data were generated and normalized as described in Leung *et al.*[11] Batch effects in gene expression were removed with probe-wise mean centering and missing data were imputed with the nearest-neighbor averaging method.[12] The array cDNA clones were annotated using SOURCE (Stanford Microarray Database) and the Entrez GeneID was used as the mapping identifier for the Affymetrix HG-U133A array. A combined data set of our training set data ($n=96$) and GSE4007 data ($n=88$) was analyzed for survival risk prediction using BRB-ArrayTools 3.6 as described above.

Results

Genes correlated with poor survival after CF therapy

As primary gastric cancer lesions cannot be reliably measured by diagnostic imaging, patient survival, not radiographic response, was used as the primary clinical covariate to which gene expression was correlated to identify a predictor of response to CF therapy. To define a gene expression signature that correlates with overall survival, we used expression array data of 96 pretreatment biopsy samples as the training set to develop a predictor (Supplementary Table 1). Ninety-five out of 96 patients (99%) in the training set cohort died with follow-up for one survivor at 39.4 months. None of the clinicopathological or treatment factors listed in Table 1, including second-line chemotherapy, were significantly correlated with survival time of the patients in the training set.

To identify a transcriptional profile related to clinical benefit from CF therapy, the survival times of patients in the array training set were correlated with the mRNA expression levels measured by microarray. One thousand five hundred and sixty-five genes were significantly correlated with the overall survival of the 96 patients (P-value <0.05). Among them, 917 genes had an HR higher than 1 (poor prognosis signature) and 648 genes had an HR lower than 1 (good prognosis signature). We performed gene ontology analyses on this 'poor prognosis signature' using Ingenuity Pathway Analysis (www.ingenuity.com). The role of *BRCA1* in DNA damage response (*BRCA2, E2F5, FANCE, MSH2, NBN, PLK1, RFC, SMARCA4, SLC19A1*), nucleotide excision repair (*ERCC2, POLR2C, POLOR2J, RAD23A, RAD23B*) and estrogen receptor signaling were highly represented canonical pathways. Many of these *poor prognosis signature* genes belonging to these three pathways are previously linked to *in vitro* cisplatin resistance.[13–15] Overexpression of *ERCC2* ($P=0.007$ in our data) is associated with cisplatin resistance in lung cancer cell lines.[13] Silencing of hHR23A ($P=0.022$ in our data) decreases the nuclear DRP1 level and cisplatin resistance in lung adenocarcinoma cells.[14] Disruption of the Fanconi anemia–BRCA pathway is reported in cisplatin-sensitive ovarian tumors.[15] Thus, this gene ontology analysis supports the clinical relevance of these DNA repair

Table 1 Clinicopathological characteristics of patients

	Training set (n=96)	*Validation set (n=27)*
Baseline clinicopathological characteristic		
Age, no. (%)		
<70 years	90 (93.8%)	25 (92.6%)
⩾70 years	6 (6.2%)	2 (7.4%)
Sex, no. (%)		
Male	73 (76.0%)	23 (85.2%)
Female	23 (24.0%)	4 (14.8%)
PS, no. (%)		
ECOG PS 0 or 1	91 (94.8%)	25 (92.6%)
ECOG PS 2 or 3	5 (5.2%)	2 (7.4%)
Histological type, no. (%)		
Lauren's intestinal	40 (41.7%)	9 (33.3%)
Lauren's diffuse	56 (58.3%)	18 (66.6%)
Location of primary lesion, no. (%)		
Upper 1/3	14 (14.6%)	2 (7.4%)
Middle 1/3	28 (29.2%)	10 (37.0%)
Lower 1/3	49 (51.0%)	15 (55.6%)
Entire stomach	5 (5.2%)	0
Distant metastasis, no. (%)	96 (100%)	27 (100%)
Tumor cell percentage in sample (%)		
Median	60	70
Interquartile range	50–70	55–80
Treatment and outcome		
Chemotherapy regimen, no. (%)		
Cisplatin/fluorouracil	96 (100%)	22 (81.5%)
Cisplatin/capecitabine	0 (0%)	5 (18.5%)
Relative dose intensity (%)		
Median	79	81
Interquartile range	73–88	72–87
Number of chemotherapy cycles		
Median	4	7
Interquartile range	3–9	5–13
Response (WHO criteria), no. (%)		
PR	38 (44.7%)	12 (48.0%)
SD	19 (22.4%)	9 (36.0%)
PD	28 (32.9%)	4 (16.0%)
Non-measurable disease	11	2
Second-line chemotherapy, no. (%)	69 (71.9%)	19 (70.4%)
Median follow-up for survivors (months)	39.4	30.4
Overall survival (months)		
Median	8.1	12.6
Interquartile range	5.6–15.9	7.4–30.4
Time to progression (months)		
Median	3.9	6.3
Interquartile range	2.2–8.3	3.9–14.6

Abbreviations: ECOG, Eastern Cooperative Oncology Group; PD, progressive disease; PR, partial response; PS, performance status; SD, stable disease; WHO, World Health Organization.

canonical pathways, which were shown to be associated with *in vitro* cisplatin resistance.

Ingenuity Pathway Analysis functional categories enriched in poor prognosis signature were: protein synthesis, DNA replication/recombination/repair and cancer (Supplementary Table 2). The protein synthesis category includes ribosomal subunit mRNAs (*RPL13, RPL18, RPL24, RPL30, RPL38, RPL5, RPL7, RPL7A, RPL8, RPS2, RPS5*) and eukaryotic translation initiation factors (*EIF1, EIF2B2, EIF2B4, EIF2S1, EIF3B, EIF3C, EIF3D, EIF3E, EIF3F, EIF3H, EIF3I, EIF4A1, EIF4A3, EIF4B, EIF4EBP1, EIF5, EIF5B*). This result suggests that the most prominent feature of poor prognosis signature is increased protein synthesis, presumably resulting from activation of oncogenes, such as *EGFR, FGFR2* and *MYC* (Supplementary Table 2). MYC-induced transcriptional activation of protein synthesis-related genes is previously shown by a microarray report that the majority of genes responsive to MYC overexpression are involved in macromolecular synthesis, protein turnover and metabolism, including 30 ribosomal protein genes.[16]

Infinitesimal perturbation analysis canonical pathways enriched in 648 genes in good prognosis signature were antigen presentation pathway, B-cell development and interleukin-15 production. Enriched functional categories were gastrointestinal disease, inflammatory disease and genetic disorder.

Development of the three-gene predictor

Although such a gene ontology analysis of the whole signature provides some insight into clinically relevant mechanisms for chemotherapy resistance, this large number of genes is not readily amenable to clinical application. Therefore, we wished to narrow down 917 genes in the whole poor prognosis signature to the smaller number of genes, which may have driven the expression of majority of genes in the signature. Focusing on such 'driver gene' candidates would also minimize the chance of including false-positive discovery in a genomic predictor. For this purpose, a second tier of genomic analysis was performed to identify genes that could be functionally important in gastric cancer cells.

Genomic DNA from samples available from the training set patients was analyzed by array CGH to identify gene amplifications. Age, sex and overall survival were similar between the 30 patients (31.3%) whose samples were analyzed by array CGH and the other patients in the training set. Using very conservative criteria (average tumor/normal $\log_2$ ratio >2.0 for $\geqslant 5$ consecutive CGH probes), nine amplicons were identified in 11 patients (Table 2). We identified genes found in both the 1565 gene expression signature whose transcriptional levels correlated with poor survival of 96 training set patients (*P*-value <0.05) and that are also located within the nine amplicons identified by the array CGH. Three genes—*MYC* (8q24.13–24.21), *EGFR* (7p11.2) and *FGFR2* (10q26)—were identified in the amplicons (Table 2) whose expression array signal values significantly correlated with the survival time of the 96 patients in the training set (Figure 1). Patients with *EGFR* and *FGFR2* amplifications had higher expression levels of each gene (8.4 and 10.2 ± 0.8 (mean $\pm$ s.d.), for *EGFR* and *FGFR2*, respectively) than tested patients without the amplification of these genes (5.9 ± 1.0 and 5.2 ± 1.1, for *EGFR* and *FGFR2*, respectively). One of the two patients with *MYC* amplification had higher expression than patients without amplification (10.9 vs 9.5 ± 0.9).

The mRNA expression array signal values of these three genes were correlated with the short survival time with *P*-values of 0.0154, 0.0096 and 0.0057, for *MYC, EGFR* and *FGFR2*, respectively. The expression patterns of these three

Table 2 Amplicons identified using array CGH[a]

Cytoband	*Start*	*End*	*Target gene*	*No. of patients*
3q27.1	185 763 900	185 763 959	*EPHB3*	1
5q33.1	149 481 646	149 514 673	*PDGFRB*	1
7p11.2	54 746 103	55 363 004	*EGFR*	1
8q24.13–24.21	126 357 675	128 822 455	*MYC*	2
9p13.3	33 745 689	33 961 753	*PRSS3, UBE2R2, UBAP2*	1
10q26	123 264 724	13 123 458 467	*FGFR2*	2
17q12	35 046 052	35 282 145	*ERBB2*	2
17q21.2	36 110 139	36 230 022	*KRT24, KRT25A, KRT25C, KRT25D, KRT10*	2
17q21.2	36 569 493	36 888 515	*KRTAP4-4, KRTAP4-10, KRTAP9-9, KRTAP9-4, KRTAP17-1, KRTHA3A, KRTHA3B, KRTHA4, KRTHA1, KRTHA7, KRTHA8, KRTHA2, KRTHA5*	1

Abbreviation: CGH, comparative genomic hybridization.
[a]Defined by aberrations with average tumor/normal $\log_2$ ratio >2.0 for $\geqslant 5$ consecutive probes.

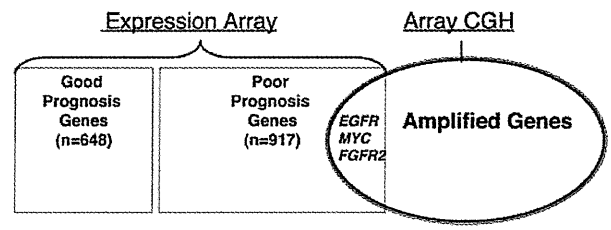

Figure 1 Three genes—*EGFR, FGFR2* and *MYC*—overlap between genes whose array expression levels correlated with survival times (96 training set patients, $P<0.05$) and gene copy number changes determined by array comparative genomic hybridization (CGH) (tumor/normal $\log_2$ ratio >2 for $\geqslant 5$ consecutive probes).

genes along with the cumulative survival data for all patients are depicted in the heatmap in Figure 2. None of the three genes had significantly different expression levels between those patients who received second-line chemotherapy and those who did not. Quantitative real-time RT-PCR and immunohistochemical staining for the three genes validated the array expression data (Supplementary Figures 1 and 2).

A three-gene predictive index percentile was then calculated for each of the 27 patients in the validation cohort, based on the weighted average of the log intensities of these three genes for each sample (designated as the three-gene predictor). Patterns of *MYC*, *EGFR* and *FGFR2* expression in these 27 patients, together with the predictive index, are graphically displayed in Figure 2. As a continuous variable, the three-gene predictive index percentile is an independent predictor for poor survival in the validation set by Cox regression analyses, after considering age, performance status, histological type and second-line chemotherapy (adjusted $P=0.017$) (Table 3). Patients predicted to have poor survival after CF using a predictive index percentile $\geqslant 67\%$ had a significantly shorter median survival than patients with a predictive index percentile $<67\%$ (7.4 months for the high-risk group vs 16.8 months for the

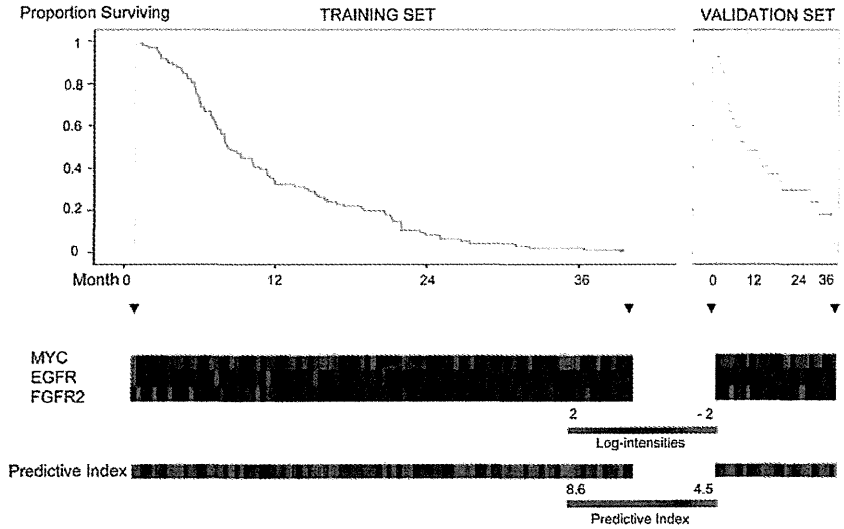

Figure 2 Affymetrix array expression levels of *MYC*, *EGFR* and *FGFR2* in 96 training set samples (left) and 27 validation set samples (right), shown with Kaplan–Meier plots for overall survival. Samples are ordered by the increasing survival period of patient from left to right, for the training and validation sets, respectively. A three-gene predictive index for each patient based on the three-gene predictor is indicated below.

Table 3 Cox regression analyses of the three-gene predictive index percentile, as a continuous variable, for 27 patients in the validation set

	Overall survival		*Time to progression*	
	P-*value*	*HR (95% CI)*	P-*value*	*HR (95% CI)*
Univariate				
Three-gene predictive index percentile[a]	0.050	1.015[b] (1.000–1.030)	0.026	1.017 (1.002–1.031)
Multivariate				
Three-gene predictive index percentile	0.017	1.023 (1.004–1.042)	0.014	1.023 (1.005–1.043)
Age $\geqslant$70 years[c]	0.027	7.614 (1.257–46.130)	0.144	3.605 (0.646–20.112)
Poor performance status (ECOG PS 2 or 3)	0.346	2.130 (0.442–10.258)	0.074	4.829 (0.861–27.086)
Second-line chemotherapy	0.041	4.231 (1.064–16.831)	0.011	5.992 (1.502–23.902)
Diffuse histological type	0.773	1.164 (0.415–3.263)	0.280	1.774 (0.626–5.025)

Abbreviations: CI, confidence interval; ECOG PS, Eastern Cooperative Oncology Group performance status; HR, hazard ratio.
[a]Computed based on weighted average of log intensities of the three genes (*EGFR*, *FGFR2* and *MYC*) obtained using a proportional hazards regression on the first two principal components of the log signal intensities of those three genes.
[b]HR for each percentile increase in three-gene predictive index percentile. For example, a predictive index percentile of 100 (the highest predictive index) is associated with an HR of 4.4 ($=1.015^{100}$), compared with a predictive index percentile of 0 (the lowest predictive index). The median predictive index (50%) is associated with HRs of 2.1 ($=1.015^{50}$), compared with the lowest predictive index.
[c]For patients aged $\geqslant$70 years, the treatment schedule for fluorouracil could be shortened at the discretion of the oncologist to 3 instead of 5 days.

low-risk cohort; $P=0.047$) (Figure 3a). As a class, the high-risk group predicted by the three-gene predictor (patient group with a predictive index percentile $\geqslant 67\%$) was associated with an adjusted HR of 3.1 (95% CI, 1.2–8.4; $P=0.022$). In addition, the three-gene predictive index percentile is also an independent predictor for the time to progression, which is a more specific indicator of the clinical responsiveness to systemic therapy than overall survival[17] (adjusted $P=0.014$) (Table 3). We therefore show that, independent of old age ($\geqslant 70$ years), poor performance status (Eastern Cooperative Oncology Group performance status $\geqslant 2$) and second-line chemotherapy, the three-gene predictive index is predictive of the benefit from CF to metastatic gastric cancer patients. An adjusted HR for time to progression according to each percentile increase in three-gene predictive index percentile was 1.023 (95% CI, 1.005–1.043) (that is, 100, 75 and 50% predictive indices are associated with an HR of 9.7 ($=1.023^{100}$), 5.5 ($=1.023^{75}$) and 3.1 ($=1.023^{50}$), respectively, compared with a 0% predictive index).

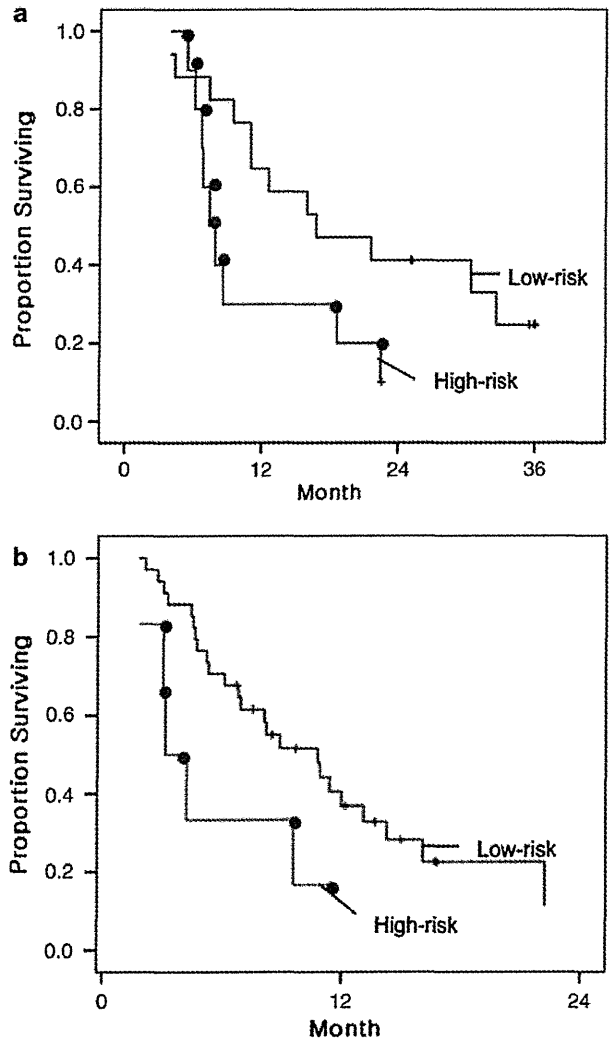

Figure 3 (**a**) Kaplan–Meier survival curves for the two risk groups of the validation cohort predicted by three-gene predictor. Patients at a high risk (predictive index percentile $\geqslant 67\%$; $n=10$) had significantly shorter median survival than patients at a low risk ($n=17$) (7.4 vs 16.8 months; log rank $P=0.047$). Green and blue lines represent overall survival curves for the predicted high- and low-risk groups, respectively. (**b**) Kaplan–Meier survival curves for the two risk groups of the published microarray data set from 40 metastatic gastric cancer patients treated with either fluorouracil-based regimens or cisplatin/irinotecan combination chemotherapy regimen. Patients at a high risk (predictive index percentile $\geqslant 67\%$; $n=6$) had shorter median survival than patients at a low risk ($n=34$), at a borderline significance (3.1 vs 10.8 months; log rank $P=0.056$). Green and blue lines represent overall survival curves for the predicted high- and low-risk groups, respectively. The color reproduction of the figure is available on the html full text version of the manuscript.

Three-gene predictor predicts survival of patients in the second validation set

To extend these results, we wished to test the predictive power of the three-gene predictor in other independent data sets. After the three-gene predictor was validated in 27 patient samples in our validation set, another microarray study with a comparable study design to our study was published in the literature.[4] These data were only one published microarray data set that could be used to determine whether the three-gene predictor could predict the outcome of metastatic gastric cancer patients treated with either cisplatin or fluorouracil. This data set contains pretreatment expression array data for 40 patients who subsequently received either fluorouracil-based chemotherapy ($n=24$) or cisplatin/irinotecan combination chemotherapy ($n=16$) and patient survival data. We applied the same three-gene predictor to this published microarray data set, just as we did to our 27 patient data in the first validation set. The three-gene predictive index percentile, as a continuous variable, was found to be significantly associated with poor survival of these 40 patients ($P=0.047$; HR according to each percentile increase in three-gene predictive index percentile = 1.014 (95% confidence interval, 1.000–1.027)). Cox multivariate analysis showed that the three-gene predictive index percentile is an independent predictor for poor survival, after considering performance status, age, sex and the chemotherapy regimen (adjusted $P=0.026$; adjusted HR = 1.017 (1.002–1.032)) (Table 4, Figure 3b). Thus, the predictive power of the three-gene predictor is consistent across two validation sets, that is, one from our study patients and the other from published data.

Interestingly, the three-gene predictor was found to be an independent predictor for poor survival, when the same Cox regression analysis was performed only on a subset of these patients ($n=16$) treated with cisplatin in combination with irinotecan, a topoisomerase I inhibitor (adjusted $P=0.011$; adjusted HR = 1.038 (1.008–1.068)). Patients treated with irinotecan were not included in the original training set patients. Hence, the predictive power of three-gene predictor may not be specifically associated with only CF therapy, although further large-scale studies need to be

performed to address the predictive value of the three-gene predictor for other therapeutic regimens.

Three-gene predictive index and radiographic response

Although the radiographic tumor response was not the main end point of this study, we also evaluated the association between the three-gene predictive index and radiographic response of patients with measurable disease. When published data[4] were also included, 104 patients had either partial response or stable disease (clinical benefit) as the best response, whereas 46 patients had progressive disease. The three-gene predictive index was significantly associated with radiographic response at a univariate *P*-value of 0.039, which is higher than the Cox regression *P*-value for the overall survival of all study patients (Table 5). This statistical association was at borderline significance in a multivariate regression analysis.

Table 4 Cox regression analyses of the three-gene predictive index percentile, as a continuous variable, for published DNA microarray data from 40 metastatic gastric cancer patients treated with either FU-based chemotherapy or cisplatin/irinotecan combination chemotherapy

	Overall survival	
	P-*value*	*HR (95% CI)*
Univariate		
Three-gene predictive index percentile	0.047	1.014 (1.000–1.027)
Multivariate		
Three-gene predictive index percentile	0.026	1.017[a] (1.002–1.032)
Performance status ⩾1	0.028	3.008 (1.129–8.016)
Age[b]	0.766	0.995 (0.961–1.030)
Male	0.538	1.359 (0.512–3.605)
FU-based chemotherapy regimen[c]	0.744	0.854 (0.332–2.199)

Abbreviations: CI, confidence interval; FU, fluorouracil; HR, hazard ratio.
[a]Adjusted HR for each percentile increase in three-gene predictive index percentile. For example, a predictive index percentile of 100 (the highest predictive index) is associated with an HR of 5.4 ($=1.017^{100}$), compared with a predictive index percentile of 0 (the lowest predictive index).
[b]As a continuous variable.
[c]As compared with the irinotecan/cisplatin combination chemotherapy regimen.

Three-gene predictor is not prognostic but predictive

Although we showed that the three-gene predictor predicted time to progression and overall survival for CF-treated patients, we wished to further address whether it represents a prognostic signature, using the published data set from 88 gastric cancer patients who were treated by surgery alone and not with chemotherapy.[11] The three-gene predictive index percentile was not a prognostic factor in this data set as a continuous variable ($P=0.506$). There was no difference in survival in the surgically treated patients between the high- and low-risk groups predicted by the three-gene predictor ($P=0.972$). These results strongly suggest that the three-gene predictor is not a predictor of prognosis for gastric cancer patients, but is predictive of the patient response to chemotherapy.

Discussion

Cytotoxic chemotherapy prolongs the median survival of metastatic gastric cancer patients from 3–5 to 9–11 months compared with best supportive care, with a response rate of 40–50%.[18–21] Combination CF constitutes the backbone for chemotherapy regimens commonly used for gastric cancers.[19,22] We also reported that CF in combination with low-dose docetaxel is active for metastatic gastric cancer with tolerable toxicity profile.[18] The ability to predict the primary resistance of common solid tumors to cytotoxic

Table 5 Logistic regression analysis on the three-gene predictive index for radiographic response of 150 patients with measurable disease, including patients represented by the published data set

	Radiographic response[a]		*Time to progression*		*Overall survival*	
	P-*value*	*OR (95% CI)*	P-*value*[b]	*HR (95% CI)*	P-*value*[c]	*HR (95% CI)*
Univariate						
Three-gene predictive index[d]	0.039	2.001 (1.036–3.864)	0.020	1.304 (1.042–1.631)	0.030	1.288 (1.026–1.618)
Multivariate						
Three-gene predictive index	0.059	1.902 (0.976–3.704)	0.019	1.309 (1.045–1.641)	0.018	1.316 (1.048–1.654)
Age ⩾70 years	0.914	1.069 (0.318–3.598)	0.791	1.119 (0.486–2.577)	0.113	1.600 (0.895–2.862)
Poor performance status (ECOG PS 2 or 3)	0.336	0.513 (0.132–1.999)	0.026	2.192 (1.097–4.381)	0.048	1.921 (1.004–3.677)

Abbreviations: CI, confidence interval; ECOG PS, Eastern Cooperative Oncology Group performance status; HR, hazard ratio; OR, odds ratio; WHO, World Health Organization
[a]No clinical benefit (progressive disease according to the WHO criteria; $n=46$) vs clinical benefit (partial response and stable disease; $n=104$).
[b]Result of Cox regression analysis on the three-gene predictive index for the time to progression of 123 patients in the training and the first validation sets.
[c]Result of Cox regression analysis on the three-gene predictive index for the overall survival of all of 163 study patients including published data set.
[d]Computed based on weighted average of log intensities of the three genes (*EGFR*, *FGFR2* and *MYC*) obtained using a proportional hazards *survival* regression on the first two principal components of the log signal intensities of those three genes.

chemotherapy is currently lacking, but would significantly improve patient care by identifying those who would best be treated by alternative strategies. This study has identified a three-gene predictor that distinguishes gastric cancer patients likely to receive a therapeutic benefit from CF from those who will not.

Most previous studies attempting to identify predictors of chemoresistance in gastric cancer have examined only individual genes such as *TS* or *ERCC1*.[23,24] High-throughput DNA microarray analyses to identify gene expression signatures predictive of chemotherapy or chemoradiotherapy resistance in gastrointestinal cancer patients have been limited by the small number of samples,[2,3] heterogeneous treatment[4] or were not prospectively designed.[5] In contrast to these previous studies, our study uses high-throughput genomic approaches, is prospective with a large, pre-defined number of training set patients, separate validation cohorts and survival data during an extended follow-up period. Although previously reported *TS* and *ERCC1* tend to be associated with poor prognosis of our patients, the association was not significant enough for them to be considered for our predictive model ($P=0.073$ and 0.076, for *TS* and *ERCC1*, respectively). Notably, the outcome discrimination predicted by the classifier was statistically significant on two validation groups, including the only available published microarray data set from chemotherapy-treated gastric cancer patients.[4] Although the sample size of our validation set is relatively small, it is nonetheless large enough to show that our three-gene predictor provides a statistically significant discrimination of patient outcome in multivariate survival analyses. The study design we employed is consistent with an allocation of two-thirds to one-third training-to-test set sample allocation as recommended by statisticians.[25]

We combined analyses of gene expression changes identified by expression profiling with the identification of DNA copy number changes using array CGH to develop a predictor composed of a much smaller number of critical genes that potentially could be of clinical utility. We identified *MYC*, *EGFR* and *FGFR2* in regions of amplification, as well as in the gene expression signature related to clinical outcome after CF therapy, suggesting that these genes might be functionally involved in determining resistance. Amplification of *MYC*, *EGFR* and *FGFR2* have previously been observed in gastric cancer at frequencies 4.8–15.5%,[26] 2.3–13.3%[27] and 3–10%,[26,28] respectively, suggesting that, in some cases, tumors amplify these regions for selective advantage. Combined expression of these three genes could predict overall survival and time to progression of CF-treated gastric cancer patients. Thus, combining array CGH analysis with relevant transcriptional changes is a feasible approach for building a predictive model using functionally important genes and reducing the likelihood of false biomarker discovery. Transcriptional levels of genes other than *MYC*, *EGFR* and *FGFR2* identified in the amplified genomic loci were not associated with the survival of the 96 training set patients (for example, $P=0.313$ for *ERBB2*).

Primary gastric tumors are not easily measurable by current radiographic techniques, and often there are no metastatic lesions that are readily quantifiable in metastatic gastric cancer patients. To develop a predictor from the general population of gastric cancer patients in an unbiased way, this study was designed to correlate gene expression profiling of the tumors with overall survival and time to progression, not radiographic response. Overall survival is the ultimate measure of the treatment benefit afforded to a patient and is a particularly appropriate gauge for patients with metastatic gastric cancer, as radiographic assessment is problematic in such patients. The fact that both the time to progression as well as overall survival are predicted by our three-gene predictor in CF-treated patients, but not surgically treated patients, suggests that the three-gene predictor is a predictive indicator for the clinical benefit from CF.

Although *EGFR* and *FGFR2* expression have been reported to have prognostic value for gastric cancer patients treated surgically,[29,30] we did not find the three-gene predictive index to be prognostic for surgically treated patients with gastric cancer. Our findings are consistent with previously reported experimental data on chemoresistance. Inhibitors of *EGFR* act synergistically with cisplatin[31] and fluorouracil,[32] whereas an *FGFR2* inhibitor is synergistic with fluorouracil.[33] MYC has been linked to cisplatin resistance in several *in vitro* models.[34–37]

Taken together, combined expression of *MYC*, *EGFR* and *FGFR2* is predictive of poor survival in CF-treated metastatic gastric cancer patients. More focused prospective trials that are designed to test the clinical utility of this three-gene predictor are warranted.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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Transcript Profiling

All expression microarray data is available at Gene Expression Omnibus (accession number GSE14210; http://www.ncbi.nlm.nih.gov/geo).

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FGFR2 gene amplification and clinicopathological features in gastric cancer

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BACKGROUND: Frequency of *FGFR2* amplification, its clinicopathological features, and the results of high-throughput screening assays in a large cohort of gastric clinical samples remain largely unclear.

METHODS: Drug sensitivity to a fibroblast growth factor receptor (FGFR) inhibitor was evaluated *in vitro*. The gene amplification of the *FGFRs* in formalin-fixed, paraffin-embedded (FFPE) gastric cancer tissues was determined by a real-time PCR-based copy number assay and fluorescence *in situ* hybridisation (FISH).

RESULTS: *FGFR2* amplification confers hypersensitivity to FGFR inhibitor in gastric cancer cell lines. The copy number assay revealed that 4.1% (11 out of 267) of the gastric cancers harboured *FGFR2* amplification. No amplification of the three other family members (*FGFR1*, *3* and 4) was detected. A FISH analysis was performed on 7 cases among 11 *FGFR2*-amplified cases and showed that 6 of these 7 cases were highly amplified, while the remaining 1 had a relatively low grade of amplification. Although the difference was not significant, patients with *FGFR2* amplification tended to exhibit a shorter overall survival period.

CONCLUSION: *FGFR2* amplification was observed in 4.1% of gastric cancers and our established PCR-based copy number assay could be a powerful tool for detecting *FGFR2* amplification using FFPE samples. Our results strongly encourage the development of FGFR-targeted therapy for gastric cancers with *FGFR2* amplification.

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Keywords: *FGFR2*; gastric cancer; gene amplification

Intensive investigations of anticancer treatments for gastric cancer have been done over the past three decades; however, the prognosis for patients with unresectable advanced or recurrent gastric cancer remains poor (Bittoni *et al*, 2010; Fujii *et al*, 2010), and new therapeutic modalities are needed.

Fibroblast growth factors (FGFs) and their receptors are considered to be associated with multiple biological activities, including fundamental developmental pathways, cellular proliferation, differentiation, motility and transforming activities (Itoh *et al*, 1994; Moffa *et al*, 2004; Grose and Dickson, 2005). Fibroblast growth factor signalling is also involved in many physiological roles in the adult organism, such as the regulation of angiogenesis and wound repair, and FGF receptors (FGFRs) are expressed on many different cell types and regulate key cell behaviours of cancer cells (Turner and Grose, 2010). Emerging evidence has demonstrated that the deregulation of FGF signalling is frequently observed in various solid cancers and haematological malignancies (Beenken and Mohammadi, 2009). The most well-known association with *FGFR* mutations is the *FGFR3* mutation observed in bladder cancer, in which somatic mutations in coding regions are observed in about 50% of all specimens (Cappellen *et al*, 1999; Turner and Grose, 2010). Other genetic alterations in *FGFR3* include gene amplification in bladder cancer and translocation in myeloma (Turner and Grose, 2010). Similarly, the deregulation of FGF signalling has been reported in various malignancies. Glioblastoma exhibits FGFR1 kinase domain gain-of-function mutations, and FGFR1 is abnormally activated in malignant prostate cells. In 8p11 myeloproliferative syndrome, translocations fuse different proteins in frame with the FGFR1 kinase domain, causing the constitutive dimerisation of the kinase (Giri *et al*, 1999; Rand *et al*, 2005; Beenken and Mohammadi, 2009). The *FGFR1* amplification has been reported in approximately 10% of breast cancers (Courjal *et al*, 1997) and oral squamous carcinomas, and has been also found at a low incidence in ovarian cancer, bladder cancer and rhabdomyosarcoma (Turner and Grose, 2010). *FGFR2* mutations are observed in 12% of endometrial cancers but are reportedly rare in gastric cancers (Jang *et al*, 2001; Dutt *et al*, 2008). The *K-sam* gene was first identified and characterised as an amplified gene in the human gastric cancer cell line KATO-III (Hattori *et al*, 1990; Ueda *et al*, 1999), and its product was later found to be identical to the bacteria-expressed kinase, or

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keratinocyte growth factor receptor, and FGF receptor 2 (FGFR2). *FGFR2* amplification has been found in diffuse-type gastric cancer-derived cell lines and the amplification was preferentially detected in diffuse-type gastric cancer. FGFR2 protein overexpression was detected using immunohistochemical staining in 20 of 38 advanced cases of diffuse-type gastric cancer (Hattori *et al*, 1996). FGFR2 protein expression was observed in 31% of the gastric carcinomas and was positively correlated with scirrhous cancer, a diffuse type, the invasion depth, the infiltration type and a poor prognosis (Toyokawa *et al*, 2009).

On the other hand, along with another group, we previously reported that *FGFR2* amplification confers hypersensitivity to FGFR inhibitor in gastric cancer cell lines both *in vitro* and *in vivo* (Nakamura *et al*, 2006; Takeda *et al*, 2007), strongly suggesting that *FGFR2* amplification may be a promising molecular target for the treatment of *FGFR2*-amplified gastric cancer. However, very limited information on *FGFR2* amplification is available regarding the frequency, the degree of the increase in the copy number, the histology and a high-throughput screening method in gastric cancer. In this report, we retrospectively studied these issues using formalin-fixed, paraffin-embedded (FFPE) samples in patients with gastric cancer who underwent surgery in an attempt to advance FGFR2-targeted therapy for gastric cancer.

MATERIALS AND METHODS

Cell culture

All of the gastric cancer cell lines used in this study were maintained in RPMI-1640 medium (Sigma, St Louis, MO, USA), except for IM95 (DMEM; Nissui Pharmaceutical, Tokyo, Japan), supplemented with 10% heat-inactivated fetal bovine serum (Gibco BRL, Grand Island, NY, USA), penicillin and streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C. IM95 and OCUM1 were obtained from the Japanese Collection of Research Bioresources (Osaka, Japan) and the others were provided from National Cancer Center Research Institute (Tokyo, Japan).

Patients

A total of 267 patients with histologically confirmed gastric cancer who had undergone surgery at the National Cancer Center Hospital between 1996 and 2006 were included in this study. All the patients in this series had an Eastern Cooperative Oncology Group performance status of 0 to 2 and had undergone surgery. Of these patients, one subject was excluded because an insufficient quantity of DNA was extracted from the patient's specimen. Thus, samples from the remaining 267 patients were analysed. This study was approved by the institutional review board of the National Cancer Center Hospital.

Isolation of genomic DNA

Genomic DNA samples were extracted from surgical specimens preserved as FFPE tissue using a QIAamp DNA Micro kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Macro-dissection of the FFPE samples was performed to select a cancer region, which was marked by a pathologist after deparaffinisation. The DNA concentration was determined using the NanoDrop2000 (Thermo Scientific, Waltham, MA, USA).

Real-time reverse-transcription PCR (RT-PCR)

cDNA was prepared from the total RNA of each cultured cell line using a GeneAmp RNA-PCR kit (Applied Biosystems, Foster City, CA, USA). Real-time RT-PCR amplification was carried out using a Thermal Cycler Dice (Takara, Otsu, Japan) in accordance with the manufacturer's instructions under the following conditions: 95 °C for 5 min, and 50 cycles of 95 °C for 10 s and 60 °C for 30 s. The primers used for the real-time RT-PCR were as follows: *FGFR2*, forward 5′-GATAAATACTTCCAATGCAGAAGTGCT-3′ and reverse 5′-TGCCCTATATAATTGGAGACCTTACA-3′; *GAPDH*, forward 5′-GCACCGTCAAGGCTGAGAAC-3′ and reverse 5′-ATGGT GGTGAAGACGCCAGT-3′. *GAPDH* was used to normalise the expression levels in the subsequent quantitative analyses.

Immunoblotting

A western blot analysis was performed as described previously (Matsumoto *et al*, 2009). The following antibodies were used: monoclonal FGFR2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), β-actin antibody and HRP-conjugated secondary antibody (Cell Signaling Technology, Beverly, MA, USA).

Cell growth inhibitory assay

To evaluate growth inhibition in the presence of various concentrations of PD173074 (Sigma), we used an MTT assay and a previously described method (Kaneda *et al*, 2010). Briefly, the cells were seeded at a density of 2×10^3 cells per well in 96-well plates. After 24 h, PD173074 was added and the incubation was further continued for 72 h at 37 °C. The assay was conducted in triplicate.

Copy number assay for four FGFR family genes

The copy numbers for *FGFR 1–4* were determined using commercially available and pre-designed TaqMan Copy Number Assays according to the manufacturer's instructions (Applied Biosystems). The primer IDs used for *FGFRs* were as follows: *FGFR1*, Hs02862256_cn; *FGFR2*, HS05182482_cn (intron 14) and Hs05114211_cn (intron 12); *FGFR3*, Hs03518314_cn; and *FGFR4*, Hs01949336_cn. The *TERT* locus was used for the internal reference copy number. Human Genomic DNA (Takara) was used as a normal control. Real-time genomic PCR was performed in a total volume of 20 μl in each well, containing 10 μl of TaqMan genotyping master mix, 20 ng of genomic DNA and each primer. The PCR conditions were 95 °C for 10 min and 40 cycles of 95 °C for 15 s and 60 °C for 1 min; the resulting products were detected using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). Data were analysed using SDS 2.2 software and CopyCaller software (Applied Biosystems).

Fluorescence *in situ* hybridisation analysis

The fluorescence *in situ* hybridisation (FISH) method was previously descried (Motoi *et al*, 2010). Probes designed to detect the *FGFR2* gene and the *CEN10p* on chromosome 10 were labelled with fluorescein isothiocyanate or Texas red and were designed to hybridise to the adjacent genomic sequence spanning approximately 0.33 and 0.64 Mb, respectively. The probes were generated from appropriate clones from a library of human genomic clones (GSP Laboratory, Kawasaki, Japan). Deparaffinised tissue sections were air dried and pre-treated with the GSP paraffin pre-treatment kit (GSP Laboratory). In all, 10 μl of fluorescent FISH probe was heated for 5 min at 73–75 °C in a waterbath for denaturation. The tissue sections were then placed in a denaturant solution (70% formamide/2 × saline sodium citrate (SSC) pH 7-8) in a 73–75 °C waterbath, denatured for 5 min, dehydrated in 70 and 100% ethanol for 1 min each at room temperature, and air-dried. Denatured probes were applied, and the specimens were covered with a coverglass and placed on a heated block at 45–50 °C. Then, the slides were sealed with rubber cement and placed in a pre-warmed humidified box overnight at 37 °C. Stringent washing was performed using 2 × SSC/0.3% NP-40 at room temperature and at 72 °C for 5 min and then with 2 × SSC at room temperature. The signals were observed using fluorescence microscopy, and the

FISH signals were evaluated by independent observers (TM and AK). After screening all the complete sections, images of the tumour cells were captured and recorded and the signals for 20 random nuclei were counted for an area where individual cells were recognised on at least 10 representative images. The positive result of copy number gain is determined as follows (FGFR2/CEN10p$\geqslant$2.0).

Statistical analysis

The statistical analyses of the clinicopathological features were performed using the Student *t*-test and the χ^2 test using PAWS Statistics 18 (SPSS Japan Inc., Tokyo, Japan). The overall survival (OS) curves were estimated using the Kaplan–Meier method.

RESULTS

FGFR2 amplification confers hypersensitivity to FGFR inhibitor in gastric cancer cell lines

We examined the growth inhibitory effect of PD173074 (0.004–80 μM) on four *FGFR2*-amplified (HSC-43, TU-KATPIII, SNU-16 and HSC-39) and four non-amplified (44As3, 58As1, IM95 and OCUM1) gastric cancer cell lines. The *FGFR2* amplification status of each cell line had already been examined using a CGH analysis (unpublished data). The mRNA and protein expressions of FGFR2 were overexpressed in the *FGFR2*-amplified cell lines (Figures 1A and B). A growth inhibitory assay showed that the IC_{50} values of the FGFR inhibitor PD173074 in *FGFR2*-amplified cells were 0.01–0.07 μM, whereas those in non-amplified cells were 2.6–13.2 μM, indicating that *FGFR2* amplification conferred an approximately 100-fold hypersensitivity to FGFR inhibitor in gastric cancer cell lines (Figure 1C).

FGFR2 amplification in clinical gastric cancer cell lines and surgical specimens

To develop a high-throughput method for detecting *FGFR2* gene amplification in a clinical setting, we verified a real-time PCR-based detection method, the TaqMan Copy Number Assay. The *FGFR2* copy number was 1.4–2.7 copies in the four non-amplified cell lines; however, the numbers in the four *FGFR2*-amplified cell lines were 28.2, 231.7, 88.2 and 36.3 copies, respectively (Figure 1D). In addition, another primer in intron 12 of *FGFR2* produced a very similar result ($R=0.99$, Figure 1D). Collectively, these results suggested that a DNA copy number assay for *FGFR2* was a sensitive and reproducible method. We also examined the copy numbers of *FGFR1*, *FGFR3* and *FGFR4*, but no obvious gene amplification was observed in all of the eight cell lines (Figure 1D). Next, *FGFR2* amplification was evaluated using the copy number assay in 267 FFPE samples of primary gastric cancer specimens. *FGFR2* amplification of more than 5 copies was observed in 11

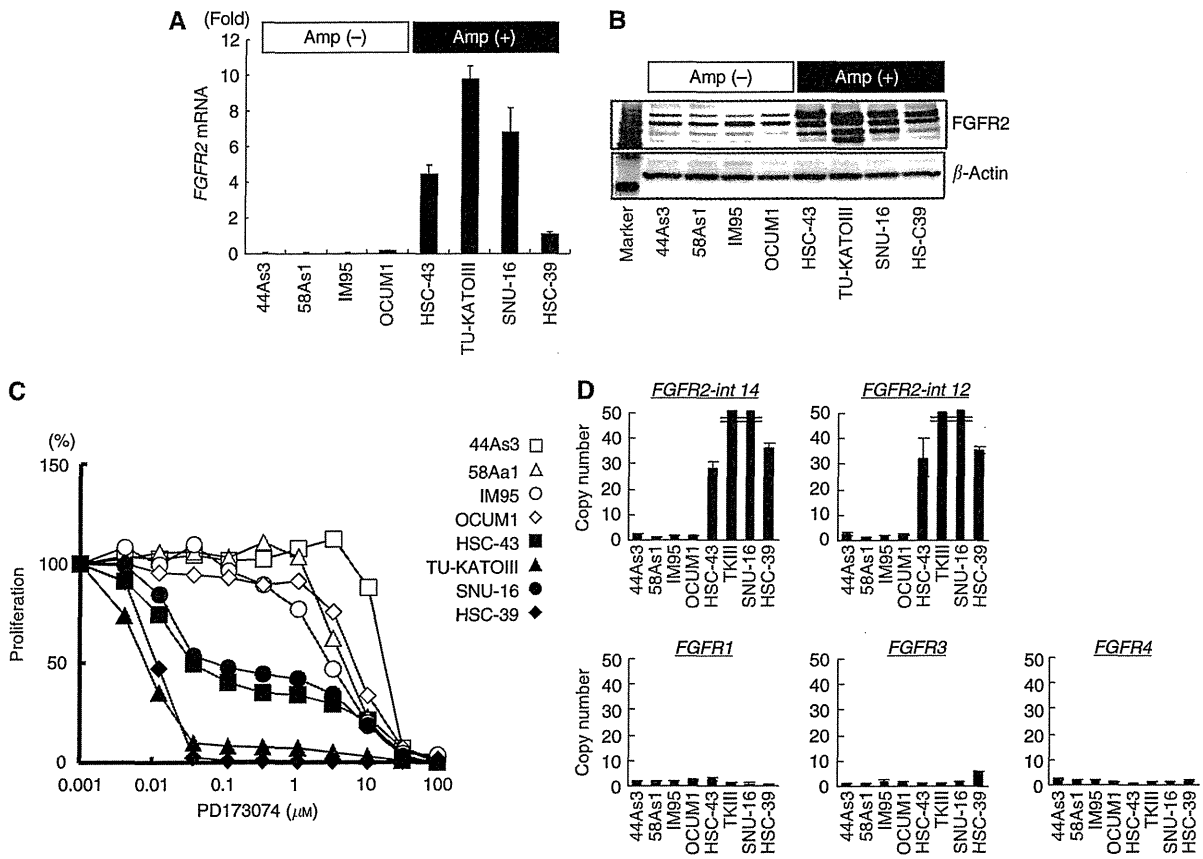

Figure 1 *FGFR2* amplification in gastric cancer cell lines. (**A**) The mRNA expression levels of *FGFR2* were determined using real-time RT–PCR for eight gastric cancer cell lines. *FGFR2* mRNA: normalised mRNA expression levels (*FGFR2/GAPDH* $\times 10^3$). (**B**) Western blot analysis for FGFR2 expression. β-Actin was used as an internal control. Marker, molecular marker. (**C**) Growth inhibition assay for the FGFR inhibitor PD173074, evaluated at the indicated concentrations using an MTT assay. (**D**) Evaluation of DNA copy number assay using gastric cancer cell lines. A TaqMan copy number assay was performed to determine the copy number using specific primers for the genomic loci of the *FGFR1–4* genes against DNA samples. Amp, gene amplification. FGFR2-int-14 and FGFR2-int-12, different primers for intron 14 or intron 12 of *FGFR2*.

cases (92.0, 63.0, 41.4, 19.9, 18.4, 13.7, 8.3, 6.2, 6.2, 5.7 and 5.6 copies), with a frequency of 4.1% (Figure 2A). The mean copy number in the non-amplified cases was 2.4 ± 0.6 copies. Meanwhile, no obvious gene amplification of *FGFR1*, *FGFR3* or *FGFR4* was observed (data not shown).

FISH analysis for *FGFR2* amplification

We used a FISH analysis to examine *FGFR2* amplification in the same samples to verify the results of the above PCR-based DNA copy number assay. Highly amplified TU-KATOIII cells showed numerous and large clustered signals, whereas non-amplified OCUM1 cells contained two normally paired signals (Figure 2B). A FISH analysis was performed on seven cases among 11 *FGFR2*-amplified cases and two non-amplified cases. The FISH analysis revealed that *FGFR2* was highly amplified in six of the seven *FGFR2*-amplified clinical samples (four showed multiple scattered signals and two showed large clustered signals), while the remaining sample exhibited a relatively low grade of amplification (FGFR2/CEN10p = 2.2, Figure 2B). The *FGFR2* signals in the G3 and G10 samples, which were determined not to be amplified based on the results of the DNA copy number assay, were not increased. These results clearly demonstrated the presence of *FGFR2*-amplified gastric cancers among clinical samples.

Clinicopathological features of *FGFR2*-amplified gastric cancer

We evaluated the clinicopathological features including age, sex, histology and pathological stage according to the *FGFR2* amplification status. Patients age with *FGFR2* amplification were significantly higher than the others, but sex and pathological stage were not associated with *FGFR2* amplification in this study (Table 1). Among the patients with *FGFR2* amplification, the

Table 1 Frequency of *FGFR2* amplification in gastric cancers and its association with clinical and pathologic factors

	FGFR2 (+)		FGFR2 (−)		
	n = 11	%	*n* = 256	%	*P*-value
Age					
Range	55–91		31–88		0.047
Median	67		63		
Gender					
Male	11	100	173	68	0.052
Female	0	0	83	32	
Pstage					
I	0	0	25	10	0.16[a]
II	0	0	32	13	
III	3	27	73	29	
IV	8	73	125	49	
Unknown	0	0	1	0	
Histology					
Tub1	0	0	41	16	0.55[b]
Tub2	2	18	51	20	
Pap	1	9	5	2	
Muc	2	18	8	3	
Sig	1	9	15	6	
Por1	0	0	28	11	
Por2	5	45	108	42	

Abbreviations: Amp = gene amplification; FGFR = fibroblast growth factor receptor; Muc = mucinous adenocarcinoma; Pap = papillary adenocarcinoma; Por = poorly differentiated adenocarcinoma; pStage = pathological stage; Sig = signet ring-cell carcinoma; Tub = tubular adenocarcinoma. [a]Comparison between pStage I+II and III+IV. [b]Comparison between intestinal (Tub1, Tub2 and Pap) and others. *P*-values were calculated using the *t*-test for age and the χ^2 test for the other variables.

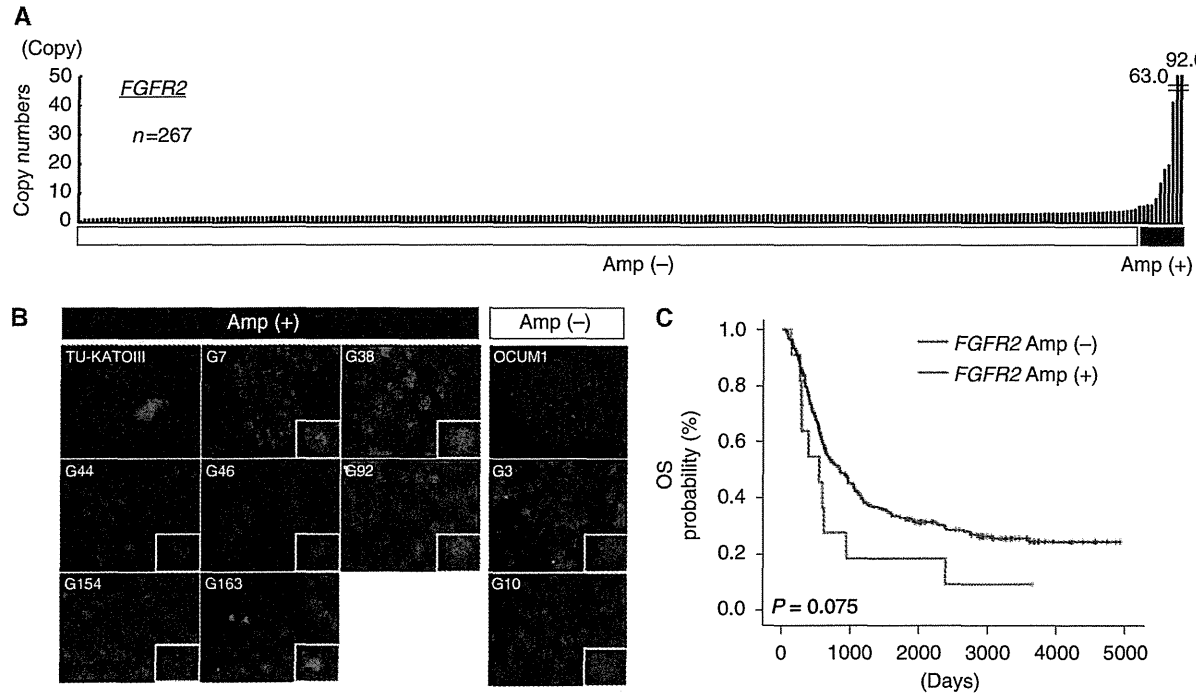

Figure 2 (**A**) Amplification of *FGFRs* in surgical specimens of gastric cancer. A TaqMan copy number assay for *FGFR2* was performed using DNA samples obtained from 267 FFPE samples. Human normal genomic DNA was used as a normal control. *FGFR2* amplification over 5 copies was observed in 11 cases (92.0, 63.0, 41.4, 19.9, 18.4, 13.7, 8.3, 6.2, 6.2, 5.7 and 5.6 copies). (**B**) Fluorescence *in situ* hybridisation analysis of *FGFR2*-amplified KATO-III cells, non-amplified OCUM1 cells and nine surgical specimens of gastric cancer. Green, signal of *CEN10P* locus; Red, signal of *FGFR2* locus; G3 ~ G92, sample numbers; Amp, gene amplification. High-power images are presented for a single cancer cell. (**C**) Overall survival in *FGFR2*-amplified gastric cancer. Kaplan–Meier curves for OS according to the *FGFR2* amplification status.

Table 2 Summary of *FGFR2*-amplified gastric cancers

No.	Age	Sex	Location	Size of lesion (cm)	Macroscopic type[a]	Lauren's classification	Histology	pStage	OS (days)	FGFR2 (CN)	FISH (type, copies)
G7	55	M	Lower	8.5 × 8	3	Diffuse	Muc > Por2, Sig	IV	612	41.4	LC, +++
G38	70	M	Upper	8.5 × 8	I + IIc	Intestinal	Pap > Tub1, Tub2, Por2	IIIa	591	92.0	MS, +++
G44	70	M	Lower	9.5 × 8	3	Diffuse	Por2 > Pap, Tub1, Muc	IIIa	938	5.6	Low, 2.2[b]
G46	75	M	Middle	10 × 6	4	Intestinal	Tub2 > Por2	IV	2380	13.7	MS, +++
G92	75	M	Middle	6.5 × 5.5	3	Diffuse	Por2 > Tub2	IV	280	19.9	MS, +++
G154	59	M	Middle	14 × 12	4	Diffuse	Por2	IV	132	5.7	MS, +++
G163	64	M	Lower	15 × 10	3	Diffuse	Muc > Sig > Tub2	IV	540	6.2	LC, +++
G203	64	M	Lower	10.5 × 6.5	4	Diffuse	Sig > Por2 > Muc	IV	283	8.3	ND
G271	91	M	Upper	7 × 6.5	2	Intestinal	Tub2 > Por1	IV	383	63.0	ND
G299	65	M	Middle	20 × 20	4	Diffuse	Por2 > Sig	IV	256	6.2	ND
G329	67	M	Middle	6.5 × 6	3	Diffuse	Por2 > Sig	IIIa	3642+	18.4	ND

Abbreviations: CN = copy number of *FGFR2* determined using a copy number assay; Diffuse = diffuse-type gastric cancer; FISH = fluorescence *in situ* hybridisation; FGFR2 = fibroblast growth factor receptor 2; Intestinal = intestinal-type gastric cancer; Location = tumor location in stomach; LC = large clustered signals; Low = low copy number gain; M = male; MS = multiple scattered signals; ND, not determined; No. = sample numbers; OS = overall survival; pStage = pathological stage; +++ = numerous *FGFR2* signals; + = patients. alive. [a]Macroscopic type, classification is based on the definitions of the Japanese Research Society for Gastric Cancer. [b]Ratio of FGFR2/CEN10p.

histologies of two cases were intestinal-type gastric cancer and one was unclassified type, while the others were diffuse-type (Table 2). The tumours were located in either the upper or lower stomach. These results are summarised in Table 2. Finally, we examined the prognostic impact of *FGFR2* amplification on OS after surgery. *FGFR2* amplification tended to be associated with a poorer outcome, compared with non-amplified cases, but no significant difference was observed in the current study (log-rank test, $P = 0.075$; Figure 2C).

DISCUSSION

To date, several studies have reported on the protein expression of FGFR2 and clinicopathological analyses using immunohistochemistry, with 20 of 49 (41%) and 42 of 134 (31%) gastric cancers expressing FGFR2 protein when evaluated using positive or negative staining (Hattori *et al*, 1996; Toyokawa *et al*, 2009). Regarding genomic alteration, the frequency of *FGFR2* amplification has been reported to be 3 out of 19 (16%, among diffuse-type gastric cancers) detected using comparative genomic hybridisation (CGH), 3 out of 57 (5%) detected using Southern blot analysis, and 2 out of 30 (7%) detected using CGH (Tsujimoto *et al*, 1997; Peng *et al*, 2003; Kim *et al*, 2010). These results suggest that the frequency of *FGFR2* amplification is around 5%, which is lower than the positive staining results obtained using immunohistochemistry. However, the frequency of amplification has not been determined in a large cohort. Our results indicated that the frequency of *FGFR2* amplification was 4.1% (11 out of 267), consistent with these previous reports on genomic alterations. To select a sub-population of gastric cancers sensitive to FGFR inhibitors in the future, gene amplification may be a more suitable biomarker than positive staining using immunohistochemistry based on the results of preclinical studies (Figure 1, Takeda *et al*, 2007).

In six cases, the copy number of *FGFR2* was larger than 10 copies and numerous signals were observed by the FISH analysis (Figure 2B), indicating that these gastric cancer cells harboured high levels of amplification, similar to the results obtained using gastric cancer cell lines. Preclinical studies suggest that these cases may be likely to respond to FGFR inhibitors. In the remaining case, *FGFR2* amplification was relatively low (4 ~ 8 copies, G44). Such cases with low levels of *FGFR2* amplification may require further investigation regarding their sensitivity to FGFR inhibitors in the future. Meanwhile, we used a copy number assay to detect gene amplification in FFPE samples. Although DNA extracted from FFPE samples was considered to be of low quality with a DNA degradation in general, a copy number assay was capable of detecting and screening amplification in the FFPE samples, which had been stored for as long as 10 years. The results were consistent with the results of FISH studies in several cell lines, with seven positive cases and two negative cases. Our findings suggest that a copy number assay is a powerful tool for detecting and screening gene amplification using FFPE samples.

Recently, trastuzumab in combination with chemotherapy has been regarded as a new standard option for patients with HER2-positive advanced gastric or gastro-oesophageal junction cancer (Bang *et al*, 2010). Therefore, the evaluation of both the HER2 and FGFR2 status before anti-cancer treatment may be needed in gastric cancer patients in the near future. Many small molecules of VEGFR2 tyrosine kinase inhibitors, categorised as anti-angiogenic agents, are now under clinical evaluation, and some of them, including sorafenib for hepatocellular carcinoma and sunitinib for renal cell carcinoma, are being clinically used as standard treatment options (Ellis and Hicklin, 2008). These compounds are also known to have a potential kinase inhibitory effect on FGFRs (Takeda *et al*, 2007; Turner *et al*, 2010), indicating that the development of these multi-kinase inhibitors may be a promising approach to the treatment of *FGFR2*-amplified gastric cancer. In addition to small molecular FGFR tyrosine kinase inhibitors, anti-FGFR antibodies, such as IMC-A1, PRO-001a and R3Mab, also offer promise as molecular-based drugs (Turner and Grose, 2010). We plan to conduct a prospective study in a cohort of Japanese patients with *FGFR2*-amplified gastric cancers.

In conclusion, we found that *FGFR2* amplification was observed in gastric cancer at a frequency of about 4.1%, and a copy number assay was a powerful tool for screening for *FGFR2* amplifications using FFPE samples. Our results warrant strong consideration of the development of FGFR inhibitors for the treatment of gastric cancers with *FGFR2* amplification.

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Conflict of interest

The authors declare no conflict of interest.

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ORIGINAL ARTICLE

First-line fluorouracil-based chemotherapy for patients with severe peritoneal disseminated gastric cancer

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Abstract
Background Treatment with an oral fluoropyrimidine plus cisplatin is widely used for advanced gastric cancer, but patients with severe peritoneal metastasis often cannot tolerate such treatment, due to inadequate oral intake or massive ascites. The aim of this study was to assess the efficacy and safety of systemic chemotherapy for advanced gastric cancer with severe peritoneal metastasis.
Methods The cases of 92 patients with advanced gastric cancer and severe peritoneal metastasis who received first-line chemotherapy at our hospital between May 2001 and February 2007 were retrospectively analyzed. Severe peritoneal metastasis was defined as massive ascites or inadequate oral intake due to peritoneal dissemination. Inadequate oral intake was defined as having required an intravenous drip infusion.
Results All 92 patients received 5-fluorouracil (5-FU)-based chemotherapy; 40 of the patients had massive ascites, 34 had inadequate oral intake, and the remaining 18 had both conditions. Among the 86 patients having assessable ascites, 23 (27%) patients showed an improvement in ascites. Of the 52 patients with inadequate oral intake, 17 (33%) patients improved to the point of ingesting without intravenous drip infusion after receiving the chemotherapy. Median time to treatment failure and overall survival time were 1.9 months [95% confidence interval (CI) 1.3–2.5 months] and 4.6 months (95% CI 3.9–5.3 months), respectively. Major grade 3 or 4 adverse events were anorexia (26%), neutropenia (26%), and anemia (22%).
Conclusion The treatment regimen of 5-FU-based chemotherapy for advanced gastric cancer with severe peritoneal metastasis was feasible, but its efficacy was not sufficient.

Keywords Gastric cancer · Peritoneal metastasis · Ascites · Oral intake

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Introduction

Gastric cancer can spread through various routes, such as by local extension of direct serosal invasion, the lymphatic pathway, and the hematogenous route. Peritoneal metastasis can be disseminated from T3/T4 gastric cancer cells breaking through the serosa. Peritoneal metastasis is reported to be a common reason for the unresectability of gastric cancer [1], and it is also reported to be a common pattern of recurrence after curative resection [2–4]. Peritoneal metastasis causes serious clinical complications such as intestinal obstruction, massive ascites, and hydronephrosis. These complications are associated with abdominal pain, abdominal fullness, and vomiting, resulting in an extremely poor quality of life for the patients.

Recently, an oral fluoropyrimidine plus cisplatin regimen was demonstrated as a standard regimen against advanced gastric cancer in several large clinical Phase III trials [5–7]. However, in these trials, patients with severe peritoneal metastasis, such as those with massive ascites and intestinal obstruction, were excluded from the

eligibility criteria because they were unable to tolerate hydration, as required during cisplatin-containing chemotherapy, or continue to receive stable oral fluoropyrimidine chemotherapy.

We retrospectively investigated a regimen of chemotherapy, and its safety and efficacy for gastric cancer patients with severe peritoneal metastasis to determine the indications for chemotherapy and to consider the most suitable regimen.

Patients and methods

Patients

Patients who received first-line chemotherapy for gastric cancer at the National Cancer Center Hospital, Tokyo, between May 2001 and February 2007 were retrospectively recruited for this study according to the following criteria: histologically proven gastric adenocarcinoma; unresectable or recurrent disease; histologically or radiologically confirmed severe peritoneal metastasis; and no prior chemotherapy (adjuvant chemotherapy completed more than 6 months before recruitment was allowed) or radiotherapy. We defined severe peritoneal metastasis as massive ascites or inadequate oral intake due to peritoneal dissemination. We defined inadequate oral intake as requiring and actually receiving an intravenous drip infusion. Patients who were administered an intravenous drip infusion for the purpose of renal protection or as a drug administration route, such as for analgesics, were excluded.

Ascites was classified in four levels: none; mild; moderate; and massive. "None" was defined as undetected by computed tomography (CT) scan; "mild" ascites was localized in only one area such as the pelvic cavity or surface of the liver; "moderate" ascites did not correspond to either mild or massive ascites; and :massive" ascites extended throughout the abdominal cavity.

This retrospective study was approved by the Institutional Review Board of the National Cancer Center and was conducted in accordance with the ethical principles stated in Japan's Ethics Guidelines for Epidemiological Research.

Assessment of response and toxicity

Response in ascites was evaluated as follows: "disappearance" was defined as being undetected by CT scan, "decrease" was defined as decreasing by more than one level, "no change" was defined as remaining at the pretreatment level, and "progression" was defined as deteriorating to a more severe level or becoming clinically worse with more frequently required drainage of ascites. We evaluated the response rate in ascites without interval confirmation. Oral intake improvement was defined as maintaining an adequate amount of intake for 7 days or more without an intravenous drip infusion. Time to treatment failure (TTF) was defined as the interval between the start of treatment and the earliest date among disease progression (either radiologic or symptomatic progression), treatment discontinuation, or death due to any cause. Overall survival (OS) was measured from the start of treatment to the date of death or last follow-up. Survival curves were estimated using the Kaplan–Meier method. All statistical analyses were performed using Dr. SPSS II software (SPSS Japan, Tokyo, Japan). Toxicity was assessed using the National Cancer Institute Common Terminology Criteria for Adverse Events version 3.0.

Results

Patient characteristics

A total of 92 patients with severe peritoneal disseminated gastric cancer received systemic chemotherapy between May 2001 and February 2007. The patient characteristics are summarized in Table 1. About half of the patients had a baseline Eastern Cooperative Oncology Group performance status of 2 or greater.

Chemotherapy

All patients received 5-FU-based chemotherapy regimens. The regimens consisted of methotrexate (MTX)/5-FU in 64 patients, 5-FU continuous infusion (ci) in 21 patients, low-dose 5-FU in 6 patients, and 5-FU/*l*-leucovorin (*l*-LV) in 1 patient. The MTX/5-FU regimen consisted of a weekly MTX dose [100 mg/m^2, intravenously (iv)] followed by 5-FU (600 mg/m^2, iv) administered 3 h after the MTX infusion. Leucovorin rescue, 15 mg orally or intravenously every 6 h for 6 times, was started 24 h after the MTX infusion. Furthermore, leucovorin rescue was continued until the serum MTX concentration was less than 5×10^{-8} mol/L if physicians judged there to be a requirement for additional doses. The low-dose 5-FU regimen consisted of a daily infusion of 5-FU (300 mg/m^2/day) [8], while 5-FU ci consisted of 800 mg/m^2/day for 5 days every 4 weeks. The 5-FU/*l*-LV regimen consisted of weekly 5-FU (500 mg/m^2, iv) plus *l*-LV (200 mg/m^2, iv).

Efficacy

Among the 92 patients, 86 (93%) had mild or more severe ascites at initial diagnosis. The remaining 6 (7%) patients had intestinal stenosis without ascites. The response rate in

Table 1 Patient characteristics

	N	%
Gender		
Male	53	58
Female	39	42
Age (years)		
Median	59	
Range	20–77	
ECOG performance status		
0	2	2
1	46	50
2	34	37
3	10	11
Primary tumor		
Present	69	75
Absent	23	25
Histological type		
Diffuse	77	84
Intestinal	10	11
Other, not specified	5	5
Severe peritoneal metastasis		
Massive ascites	40	43
Inadequate oral intake	34	37
Both	18	20
Ascites[a]		
None	5	5
Mild	24	26
Moderate	4	4
Massive	58	63
Intravenous drip infusion		
Peripheral venous infusion	24	26
Central venous infusion	28	30

ECOG Eastern Cooperative Oncology Group

[a] One patient did not have pretreatment computed tomography (CT)

patients with ascites was 27% (95% CI 17.8–37.4%): 2 patients achieved disappearance of ascites and 21 patients had a decrease of ascites, 20 patients showed no change of ascites, and 33 patients had an increase of ascites. The remaining 10 patients could not be assessed. Among the 52 patients with inadequate oral intake, improvement was observed in 17 patients (33%, 95% CI 20.3–47.1%), and 2 patients who had undergone endoscopic stent placement or ileostomy during chemotherapy could not be evaluated. The reasons for treatment discontinuation were disease progression in 77 (84%) patients, loss of follow-up in 8 (9%), death in 2 (2%), unacceptable toxicity in 3 (3%), patient refusal without causal relationship to treatment in 1 (1%), and physician decision in 1 (1%). Among the 77 patients with disease progression, 57 (74%) patients had progressive disease associated with peritoneal metastasis.

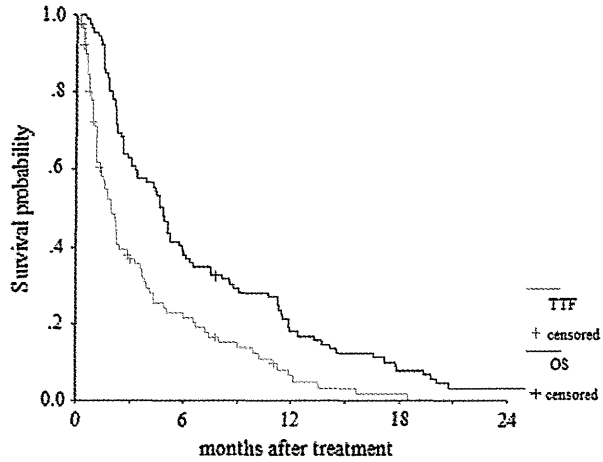

Fig. 1 Overall survival (*OS*; *black line*) and time to treatment failure (*TTF*; *gray line*) in the 92 patients. The *marks* on the curves indicate censored cases

At the time of the analysis, 90 patients had died, at a median follow-up time of 4.7 months (range 0.5–45.9 months). Median TTF and OS for the 92 patients in total were 1.9 months (95% CI 1.3–2.5 months) and 4.6 months (95% CI 3.9–5.3 months), respectively (Fig. 1).

Second-line treatment

After discontinuation of the first-line treatment, 42 (46%) patients received supportive care only, 37 (40%) patients received second-line chemotherapy, 1 (1%) patient each received immunotherapy and palliative gastrectomy, and 11 (12%) patients were lost to follow-up. Second-line chemotherapy regimens consisted of taxanes for 28 patients, S-1 (tegafur, 5-chloro-2,4-dihydroxypyridine, and potassium oxonate) for 4 patients, MTX/5-FU for 2 patients, 5-FU ci for 2 patients, and mitomycin for 1 patient. Among the 8 patients who received third-line chemotherapy, 3 received weekly paclitaxel as taxane-containing regimens. A total of 32 (35%) patients underwent taxane-containing chemotherapy as additional treatment.

Adverse events

Adverse events in all 92 patients are shown in Table 2. The most common hematological toxicities were neutropenia and anemia. Grade 3 or 4 leukopenia and neutropenia occurred in 17 (18%) and 24 (26%) patients, respectively, and febrile neutropenia occurred in 7 patients. As to non-hematological toxicities, all grades of anorexia were seen in 95% of patients, while grade 3 nausea and vomiting occurred in 5 and 4% of patients, respectively. These

Table 2 Adverse events

	Number of patients				Gr 3/4 (%)
	Gr 1	Gr 2	Gr 3	Gr 4	
Hematological toxicity					
Leukocytes	26	19	12	5	18
Neutrophils	11	18	13	11	26
Hemoglobin	31	33	17	3	22
Platelets	14	2	2	1	3
Nonhematological toxicity					
Anorexia	33	30	19	5	26
Nausea	50	12	5	0	5
Vomiting	34	4	4	0	4
Mucositis	20	2	1	0	1
Diarrhea	34	6	3	0	3
Constipation	14	7	2	1	3
Fatigue	56	27	5	0	5
Febrile neutropenia	–	–	6	1	8
Bilirubin	12	4	8	2	11
AST	27	11	13	0	14
ALT	26	12	10	0	11
Creatinine	12	8	1	0	1

Gr grade, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase

gastrointestinal adverse events were frequently attributed to the underlying gastric cancer and peritoneal metastasis. Moreover, elevated levels of bilirubin and transaminase were frequently observed due to progressive disease of peritoneal metastasis. No other unexpected severe toxicities were observed during the treatment. Ten (11%) patients died within 30 days from the last administration of 5-FU-based chemotherapy, 7 patients died of disease progression, 2 patients died of treatment-related causes, and 1 patient died of intestinal perforation clinically diagnosed as undeniably treatment-related. As to the treatment-related deaths, one patient developed septic shock with febrile neutropenia and another patient had septic shock with no apparent source of infection or neutropenia.

Discussion

The present study demonstrated that gastric cancer patients with severe peritoneal metastasis receiving 5-FU-based chemotherapy as the first-line treatment had median TTF and OS of 1.9 and 4.6 months, respectively. Early death within 30 days from the last administration of this treatment occurred in 10 (11%) patients, but in the majority of cases, death was caused by disease progression, and treatment-related deaths were observed in only 3 (3%) patients. Therefore, the 5-FU-based chemotherapy for gastric cancer patients with severe peritoneal metastasis was feasible, but its efficacy was unsatisfactory.

Recent Phase III trials have demonstrated the noninferiority of chemotherapy using oral fluoropyrimidines, such as capecitabine or S-1, compared to 5-FU-based chemotherapy [6, 7, 9]. Moreover, oral fluoropyrimidine and cisplatin combination therapy is a standard regimen worldwide [5, 7]. In these clinical trials, gastric cancer patients with severe peritoneal metastasis were excluded from the eligibility criteria, due to the absence of measurable lesions and potential severe complications such as massive ascites, intestinal obstruction, hydronephrosis, and obstructive jaundice.

In a recent Phase III trial (Japan Clinical Oncology Group [JCOG] 0106) comparing 5-FU ci with MTX/5-FU for gastric cancer patients, limited peritoneal metastasis was reported [10]. In that trial, the median survival of 10.6 months in the MTX/5-FU experimental arm did not show a significant advantage over the 9.4 months in the 5-FU ci control arm. Moreover, several Phase II trials of MTX/5-FU, weekly paclitaxel (wPTX), and modified FOLFOX4 for gastric cancer with malignant ascites demonstrated a response rate in patients with ascites of about 35%; median treatment durations of 8, 12, and 10 weeks; and median survivals of 5.1, 5.2, and 8.4 months, respectively [11–13]. Even in these Phase II trials, patients with severe peritoneal metastasis such as massive ascites or inadequate oral intake were almost always excluded from the eligibility criteria. Therefore, we conducted the present retrospective study for patients with severe peritoneal metastasis to determine the indications for chemotherapy and to consider the most suitable regimen.

The prognosis in our patient population was much worse than those in the above-mentioned previous phase II studies for gastric cancer with malignant ascites [11–13], and the efficacy of the 5-FU-based chemotherapy for gastric cancer with severe peritoneal metastasis was not sufficient. This poor result was mainly due to the inferior condition of the patient population (with poor performance status, massive ascites, or inadequate oral intake) compared with the condition of the patients in the previous Phase II studies. Moreover, we consider the low proportion of patients (40%) receiving second-line chemotherapy as another reason for the poor result in our study. Previous Phase II studies for gastric cancer with malignant ascites did not refer to the post-treatment, while in recent Japanese Phase III trials for advanced gastric cancer, the proportion of patients receiving second-line chemotherapy was 75% in the SPIRITS (S-1 vs. S-1/cisplatin) trial and 78% in the JCOG 9912 (5-FU ci vs. S-1 vs. irinotecan/cisplatin) trial [5, 9]. Although we chose the study patients after May 2001, when paclitaxel was approved for treating gastric cancer in Japan, the present study had a much smaller