and some European countries, preoperative and postoperative chemotherapy with epirubicin, cisplatin, and 5-fluorouracil is used based on evidence from the MAGIC trial;[2] and in Japan, standard adjuvant treatment is single-agent postoperative chemotherapy with the oral fluoropyrimidine S-1 after D2 surgery based on results of the ACTS-GC trial.[3] These different approaches produced different survival results, which could not be explained by the difference of tumor biology based on varying ethnicity. Indeed, during the past decade, studies have elucidated the benefits of D2 gastrectomy and surgical undertreatment negatively affected the survival results of adjuvant treatment.[4,5] Thus, D2 gastrectomy is now the globally accepted surgical standard.[6,7] However, the optimal adjuvant therapy to use with D2 surgery has not been established.

Now, a study published in *The Lancet* (CLASSIC trial) by Bang *et al.*[8] reports that adjuvant chemotherapy with capecitabine and oxaliplatin after D2 surgery for patients from Asia with stage II or III gastric cancer resulted in significantly improved disease-free survival (DFS) compared with D2 surgery alone ($P<0.0001$). This trial is the first positive phase III study to show that doublet combination chemotherapy that included a platinum-based compound after D2 surgery is effective for gastric cancer.

> **"...evidence supports two approaches to adjuvant chemotherapy after D2 gastrectomy..."**

This randomized phase III study was well designed. The primary end point was DFS, a surrogate end point for overall survival. A total of 1,035 patients were enrolled for 37 months in 37 centers in South Korea (nearly 90% of the patients), China, and Taiwan. The study data were made available at the planned interim analysis time point with a median follow-up period of 2.9 years. The patient populations in the two arms were well balanced. Less than half of the patients had T3 tumors (T4 in Union for International Cancer Control TNM seventh edition), whereas 90% had nodal metastases. The tumors were located mostly in the gastric antrum and body (more than 80%), suggesting classic gastric cancer, and less than 3% of the participants had gastroesophageal junction cancer.

Toxic effects related to capecitabine and oxaliplatin were considered to be acceptable.[8] Major grade 3 or 4 toxic effects included neutropenia (22%), thrombocytopenia (8%), and nausea (8%). The therapy completion rate in the chemotherapy arm was 67%, which is quite high for gastric adjuvant chemotherapy that included a platinum-based compound; this high compliance likely contributed to the positive outcomes.

Table 1 | Comparisons between CLASSIC[8] and ACTS-GC[3]

Comparator	CLASSIC	ACTS-GC
Number of patients	1,035	1,059
Median age	56 years	63 years
Accrual period	37 months	39 months
Median follow-up period	2.9 years	2.9 years
Primary end point	DFS	OS
Tumor stage	T2 (54%), T3 (44%), node positive (90%)	T2 (54%), T3 (43%), node positive (89%)
Completion of chemotherapy	67% (6 months)	65.8% (12 months)
3-year DFS or RFS* with vs without chemotherapy (HR)	74% vs 59% (0.56 [95% CI 0.44–0.72])	72.2% vs 59.6% (0.62 [95% CI 0.50–0.77])
3-year OS with vs without chemotherapy (HR)	83% vs 78% (0.72 [95% CI 0.52–1.00])	80.1% vs 70.1% (0.68 [95% CI 0.52–0.87])

*RFS for ACTS-GC. Abbreviations: DFS, disease-free survival; OS, overall survival; RFS, recurrence-free survival.

Over the course of the study, the DFS curves clearly separated between the two arms.[8] In the chemotherapy group, 3-year DFS was 74%, which compared with 59% for the surgery-alone arm. The overall hazard ratio was 0.56. The hazard ratio was relatively constant in stage II (0.55), IIIA (0.57), and IIIB (0.57) disease, suggesting that capecitabine and oxaliplatin therapy was effective regardless of tumor stage. However, overall survival curves did not markedly separate between the two arms. The overall survival rate at 3 years was 83% in the chemotherapy arm and 78% in the surgery-alone arm; this similarity may have resulted from a number of factors. First, follow up for assessing overall survival as an end point has not been completed; nearly half (48%) of patients with recurrent disease in the surgery-alone arm were still alive at the time of the analysis. Second, survival after recurrence may differ between the two arms owing to the different rescue regimens available to the patients. Four types of cytotoxic drugs are used for the treatment of gastric cancer: fluoropyrimidine, platinum-based compounds, taxanes, and CPT-11. In the capecitabine and oxaliplatin arm, the patients had already been exposed to two of these key drugs during the adjuvant therapy; therefore, there were only two classes of drug available for chemotherapy after recurrence. By contrast, patients in the surgery-alone arm could receive all four drugs after recurrence. This difference may have affected survival after recurrence, which could shift the overall survival curve in favor of the surgery-alone arm. Third, DFS may be associated with event bias. Although imaging was performed every 6 months during the first 3 years and every year thereafter, none of the presented data show that the intervals between imaging were consistent in the two arms. DFS could be shortened in the control arm if the patients were checked earlier—for example, in response to small increases of tumor marker levels or subtle clinical signs suggestive of recurrence.

There are several differences between the two trials (ACTS-GC and CLASSIC) that have assessed adjuvant chemotherapy in this patient population (Table 1).[3] The two trials were similar in a large number of areas: the number of patients, accrual period, median follow-up period at the time the analysis was performed, surgery, and tumor and nodal stage; however, the median patient age was 7 years younger in the CLASSIC than in the ACTS-GC trial. Toxic effects were mostly mild, although the duration of chemotherapy was longer in the ACTS-GC study. DFS curves from the CLASSIC study and recurrence-free survival (RFS) curves from the ACTS-GC study clearly separated between the chemotherapy and the surgery-alone arms; hazard ratios for DFS (CLASSIC) or RFS (ACTS-GC) were similar and relatively low. Interestingly, the hazard ratios were similar regardless of tumor stage in the CLASSIC study, whereas the hazard ratio increased for patients with more-advanced stage disease in the ACTS-GC trial. The primary difference was that the overall survival curves clearly separated in the

Practice point

In patients treated with D2 surgery for stage II or III gastric cancer, capecitabine and oxaliplatin without radiotherapy is an appropriate option.

ACTS-GC data but not in the CLASSIC data despite a similar median follow-up period.

Although D2 gastrectomy is globally accepted among surgeons as the surgical standard of care for curable gastric cancer, some medical oncologists claim that the difference in the overall survival between the Asian[3,8] and INT-0116[1] studies could be attributable to putative East–West differences in tumor biology that have yet to be documented for gastric cancer. In the editorial regarding the published data of the 5-year results of ACTS-GC,[8] Macdonald[9] completely ignored the significant and well-documented survival impact of surgical undertreatment. Indeed, recently reported results of a Korean study on postoperative chemoradiotherapy after D2 gastrectomy did not show a benefit from chemoradiotherapy over postoperative chemotherapy alone,[10] which confirmed that radiation simply compensated for the effect of D2 surgery in patients who underwent surgical undertreatment.

In conclusion, evidence supports two approaches to adjuvant chemotherapy after D2 gastrectomy for resectable gastric cancer: S-1 for 1 year, and capecitabine and oxaliplatin for 6 months. With capecitabine and oxaliplatin, the chemotherapy period is shorter but the treatment is more toxic compared with S-1. Balancing the risks and efficacy of treatment, S-1 seems to be appropriate for patients with stage II disease and capecitabine and oxaliplatin seems attractive for patients with stage III resectable gastric cancer. Direct comparison of these approaches should be undertaken after the long-term data from the CLASSIC trial are available.

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Competing interests

M. Sasako declares an association with the following companies: Chugai Pharmaceuticals, Sanofi-Aventis, Taiho Pharmaceuticals. See the article online for full details of the relationships. T. Yoshikawa declares no competing interests.

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GYNECOLOGICAL CANCER

First-line bevacizumab for ovarian cancer—new standard of care?

Susana Banerjee and Stan B. Kaye

Demonstration of the clinically significant activity of bevacizumab in advanced-stage ovarian cancer has attracted a great deal of interest. Here, we summarize the two positive phase III trials that led to EMA approval of bevacizumab as first-line therapy and discuss the optimum use of the drug in this disease.

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In December 2011, two positive phase III trials[1,2] that assessed bevacizumab in patients with ovarian cancer were reported in the *New England Journal of Medicine*; these results led to the EMA approval of the drug as first-line treatment in combination with carboplatin and paclitaxel for this disease.[3] Bevacizumab is currently the most widely tested antiangiogenic agent for the treatment of cancer. Bevacizumab is a monoclonal antibody that targets the VEGF pathway, which has a critical role in ovarian function as well as in the spread of ovarian cancer.[4] Therefore, positive results from clinical trials assessing bevacizumab in this notoriously difficult-to-treat disease have been eagerly anticipated.

The first study (GOG-0218) was reported by Burger *et al.*[1] and was a double-blind, three-arm, placebo-controlled study in 1,873 patients with newly diagnosed stage III (incompletely resected with residual disease >1 cm) or stage IV epithelial ovarian cancer. Patients were randomly assigned to one of three treatments: combination chemotherapy (carboplatin–paclitaxel), carboplatin–paclitaxel chemotherapy plus concurrent bevacizumab or carboplatin–paclitaxel chemotherapy plus concurrent and maintenance bevacizumab. The bevacizumab dose was 15 mg/kg for up to 22 cycles (15 months total). After a protocol amendment, stage III patients with macroscopic residual disease of ≤1 cm were also included. Nevertheless, all patients enrolled had advanced-stage disease and their overall outlook was worse than those patients assessed in the second study, ICON7.[2]

Perren *et al.*[2] published the results from the ICON7 study. The trial randomly assigned patients to one of two arms: 1,528 patients received carboplatin–paclitaxel chemotherapy with or without concurrent and maintenance bevacizumab. Bevacizumab was given at 7.5 mg/kg (half the dose used in GOG-0218) for a total of 18 cycles (12 months total). In this trial, 9% of patients had high-risk, early stage disease (FIGO stage I or IIA, clear cell or grade 3 histology) whereas 30% were at the

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PLOS ONE

Molecular Marker Identification for Relapse Prediction in 5-FU-Based Adjuvant Chemotherapy in Gastric and Colorectal Cancers

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Abstract

To confirm the clinical significance of NF-κB and JNK protein expression from experimentally identified candidates for predicting prognosis for patients with 5-FU treatment, we evaluated the protein expression of surgically removed specimens. A total of 79 specimens were obtained from 30 gastric and 49 colorectal cancer patients who underwent R0 resection followed by postoperative 5-FU based adjuvant chemotherapy. Immunohistochemical examinations of NF-κB and JNK on tissue microarrays (TMAs) revealed that significantly shorter time-to-relapse (TTR) in both NF-κB(+) and JNK(−) subgroups in both gastric (NF-κB(+), $p = 0.0002$, HR11.7. 95%CI3 3.2–43.4; JNK(−), $p = 0.0302$, HR4.4, 95%CI 1.2–16.6) and colon (NF-κB(+), $p = 0.0038$, HR36.9, 95%CI 3.2–426.0; JNK(−), $p = 0.0098$, HR3.2, 95%CI 1.3–7.7) cancers. These protein expression patterns also show strong discriminately power in gastric cancer patients for overall survival rate, suggesting a potential utility as prognostic or chemosensitivity markers. Baseline expression of these proteins using gastric cancer cell lines demonstrated the reciprocal patterns between NF-κB and JNK, while 5-FU exposure of these cell lines only induced NF-κB, suggesting that NF-κB plays a dominant role in the response to 5-FU. Subsequent siRNA experiments confirmed that gene knockdown of NF-κB increased 5-FU-specific sensitivity, whereas that of JNK did not affect the chemosensitivity. These results suggest that the expression of these proteins may aid in the decisions involved with adjuvant chemotherapy for gastrointestinal tract cancers.

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Introduction

Although several standard chemotherapeutic regimens have been established, there is still a great need to identify chemosensitivity or prognostic markers that allow for the prediction of cancer chemotherapy efficacy. The application of biomarkers with high discriminatory power can help clinicians avoid difficult chemotherapy regimens with unnecessary adverse effects as well as allow for an earlier decision to use alternative regimens. However, despite the use of several high throughput screening methods in this context, the identification of biomarkers has been difficult [1].

During the characterization of molecular and cellular characteristics of a panel of 12 human cancer cell lines, we developed a system in which a conventional *in vitro* chemosensitivity assay using clinically approved drugs combined with quantitative protein expression profiling using a 'reverse-phase' lysate array (RPA) was used to identify proteins that may be relevant to the activity of the chemotherapeutic agents [2]. Both technologies produce a quantitative output, which allows for the analysis of a large number of combinations between drug potency and protein expression [2,3]. Moreover, this system hypothesizes that the expression profile of a protein may be a predictor of chemosensitivity to a given drug. Subsequent validation is then required to determine if the markers are clinically relevant with regard to chemosensitivity.

A recent collection of individual patient data from colon cancer cases has revealed that 5-FU-based adjuvant chemotherapy provides a significant disease-free survival (DFS) benefit by reducing

the recurrence rate, which leads to a long-term overall survival (OS) benefit [4]. In East Asian countries, it has been well-accepted that resectable, locally advanced gastric cancer will benefit from 5-FU-based adjuvant chemotherapy such as S-1, which is an oral fluoropyrimidine, for prolonged OS and recurrence-free survival (RFS) [5,6]. However, approximately 30–40% of patients experience recurrence even after receiving a curative operation and 'standard' adjuvant chemotherapy [7,8]. Despite the intensive use of 5-FU for gastrointestinal cancers, to-date the markers for 5-FU have not achieved standard-of-practice usefulness [9].

In the present study, we collected 79 surgically removed cancer specimens from gastric and colon cancer patients who had not received any chemotherapy at the time of operation and later received 5-FU based adjuvant chemotherapy to determine if any of the markers were associated with TTR. We produced a tissue microarray (TMA) representing all 79 specimens on a glass slide and probed them with primary antibodies [2] that recognized a specific protein identified as a candidate marker for relapse. To confirm a direct association of protein expression and the 5-FU anti-tumor effect, we also performed gene knockdown by siRNA in several human cancer cell lines.

Materials and Methods

Ethics Statement

The study has been approved by Institutional Review Board at Iwate Medical University in compliance with the Helsinki declaration. An individual written consent was obtained from all patients and the absolute confidentiality was preserved even after the patient has died. All analyses were performed anonymously so individual patients were not identified.

Prediction of Proteins as Candidate Markers of Prognosis

We first performed a conventional chemosensitivity assay whereby 144 combinations of 12 anticancer drugs and 12 cell lines were evaluated for chemosensitivity using a 50% growth inhibition (GI_{50}) value (A matrix, Fig. 1A) [2]. The baseline expression level of 50 proteins from the cell line panel was quantitatively analyzed using a 'reverse-phase' lysate microarray (RPA) [10,11], which is a western blot in microscale dot format, followed by quantitative immunodetection (P matrix) [12], where each matrix is visualized based on average-linkage hierarchical clustering (Fig. 1B) [2,13]. A correlation of correlation between A and P matrices (AP matrix) was then established using the algorithm reported by Scherf et al. [14] (Fig. S1, S2). The AP matrix allows us to predict an association between protein expression and chemosensitivity. Using this method, we identified eight proteins based on 5-FU chemosensitivity (Fig. 1C, Table S1) [2].

Surgically Removed Specimens

Seventy-nine surgically removed specimens, including 30 gastric and 49 colorectal cases, were collected from patients who had not received any anticancer agents by the time of surgery. All surgical cases were conducted at the Department of Surgery at Iwate Medical University Hospital between 1997 and 2008. After the surgery, all cases were confirmed to meet the criteria for 5-FU-based adjuvant chemotherapy together with a final clinicopathological diagnosis (Table 1).

TMAs

A tumor-rich area of each tissue specimen was marked on a hematoxylin and eosin (H&E) stained section under a microscope. The core cylinder of the tumor-rich area from each specimen was punched out from the paraffin block using a manual tissue microprocessor (KIN-1, Azumaya, Japan) with a steel needle having an inner diameter of 2 mm [15]. The cylindrical cores were arrayed in recipient paraffin blocks. TMA sections (4 μm thick) were obtained using a standard preparation. In the present study, a core was punched out for each sample in the paraffin block, but some adjacent sections of strongly positive or negative samples were also examined to assess for any considerable heterogeneity.

Immunohistochemistry

The tissue specimens were incubated at 97°C in 1 mM EDTA pH 9.0 for 30 min in a microwave oven for antigen retrieval. They were then incubated with the primary antibodies NF-κB p65 (Cell Signaling Technology, Danvers, MA) and JNK/SAPK1 (BD BioSciences, Franklin Lakes, NJ) overnight at 4°C. Immunostaining was performed using a DAKO Envision+ system (DakoCytomation, Denmark) and an autostainer. For NF-κB staining evaluation, samples with more than 10% clear nuclear staining were designated as positive (Fig. 1D). For JNK staining evaluation, we first divided the staining strength into 4 grades, and subsequently divided them into two groups (negative and positive). The staining evaluation was focused on the nucleus for NF-κB, and on the cytoplasm for JNK, in epithelial components for both stainings [16]. Information on the primary antibodies used in the study is provided in Table S2. The final staining score was tabulated in a binary manner for statistical analyses.

Statistical Analysis

Associations between clinicopathological characteristics and immunohistochemical data were used to evaluate the significance among categorical variables by the Fisher's exact test or χ^2 test. TTR and OS were calculated from the date of operation to either the date of relapse, death, or censoring with Kaplan-Meier estimation by grouping protein expression scores. A Kaplen-Meier estimator between groups of immunohistochemical grades was compared with a log-rank test. Multivariate subset analysis using a Cox proportional hazard model was performed to explore the interaction between TTR/OS and to identify independent factors. All statistical analyses were performed using JMP 7.0 (SAS Institute, Cary, NC).

Molecular Marker Induction by 5-FU

Five human gastric cancer cell lines (GSS, HuG1-PI, KATOIII, KE39, and MKN45) were grown in RPMI1640 supplemented with 10% FBS. Baseline protein expression levels of the identified markers (NF-κB/p65 and JNK) were measured by western blot. Cells were trypsinized for cell lysate preparation according to a published protocol [17]. Nitrocellulose membranes were then incubated with the primary antibodies used for immunohistochemistry followed by chemoluminescent signal development (SuperSignal West Pico, Thermo Scientific, USA). To determine if the respective protein levels were enhanced by 5-FU, two different concentrations of 5-FU (50 and 100 μM) were added in the culture medium for four hours. Immunocytochemistry was also performed on four-chamber cell culture slides using the same primary antibodies described above followed by either Alexa488 or 564-conjugated secondary antibodies, respectively. A standard fluorescent microscope was used to examine the cellular localization of the proteins.

Target Gene Knockdown

Five human gastric cancer cell lines were grown to 70% confluency in RPMI1640 supplemented with 10% FBS in a 96-well plate. The cells were then treated with a cationic-lipofection reagent (*Trans*IT-TKO Transfection Reagent, Mirus, WI) in the

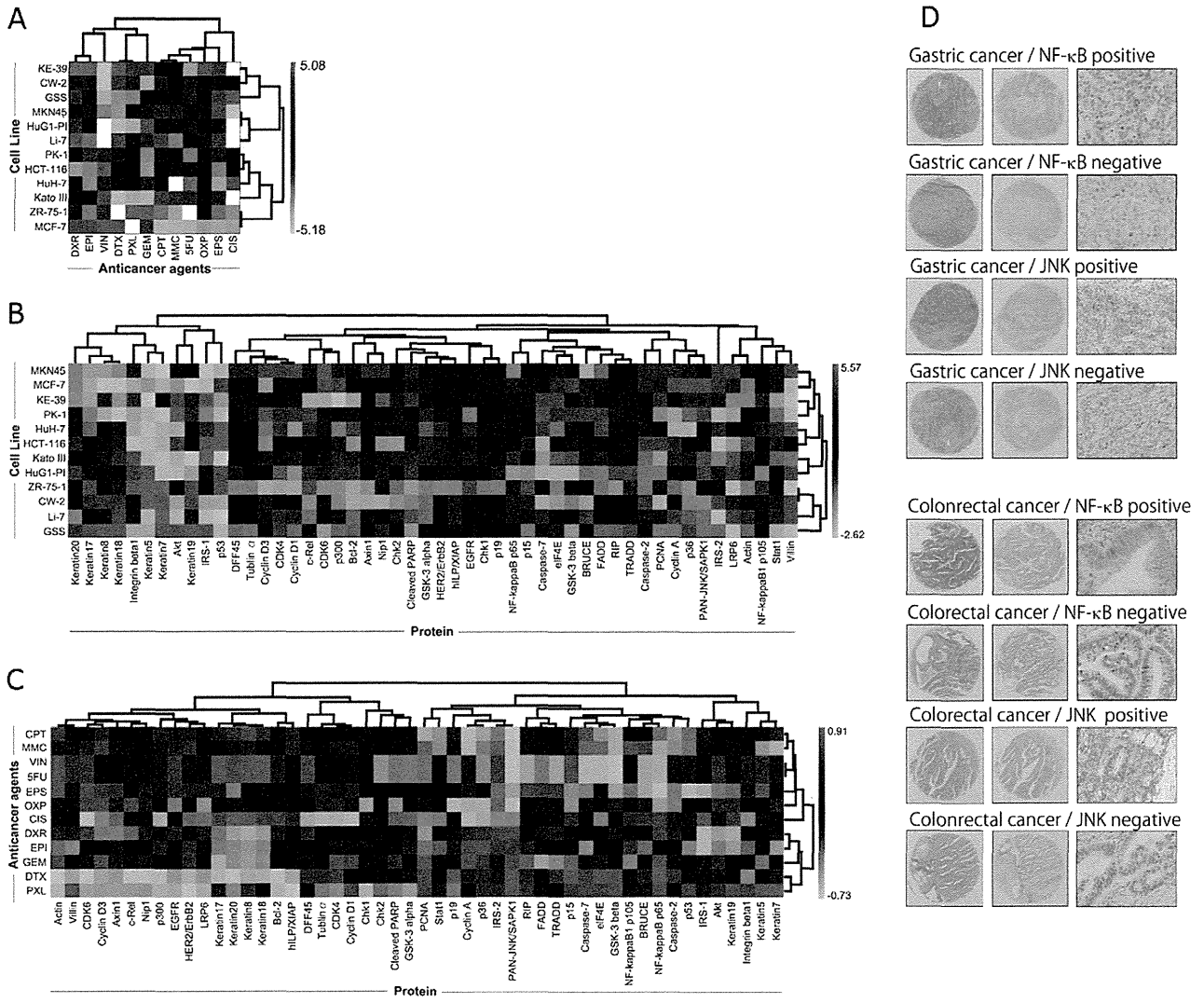

Figure 1. Hierarchical clustering of three different matrices and results of immunohistochemical examinations of candidate markers. (A) Based on a chemosensitivity assay of a cancer cell line panel, the A (activity) × C (cells) = AC matrix was created. (B) Quantitative protein expression data of each cell line determined by "reverse-phase" lysate microarray generates the C×P (protein) = CP matrix. (C) A heatmap with hierarchical clustering representation of the AP matrix, which is generated from AC and CP matrices. (D) Immunohistochemical stainings of candidate markers for 5-FU treatment.
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presence of siRNA specific for either NF-κB p65 or JNK gene transcripts (Signal Silence, Cell Signaling Technologies, MA) for 48 h. After the siRNA transfection, each drug (5-FU, cisplatinum, docetaxel, and paclitaxel) was added at a concentration that inhibited 50% cell growth (GI_{50}) for each cell line [2] and then incubated for an additional 48 h for the growth inhibitory assay (WST-1, Dojindo, Japan). The effect of NF-κB on growth suppression was evaluated if the growth was reduced to less than 50% by siRNA with a drug concentration at the GI_{50}. The effect of JNK siRNA was evaluated if cell growth was more than 50% of the control. All experiments were repeated at least three times. To verify the gene specific effect of siRNA, we have also performed an experiment using different siRNAs targeting p65 and JNK (SignalSilence siRNAI and siRNAII for NF-κB and SAPK/JNK, respectively; Cell Signaling Technology). The same trend was obtained for both siRNAs. Control samples were corresponding cell lines with siRNA transfection without anticancer drugs.

Results

Patients

The median age of the 79 patients was 68 years (range, 37–83 years), with 30 gastric cancer and 49 colorectal cancer patients. All 79 patients underwent either a gastrectomy or colorectomy with lymph node dissection. The operational curability was no residual tumor (R0) for all cases. Pathological findings revealed that all tumors invaded beyond the mascularis propria. Twenty-six (33%) of the patients had pathologically negative regional lymph node metastases, while no patients showed distant metastases. All patients satisfied the following criteria for 5-FU-based adjuvant chemotherapy: Histologically confirmed gastric (> Stage II) or colorectal (>T2) cancer with apparent R0 surgery; no hepatic, peritoneal, or distant metastasis; patient age between 20 and 85; no prior chemotherapy; and adequate organ function. Chemotherapy was considered to be completed if a patient was able to

Table 1. Postoperative Clinicopathological Characteristics in Cancer Patients for 5-FU Based Chemotherapy.

Characteristic	Total (n = 79)		Stomach (n = 30)		Colorectal (n = 49)	
	No.	%	No.	%	No.	%
Age, years						
Median	68		68		68	
Range	37–83		45–83		37–80	
Sex						
Male	49	62	19	63	30	61
Female	30	38	11	37	19	39
T factor						
High	53	67	16	53	37	76
Low	26	33	14	47	12	24
N factor						
High	53	67	28	93	25	51
Low	26	33	2	7	24	49
Stage						
High	42	53	17	57	25	51
Low	37	47	13	43	24	49
†Chemotherapy Completed						
Completed	60	76	25	83	35	72
Suspended	15	19	5	17	10	20
Unknown	4	5	0	0	4	8

TN factors and Stages are divided into the following binary categories: High (≥T3), and Low (T2); High (≥N1), and Low (N0); and High (≥Stage III), and Low (Stage I, II).

†Chemotherapy completed, continued chemotherapy for 0.5 years for colorectal and 1 year for stomach. Information on chemotherapy completion was not available in four cases. NA, not applicable.

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continue the following 5-FU based regimens: (i) S-1 (60 mg/m^2/body) for 1 year for gastric cancer; and (ii) either doxifluridine (800–1200 mg/day), 5-FU (370 mg/m^2/day), or UFT-E granules (300 mg/m^2/day) for six months for colorectal cancer. Sixty (76%) patients completed chemotherapy, 15 (19%) patients suspended treatment, and 4 (5%) patients had missing information.

The median observation time after operation was 3.41 and 5.04 years in stomach and colon, respectively (range, 1.41–7.00 years in stomach; and 0.99–10.24 years in colon). In non-relapsed cases, the minimum observation time was 2.43 and 2.36 years in stomach and colon, respectively. The median TTR was 1.56 and 1.52 years in stomach and colon, respectively (range, 0.72–3.33 years in stomach; and 0.27–4.47 in colon). Among 77 cases of which survival status was confirmed, the 3-year overall survival rate in the relapsed and non-relapsed groups was 0.60 and 0.65 in stomach and 0.97 and 0.96 in colon, respectively. The clinicopathological parameters on the basis of relapse status are shown in Table S3.

Immunohistochemistry

The immunostaining scores of all candidate proteins were evaluable (Fig. 1D and Fig. S3). NF-κB showed distinct nuclear staining that was scattered throughout the nuclei and did not form clusters. Some cells showed cytoplasmic staining, but it was not as distinct as those with nuclear staining. The staining of JNK was not as strong as that of NF-κB but was clearly localized in the cytoplasm. The remaining six candidate proteins and three proteins of interest were in their expected subcellular locations (Fig. S3). After determining the subcellular localization of the proteins, the strength of staining was scored in a binary manner.

Correlation between Protein Expression and Clinicopathological Findings

A contingency parameter analysis of each protein in terms of relapse revealed that the protein levels of NF-κB and JNK were significantly associated with relapse in stomach ($p = 0.0004$ and 0.029 for NF-κB and JNK, respectively; Table S4). When the expression of these two proteins was combined, the contingency analysis demonstrated a stronger discriminating power than each individual protein.

Based on a Kaplan-Meier analysis, JNK and NF-κB expression levels were associated with significant differences in the non-relapse rate (Fig. 2). A log-rank test of the Kaplan-Meier analysis showed a significant difference in the non-relapse rates between the JNK(+) and JNK(−) groups in both stomach ($p = 0.0302$, HR4.4, 95%CI 1.2–16.6) and colon ($p = 0.0098$, HR3.2, 95%CI 1.3–7.7); and also between the NF-κB(+) and NF-κB(−) groups in both stomach ($p = 0.0002$, HR11.7, 95%CI 3.2–43.4) and colon ($p = 0.0038$, HR36.9, 95%CI 3.2–426.0, Fig. 2). Interestingly, in stomach, all NF-κB(+) cases were JNK(−), whereas 58% of JNK(−) cases were NF-κB(+). The probability of relapse when these markers were combined showed greater difference than using the individual markers in both stomach and colon (Fig. 2).

We also screened p53, Tymidine Synthetase, and MDR-1 expression in pooled stomach and colon samples because it has been suggested that these proteins or encoding genes may be associated with 5-FU drug potency [18,19,20,21]. However, no significant association was observed between the relapse rate and the expression level of these proteins (Fig. S4).

Of the 3-year OS rate of gastric cancer, NF-κB(−) and (+) cases was 0.94 and 0.77, respectively, and 0.82 and 1.00 for JNK(−) and (+), respectively. Of the 3-year OS rate of colon cancer, NF-κB(−) and (+) cases was 0.79 and 1.00, respectively, and 0.72 and 0.90 for JNK(−) and (+), respectively. However, there was a significant difference in the OS rate by Kaplan-Meier estimation in gastric cancer (NF-κB(+), HR 7.9, 95%CI 2.1–30.3; JNK(−), HR 0.25, 95%CI 0.06–1.09, Fig. S5).

Subset Analysis

To identify general relationships between markers and clinicopathological findings, a subset analysis was performed with stomach and colorectal pooled samples. There was a significant association between NF-κB status and T-factor/Stage for TTR (Fig. S6), but no significant association was observed between JNK status and any variables for TTR (Fig. S7). Interestingly, however, there was a significant association between the combined marker status: and T-factor/Stage for TTR, and chemotherapy completion status for OS (Fig. S8, S9). The association between OS and the status of each factor was also analyzed according to sex, age, lesions, TNM classifications, and the status of chemotherapy completion (Fig. S10, S11). A multivariate analysis revealed that NF-κB expression and the T factor were independent factors for both relapse and survival.

Molecular Responses by 5-FU

Baseline protein expression of NF-κB and JNK was measured by western blot. Interestingly, the expression pattern was reciprocal; cell lines with high NF-κB expression showed relatively low JNK expression (Fig. 3A). The reciprocal expression pattern

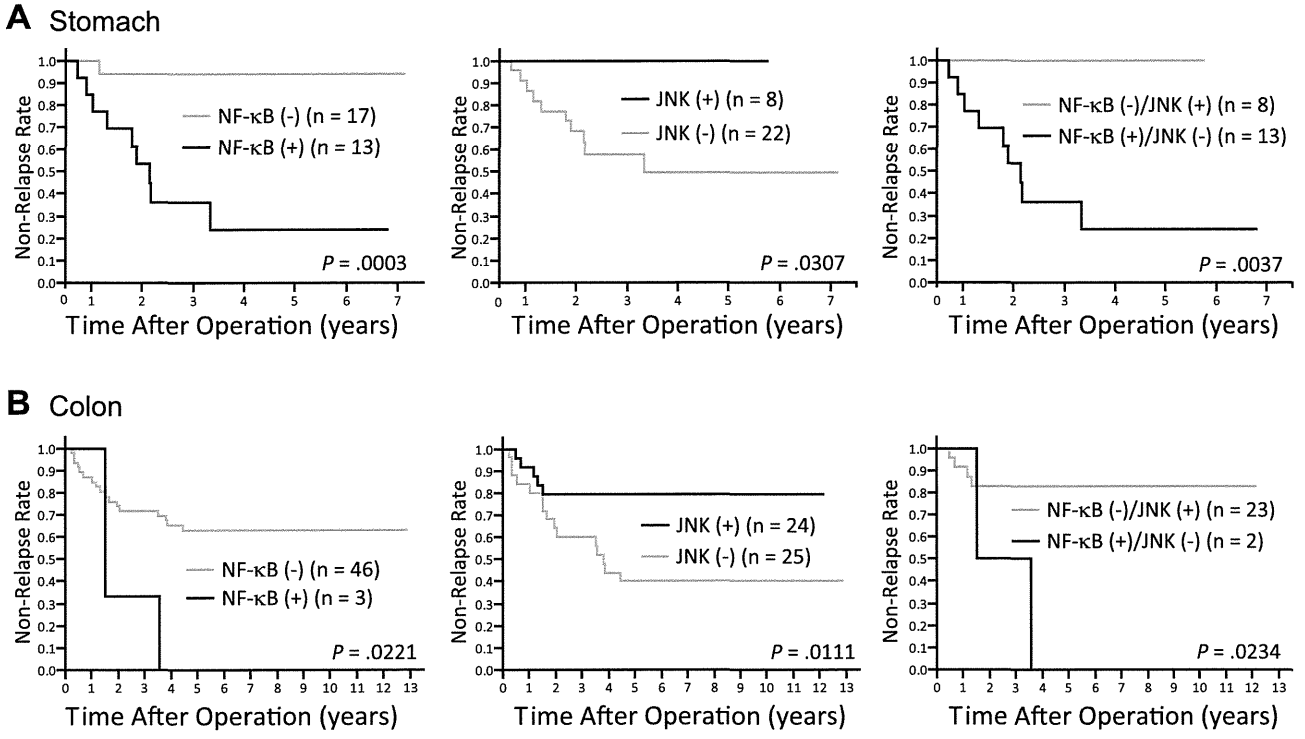

Figure 2. Time-to-relapse (TTR) rates on the basis of NF-κB and JNK protein expression in gastric and colon carcinomas.
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was concordant with the directionality of these proteins as biomarkers. We also tested the protein induction by 5-FU. NF-κB expression was induced and increased in the total protein fraction by 5-FU in a dose-dependent manner (Fig. 3B). Subsequent immunocytochemical analysis revealed that nuclear NF-κB was prominently visualized after 5-FU exposure, while JNK did not exhibit a noticeable change in localization (Fig. 3C).

The Effect of NF-κB p65 and JNK Gene Knockdown on Cell Growth

Four out of five cell lines (GSS, KATOIII, KE39, and MKN45) exhibited significant growth suppression after NF-κB siRNA transfection and 5-FU treatment ($p<0.05$, Student t-test; Fig. 3D, Fig. S12). The 5-FU-dependent, statistically significant growth suppression was seen in GSS, KATO-III, and MKN45. JNK siRNA treatment was expected to induce an anti-apoptotic effect based on previous studies [22], which was hypothesized to increase the growth rate in the presence of anticancer drugs. Four out of five cell lines treated with JNK siRNA demonstrated 5-FU-mediated growth induction or no change. These siRNA experiments revealed that NF-κB and JNK seem to have reciprocal roles in terms of 5-FU-mediated growth suppression.

Discussion

Although the choice of adjuvant chemotherapy after resection of gastric cancer is slightly different between countries, 5-FU has been shown to be the most effective treatment option. Validation of postoperative chemotherapy and surgery alone has demonstrated 3-year OS rates of 80.1% and 70.1%, respectively, in Japan [23]. Postoperative chemoradiotherapy (CRT) has been conducted in the United States for advanced gastric cancer, and the OS after CRT has been reported to be 50%, while that of surgery alone was 41% [24]. In Europe, the MAGIC trial revealed the efficacy of perioperative chemotherapy and demonstrated that the 5-year OS was 36.3% and 23.0% in the perioperative chemotherapy and surgery alone groups, respectively [25]. On the other hand, adjuvant chemotherapy (5-FU/LV) for advanced colorectal cancer was shown to improve OS in the 1990s, and more recently a further improvement of DFS and OS has been observed with the addition of oxaliplatin [26]. In general, adjuvant chemotherapy for colorectal cancer is only administrated for six months, but has been shown to provide long-term benefits, including prolonged 5-year DFS and OS [4,26].

With a standard of care established, we now face the issue of how to treat patients who fail these standard therapies. In fact, 30–40% of patients treated with the standard adjuvant chemotherapy experience recurrence after surgery within five years in advanced gastric and colon carcinomas [8,27]. Therefore, it has been an important goal to improve treatment regimens for the subset population where standard therapy may be ineffective.

In the present study, to validate the utility of identified markers, we collected archived tissues from patients that had undergone curative resection for gastric and colorectal cancer. All patients were eligible to receive adjuvant chemotherapy, including patients with stage I/II colorectal cancer. A previous report showed that among 769 mp cases from a retrospective cohort of Japanese patients, the recurrence rate of stage I (mp, N0) patients was 7% [28]. Although the number of patients was limited, the QUASAR study reported that a higher percentage of stage I patients (25%) who had received surgery alone died within five years compared to patients who received adjuvant chemotherapy [29]. Moreover, it has been reported that circulating tumor cells were found in 6% of stage I colorectal cancer patients after curative operation [30].

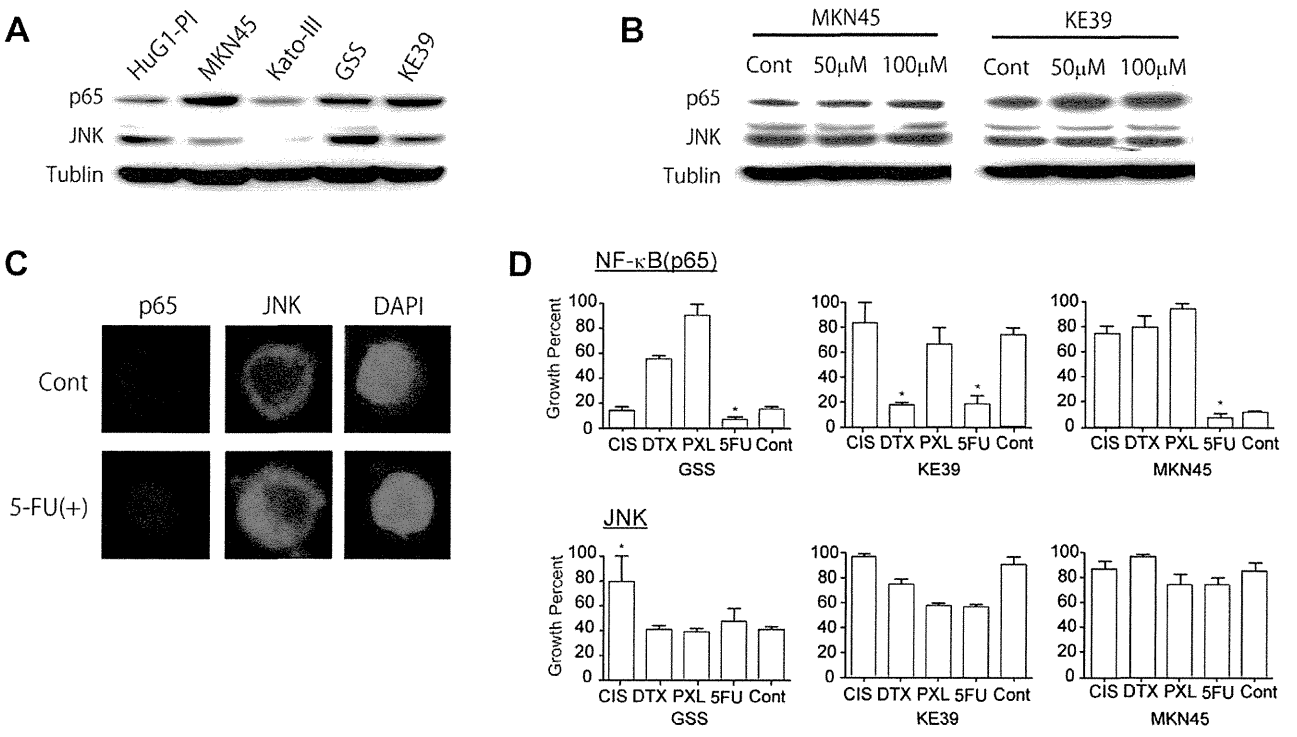

Figure 3. Induction of biomarkers by 5-FU treatment. (A) Baseline protein expression of NF-κB and JNK in five gastric cancer cell lines. Tublin was used as a loading control. (B) Induction of candidate biomarkers in response to 5-FU treatment in different concentrations in MKN45 and KE39. (C) Examination of protein localization by fluorescent immunocytochemistry using MKN45. (D) Increased inhibitory growth effect by anticancer agents in gastric cancer cell lines after transfection of siRNA for NF-κB p65 and JNK transcripts. Control samples are the corresponding cell lines transfected with the indicated siRNAs without anticancer agents. Abbreviations are: CIS, cisplatinum; DTX, docetaxel; and PXL, paclitaxel; and 5FU, 5-fluorouracil. *$p<0.05$, Student *t*-test.
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These clinical and biological findings should make it reasonable the inclusion of Stage I in the present study.

It has been reported that approximately 25% of patients with stage II tumors are considered to have an increased risk of recurrence because of: (a) penetration of the serosa (T4); (b) extramural venous invasion; (c) poorly differentiated histology; (d) presentation of an obstruction; or (e) having a yield of less than 10–12 lymph nodes [31]. In the present study, most of the stage II colorectal cases possessed one of these risk criteria, except in one case where the patient was young (37-years-old), for which the QUASAR study justified the eligibility. Although the use of chemotherapy in stage I is not recommended and Stage II cases has been controversial, we conducted the validation because it aimed to select an individual who may not benefit from "standard" chemotherapy or have a potential risk in lower stages, which is in contrast to the approach of epidemiological studies.

Previous reports have demonstrated the significance of NF-κB in prognosis, angiogenesis, and chemoresistance in stomach and colon carcinomas [32,33,34,35,36]. Our present data demonstrated that the nuclear localization of NF-κB could predict the outcome of patients at the time of operation who subsequently receive adjuvant chemotherapy. Interestingly, NF-κB(+) tumor cells were found scattered in the sections in which there was a clear distinction between positive and negative cells. Molecular experiments revealed a clear reciprocal relationship between NF-κB and JNK expression, which suggested a potential association with 5-FU therapy and the pathological findings. To clarify the role of NF-κB and JNK in the tumor response to 5-FU, we conducted gene knockdown experiments. Knockdown of the NF-κB (p65) gene revealed that the majority of cancer cell lines tested demonstrated clear 5-FU-specific growth suppression, while other drugs even induced cell growth. This result suggests a direct association between 5-FU sensitivity and NF-κB expression and supports the diagnostic application of this analysis for 5-FU-based adjuvant chemotherapy. It should also be noted that taxans and topoisomerase inhibitors activate the NF-κB pathway, which leads to cell proliferation through MYC and IKK activation, respectively [33]. The clinical implications of these mechanisms remain to be elucidated.

NF-κB has been implicated in the development of drug resistance in a wide range of cancer cells. Inhibition of NF-κB activation reduced chemoresistance in gastric and colorectal cancer cell lines, which is consistent with our present results [6,32,37,38]. Constitutive activation of NF-κB has been suggested as a potential prognostic factor in gastric cancer [39,40,41] and correlates with the progression and chemotherapy resistance of colorectal carcinomas [42,43].

JNK proteins have diverse functions on cell proliferation and on the induction of apoptosis through stress-activated protein kinase pathways, and are often down-regulated in cancers [44,45]. In the present study, the role of JNK in the context of 5-FU response seems to be passive with respect to chemosensitivity, according to our siRNA experiment. The immunohistochemical analysis in this study showed that NF-κB and JNK were reciprocal indicators of prognosis. However, knockdown of NF-κB sensitized 5-FU, while JNK did not make cells resistant to 5-FU. NF-κB activation by TNF-α is tightly regulated by JNK in the context of a proinflammatory response, which occurs immediately after stim-

ulation [46,47,48,49]. On the other hand, activation of NF-κB in malignancy or chemoresistance seems to be constitutive in a part of gastrointestinal tumor progression [47,50]. In the present study, although NF-κB nuclear staining was only seen in a small fraction of tumor cells, JNK was stained relatively ubiquitously throughout the tissue. Therefore, our current results may indicate that JNK staining reflects a degree of background chronic inflammatory or stress responses of gastric mucosa [51], while NF-κB constitutive activation is associated with the malignant potential of the tumor cells [39,40,41,52]. In addition to the intrinsic malignant potential, our results also demonstrated that NF-κB plays a specific role in 5-FU response. Taken together, although a larger clinical research is required, NF-κB nuclear expression may be a good candidate as a 5-FU chemosensitivity prediction marker, while JNK may be a supportive marker that reflects the background mucosal information.

Although the present result is still preliminary from a practical point of view, these results may provide an opportunity for alternative regimens to be considered for cases that indicate a low probability of a 5-FU-based chemotherapeutic response. A larger immunohistochemical study that includes NF-κB/JNK analyses will be necessary to prove the utility in gastric and colorectal cancers.

Supporting Information

Figure S1 Flow of Chemosensitivity Marker Identification. (A) Based on a chemosensitivity assay of a cancer cell line panel, the A (activity) × C (cells) = AC matrix was created. The left two panels show cell growth curves on the basis of drug concentration. The middle panel shows the 50% growth inhibition (GI_{50}) values in a bar graph. All data are centered by Peak Plasma Concentration (PPC) values that are unique for each drug. The right panel represents the GI_{50} values and the cells in a heatmap with a hierarchical clustering format. (B) "Reverse-phase" protein lysate microarray (left) and C (cells) × P (protein) = CP matrix in a heatmap with a hierarchical clustering format (right). (C) A heatmap with hierarchical clustering representation of the AP matrix, which is generated from AC and CP matrices. The dendrogram indicates the distance based on the correlation coefficient of the data set next to each other. Hence, the AP matrix shows the correlation between protein expression and drug efficacy across all cell lines. Cited with permission from reference #2.
(TIF)

Figure S2 Correlation between candidate proteins and drug sensitivity. Left: Scattergram based on 5-FU sensitivity and NF-κB expression. The correlation coefficient is positive, but is negative ($r = -0.304$) when the gastrointestinal cell lines (CW2, HCT116, GSS, KATOIII, MKN45, HuG1-PI, and KE39) were analyzed, which is consistent with the validation result from the TMAs. Right: Scattergram based on 5-FU sensitivity and JNK expression. It has been well-accepted that screening tools, such as microarray-based techniques, can discover useful biomarkers, but may also isolate false-positives. The correlation coefficient of NF-κB and drug sensitivity was positive for the screening, which was expected to identify a trend whereby higher protein expression correlated with higher drug sensitivity; however, the result was opposite. A possible explanation for the discrepancy is that the number of cell lines for the screening may be too small. In fact, most of the gastrointestinal cell lines lined up as a "negative slope", which is consistent with the clinical result. As expected, subsequent confirmation molecular analysis revealed the association between NF-κB and 5-FU.
(TIF)

Figure S3 Immunohistochemical staining of candidate proteins on TMAs. The TMAs were used to validate expression of 9 proteins. Each protein shows a set of 6 panels. The top rows represent positive staining, while the bottom row represents the corresponding negative samples. From the left, H&E staining (40x), a low power immunohistochemical image (40x), and a high power immunohistochemical image (400x). The level of staining for each specimen was scored in a binary manner.
(TIF)

Figure S4 Time-to-relapse (TTR) on the basis of candidate protein expression. TTP was compared on the basis of candidate protein expression in a binary manner from immunohistochemical staining of the TMAs. There were 79 patients assessed, including both gastric and colorectal cancer patients.
(TIF)

Figure S5 Kaplan-Meier estimation of the non-relapse rate and Overall Survival (OS), depending on the lesions, based on NF-κB and JNK expression. (A) Stomach, and (B) Colon.
(EPS)

Figure S6 Hazard ratio for relapse and p values for the interaction of NF-κB status and clinical subgroup categories.
(TIF)

Figure S7 Hazard ratio for relapse and p values for the interaction of JNK status and clinical subgroup categories.
(TIF)

Figure S8 Hazard ratio for relapse and p values for the interaction of NF-κB/JNK status and clinical subgroup categories.
(TIF)

Figure S9 Hazard ratio for death and p values for the interaction of NF-κB/JNK status and clinical subgroup categories.
(TIF)

Figure S10 Hazard ratio for death and p values for the interaction of NF-κB status and clinical subgroup categories.
(TIF)

Figure S11 Hazard ratio for death and p values for the interaction of JNK status and clinical subgroup categories.
(TIF)

Figure S12 Enhanced growth inhibitory effect by p65 gene knock down. Growth inhibitory effect of anticancer drugs at a concentration that elicits a 50% growth inhibitory (GI50) effect after 48 h of incubation in gastric cancer cell lines after transfection of siRNA for NF-κB p65 subunit (A) and JNK (B).
(TIF)

Table S1 Candidate Markers Identified From Quantitative Protein Expression Analysis and Chemosensitivity Assay
(DOC)

Table S2 Primary antibodies Used for Candidate Marker Validation on TMAs
(DOC)

Table S3 Clinicopathological Features of the State of Relapse
(DOC)

Table S4 Clinicopathological Features of Immunohistochemical Status
(DOC)

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Author Contributions

Conceived and designed the experiments: SSN CM GW. Performed the experiments: KI SSN TC MI KK FE HK TM HN T. Iwaya NY GT. Analyzed the data: KI SSN. Contributed reagents/materials/analysis tools: HF MT T. Itabashi NU TS KO KK. Wrote the paper: KI SSN.

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Gastric Cancer
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ORIGINAL ARTICLE

Overweight is a risk factor for surgical site infection following distal gastrectomy for gastric cancer

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Abstract
Background Our objective was to assess the risk factors for surgical site infections (SSIs) in gastric surgery using the results of the Osaka Gastrointestinal Cancer Chemotherapy Study Group (OGSG) 0501 phase 3 trial.
Methods The OGSG 0501 trial was conducted to compare standard prophylactic antibiotic administration versus extended prophylactic antibiotic administration in 355 patients who underwent open distal gastrectomy for gastric cancer. Various risk factors associated with the incidence of SSI following gastrectomy were analyzed from the results of this multi-institutional randomized controlled trial.
Results Among the 355 patients, there were 24 SSIs, for an overall SSI rate of 7 %. Multivariate analysis using eight baseline factors (administration of antibiotics, age, sex, body mass index [BMI], prognostic nutritional index, tumor stage, lymph node dissection, reconstructive method) identified that BMI $\geq$25 kg/m^2 was an independent risk factor for the occurrence of SSI (odds ratio 2.82; 95 % confidence interval [CI] 1.05–7.52; $P = 0.049$). BMI also showed significant relationships with the volume of blood loss and the operation time ($P = 0.001$ and $P < 0.001$, respectively).
Conclusion Compared with patients of normal weight, overweight patients had a significantly higher risk of SSI after distal gastrectomy for cancer.

Keywords Overweight · SSI · Gastric cancer · Gastrectomy · Obesity

Introduction

Surgical site infection (SSI) is one of the most common nosocomial infections, accounting for 14–16 % of nosocomial infections overall, and 38 % of nosocomial infections among surgical patients [1]. Previous studies on SSIs have provided feedback to surgeons and healthcare workers, and are important contributors to strategies for reducing the risk of SSI. Several studies concerning SSIs following gastric surgery have been conducted and reported. Prospective trials involving patients undergoing gastrointestinal surgery have reported some factors, such as overweight and hypo-albuminemia, which increase the risk of deep or organ SSI [2, 3].

Previously, we conducted a phase 3 randomized controlled trial (RCT), the Osaka Gastrointestinal Cancer Chemotherapy Study Group (OGSG) 0501, to compare standard antimicrobial prophylaxis administration versus extended antimicrobial prophylaxis administration in patients receiving open distal gastrectomy for gastric

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cancer, and the results concerning the primary and secondary endpoints have been reported [4]. Because the OGSG 0501 trial was based on a single elective surgical procedure performed under uniform conditions, it is worthwhile to analyze the risk factors associated with SSIs following gastrectomy, using the data of the OGSG 0501.

Patients and methods

From June 2005 to December 2007, 355 gastric cancer patients underwent open distal gastrectomy under general anesthesia at multiple institutions. All 355 patients, 174 with Billroth-I reconstruction, 165 with Roux-en-Y reconstruction, and 16 with other methods of reconstruction following gastrectomy were included in the statistical analysis.

We defined SSI according to the surgical patient component of the 1999 Centers for Disease Control and Prevention (CDC) National Nosocomial Infection Surveillance (NNIS) System manual [1, 5, 6]; this definition includes superficial, deep, and organ/space SSIs. The patients were monitored for SSI according to the NNIS criteria until 30 days after the operation at each institution. The definitions of SSI are listed below [4, 5].

Superficial incisional SSI

Infection involves only skin or subcutaneous tissue of the incision and at least one of the following: purulent drainage, with or without laboratory confirmation, from the superficial incision; organisms isolated from an aseptically obtained culture of fluid or tissue from the superficial incision; and at least one of the following signs of infection: pain or tenderness, localized swelling, redness or heat, and superficial incision that has been deliberately opened by the surgeon, unless the incision is culture-negative.

Deep incisional SSI

Infection involves deep soft tissues (e.g., fascial and muscle layers) of the incision and at least one of the following: purulent drainage from the deep incision but not from the organ or space component of the surgical site; a deep incision spontaneously dehisces or is deliberately opened by a surgeon when the patient has at least one of the following signs or symptoms: fever (>38 °C localized pain, or tenderness, unless the site is culture-negative; or an abscess or other evidence of infection involving the deep incision is found on direct examination, during reoperation, or by histopathological or radiological examination.

Organ or space SSI

Infection involves any part of the anatomy (e.g., organs or spaces), other than the incision, which was opened or manipulated during an operation and at least one of the following: purulent drainage from a drain that is placed through a stab wound into the organ or space; organisms isolated from an aseptically obtained culture of fluid or tissue in the organ or space; or an abscess or other evidence of infection involving the organ or space that is found on direct examination, during reoperation, or by histopathological or radiological examination.

Risk factors considered in the present study included age, sex, body mass index (BMI), preoperative laboratory data (white blood cell number, lymphocyte number, albumin, and prognostic nutritional index [PNI]), gastric carcinoma stage, and operative characteristics (duration of surgery, operative blood loss, extent of lymph node dissection, operative curability, and method of reconstruction following gastrectomy). According to the World Health Organization classification, BMI $\geq$25 is considered as overweight and BMI <25 as non-overweight [7]. The operation and disease staging were performed according to the guidelines for clinical and pathologic studies in the 13th edition of the *Japanese classification of gastric carcinoma* [8]. PNI was calculated as follows: PNI = 10 $\times$ albumin (mg/dl) + 0.005 $\times$ lymphocyte number (cells/mm^3) [9]. There were no patients who underwent neoadjuvant chemotherapy.

Outline of OGSG 0501, as the original trial

The protocol for the prospective study OGSG 0501 was reviewed and approved by the ethics review board of each participating institution. Eligible patients at each institution participating in the Osaka Gastrointestinal Cancer Chemotherapy Study Group (OGSG) provided written consent to participate in the trial, clinical follow up, and data collection. The OGSG 0501 was a multi-institutional RCT to evaluate the optimal duration of prophylactic antibiotic administration in patients initially planned to have distal gastrectomy with D2 lymphadenectomy for gastric cancer. Patients were randomly assigned to either the standard antibiotic prophylaxis group (standard group) or the extended prophylactic antibiotic group (extended group). The standard group received 1 g of cefazolin less than 30 min before the incision and every 3 h intraoperatively. The extended group received 2 g/day of cefazolin on postoperative days 1 and 2 in addition to receiving the same dose as that given to the standard group. The primary endpoint of OGSG 0501 was the incidence of SSIs. Analysis was based on the intention-to-treat principle. The

results concerning the endpoints and other details of the study design have been reported [4].

The OGSG 0501 trial was registered with the University Hospital Information Network (UMIN-CTR) (http://www.umin.ac.jp/ctr/) under identification number UMIN000000631.

Statistical analysis

All enrolled patients were divided into two groups according to whether or not they developed SSI postoperatively. All factors were compared between the two groups by univariate analysis, i.e., the χ^2 test or Fisher's exact test for categorical variables, or a two-sided Mann–Whitney *U*-test for continuous variables.

Multivariate analysis was also performed using a logistic regression model to assess the effects of the factors on SSI. A P value of <0.05 was considered to be statistically significant. Statistical analyses were performed with SPSS version 17.0 (SPSS Japan, Tokyo, Japan).

Results

There were 355 distal gastrectomies (176 patients in the standard group, 179 patients in the extended group) performed as inpatient procedures for gastric cancer (Fig. 1). The baseline patient and operative characteristics are shown in Table 1. The results concerning the detailed patient characteristics can be referred to in the previously reported data [4]

The overall SSI rate for open distal gastrectomy was 7 % (24/355), 5 % (8/176) for the standard group, and 9 % (16/179) for the extended group. Six patients had superficial type SSIs and 18 had organ/space type SSIs. There were no deep SSIs.

Univariate analysis of risk factors for SSI demonstrated that extended administration of antibiotics, male sex, BMI $\geq$25 kg/m^2, and duration of operation >200 min were associated with a higher, but non-significant, incidence of SSIs ($P = 0.105$, $P = 0.098$, $P = 0.158$, and $P = 0.076$, respectively). However, multivariate analysis revealed that only BMI $\geq$25 kg/m^2 was independently associated with an increase in the incidence of SSIs (odds ratio 2.82; 95 % confidence interval [CI] 1.05–7.52; $P = 0.049$) (Table 2). For the risk factors in the multivariate analysis, we included only the baseline factors, because if operative data, such as duration of operation and blood loss, had been added for the analysis, the results would have been confusing.

Subgroup analysis showed that surgery for the patients with BMI $\geq$25 resulted in a larger volume of blood loss and a longer duration of operation when compared with finding in patients with BMI <25 ($P = 0.001$ and $P < 0.001$, respectively) (Table 3).

The relationship between BMI and the type of SSI (superficial or organ/space) was not significant (Table 4).

Discussion

The present study focused on the risk factors for SSI. The risk of SSI after gastric surgery for gastric cancer was

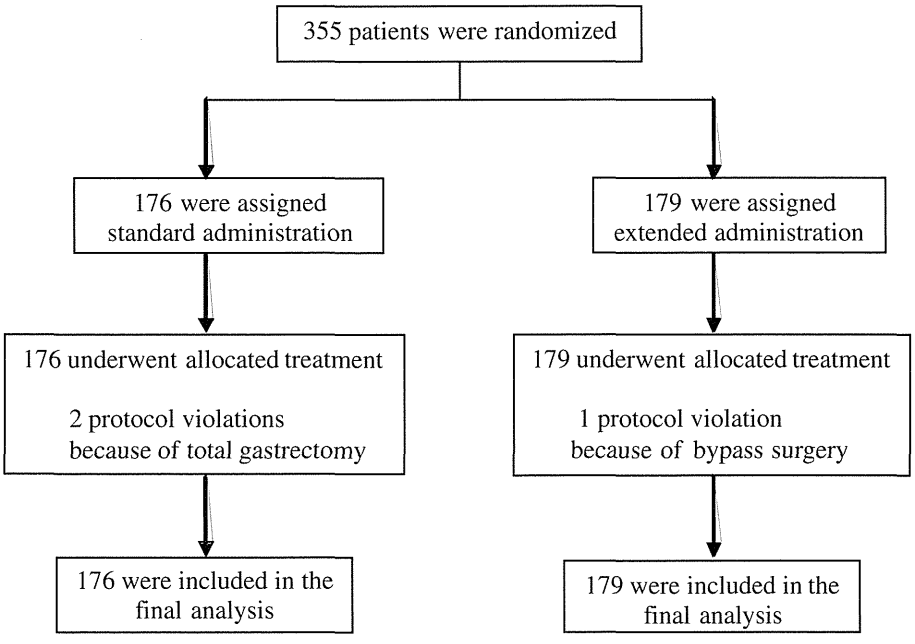

Fig. 1 CONSORT flowchart of Osaka Gastrointestinal Cancer Chemotherapy Study Group (OGSG) 0501 trial. *Administration* administration of antibiotics

statistically evaluated using a logistic regression model. Our data suggest that the risk of SSI depends on whether the patient's BMI is less than 25 or 25 or greater.

Investigators have reported the overall SSI incidence for open distal gastrectomy to be in the range of 10–16 % [3, 10]. The incidence was 7 % in the present study. Watanabe et al. [10] reported a higher incidence of organ/space SSIs than superficial and deep incisional SSIs in gastric surgery. However, many investigators have reported that colorectal surgery is more frequently associated with superficial incisional SSIs than with deep incisional or organ/space SSIs [10–12]. Complications specific to gastric surgery with lymphadenectomy, such as pancreatic fistula, may affect the incidence of organ/space SSIs. The difference in thickness between upper and lower abdominal subcutaneous tissues may also affect the incidence of various types of SSIs. In our study, the relationship between BMI and the type of SSI (superficial, deep, and organ/space) was not significant.

The impact of BMI on specific complications after elective abdominal or general surgery, especially colorectal surgery for cancer, has been assessed. SSI is the most common complication after colectomy, and obesity or overweight is thought to increase this risk by 2.5- to 5-fold as compared with patients of normal weight [13–16]. This risk may be related to the decreased oxygen tension in relatively avascular adipose tissue, differences in wound healing, greater wound size, or technical difficulties [13, 17]. However, another report suggests that obesity or overweight is not a risk factor for SSI after colectomy [18].

Recently, risk factors associated with SSI in upper gastrointestinal surgery have been reported. Watanabe et al. [10] reported that in upper alimentary tract surgery, significant relationships were observed between the incidence of SSI and both intraoperative blood loss and combined resection procedures, but BMI was not associated with the incidence of SSI. Imai et al. [19] found, in a retrospective study, that diabetic gastric surgery patients had a 2.7-fold higher risk of SSI as compared with the patients without diabetes, open surgery had a 1.9-fold higher risk of SSI as compared with laparoscopic surgery, and operations lasting for 6 h or longer had a 2.8-fold

Table 1 Baseline and operative characteristics of study patients ($n = 355$)

Age (years)[a]	65 (35–84)
Sex, male/female	240/115
BMI (kg/m^2)[a]	22.4 (12.4–33.0)
Stage IA/IB/II/IIIA/IIIB/IV	189/58/47/24/17/20
Antimicrobial prophylaxis administration	
Extended/standard	179/176
White blood cell number (cells/mm^3)[a]	5700 (2890–10800)
Lymphocyte number (cells/mm^3)[a]	1814 (510–4679)
Hemoglobin (mg/dl)[a]	13.4 (7.0–18.4)
Albumin (mg/dl)[a]	4.2 (2.0–5.3)
PNI[a,b]	51.42 (25.1–68.9)
Duration of surgery (min)[a]	204 (58–428)
Operative blood loss (ml)[a]	200 (0–1700)
Lymph node dissection D0/1/2/3	16/96/240/3
Curability R0–1/2	332/23
Reconstruction method BI/BII/RY/other	174/4/165/12

BMI body mass index, *BI* Billroth-I reconstruction, *BII* Billroth-II reconstruction, *RY* Roux-en-Y reconstruction

[a] Values are expressed as medians (ranges)

[b] PNI (prognostic nutritional index) was calculated as follows: PNI = 10 × albumin (mg/dl) + 0.005 × lymphocyte number (cells/mm^3)

Table 2 Univariate and multivariate analysis for the risk of SSI ($n = 355$)

	SSI present ($n = 24$)	SSI absent ($n = 331$)	Univariate logistic	Multivariate logistic	
			P value	Odds ratio (95 % CI)	*P* value
Extended administration of antibiotics	16	163	0.105	1.89 (0.72–4.93)	0.167
Age >65 years	14	173	0.566	1.15 (0.46–2.89)	0.535
Male sex	20	220	0.098	2.22 (0.69–7.09)	0.179
BMI $\geq$25 kg/m^2	8	69	0.158	2.82 (1.05–7.52)	0.049*
PNI >50	14	169	0.531	3.70 (0.61–22.7)	0.412
Stage >III	4	57	0.945	1.06 (0.29–3.88)	0.516
Lymph node dissection D2 or 3	16	227	0.846	1.08 (0.41–2.85)	0.885
Reconstruction BII or RY	13	156	0.506	1.33 (0.52–3.41)	0.528
Duration of surgery >200 min	17	171	0.076	–	
Operative bleeding >200 ml	15	174	0.349	–	

All factors in the two groups were compared by univariate analysis. Multivariate analysis was performed using a logistic regression model

CI confidence interval, *BII* Billroth-II reconstruction, *RY* Roux-en-Y reconstruction

* *P* value of <0.05 was considered to be statistically significant

Table 3 Relationship between overweight and surgical outcome ($n = 355$)

	BMI <25 ($n = 278$)	BMI ≥25 ($n = 77$)	*P* value
Operative blood loss (ml)			0.001*
<200	143 (51)	23 (30)	
≥200	135 (49)	54 (70)	
Operation time (min)			<0.001*
<200	147 (53)	20 (26)	
≥200	131 (47)	57 (74)	

Compared by χ^2 test. Values in parentheses are percentages

* *P* value of <0.05 was considered to be statistically significant

Table 4 Relationship between BMI and type of SSI ($n = 24$)

	BMI <25 ($n = 16$)	BMI ≥25 ($n = 8$)	*P* value
Type of SSI			0.317*
Superficial	3 (19)	3 (38)	
Organ/space	13 (81)	5 (62)	

Compared by χ^2 test. Values in parentheses are percentages

* *P* value of <0.05 was considered to be statistically significant

higher risk of SSI compared with shorter operations, but high BMI was not associated with the risk of SSI. On the other hand, a prospective trial found that among overweight and hypo-albuminemic patients undergoing gastrointestinal surgery, there was an increased risk of deep/organ SSI [2, 3]. The data from our present multivariate analysis suggested that BMI ≥25 kg/m^2 was independently associated with an increased incidence of SSI after distal gastrectomy for gastric cancer. However, other clinical baseline characteristics (such as PNI), operative characteristics (such as duration of surgery, operative blood loss, lymph node dissection, the method of reconstruction following gastrectomy), and the extended administration of antibiotics had no significant association with the incidence of SSI. Moreover, in our study, because surgery for overweight patients required more time and incurred a larger volume of blood loss, it appeared that the incidence of SSI for overweight patients was higher than that in patients of normal weight. Our data are comparatively reliable and noteworthy, because this study was derived from the data of a phase 3 prospective randomized trial that was based on a single elective surgical procedure performed under uniform conditions

In conclusion, the present study has revealed that, compared with patients of normal weight, overweight patients have a significantly higher risk of SSI after distal gastrectomy for cancer, and the SSIs in overweight patients may not be prevented by the extended administration of antibiotics. Quality improvement initiatives for overweight patients undergoing gastric surgery should focus on the complication of SSI.

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Conflict of interest We declare that we have no conflicts of interest.

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Severity of Complications After Gastrectomy in Elderly Patients With Gastric Cancer

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Abstract

Background The risk of surgery for gastric cancer has not been fully evaluated, and this study aimed to assess the severity of postoperative complications after D2 or modified D2 gastrectomy in elderly patients.

Methods Eligible patients were retrospectively selected from the Kanagawa Cancer Center database between 1990 and 2009 based on the following criteria: age ≥80 years and D2 or modified D2 gastrectomy as a primary treatment for gastric cancer. The severity of complications was evaluated using the Clavien–Dindo classification.

Results A total of 83 patients with a median age of 82 years (range 80–88 years) were entered in this study. Sixty (72 %) had at least one co-morbid condition. American Society of Anesthesiologists scores were 2 in 66 patients and 3 in 17 patients. The extent of gastrectomy was distal in 65 (78 %) and total in 18 (22 %) patients. The procedure used for lymphadenectomy was modified D2 in 38 (46 %) and D2 in 45 (54 %) patients. Altogether, 18 complications were observed in 15 patients. The overall morbidity rate was 18 % [95 % confidence interval (CI) 9.7–26.2 %], and the mortality rate was 3.6 % (95 % CI 0–7.6 %). Complications were classified as grade 2 ($n = 9$), grade 3a ($n = 1$), grade 3b ($n = 4$), grade 4 ($n = 1$), and grade 5 ($n = 3$). Severe complications (≥ grade 3) occurred in 8.4 % (95 % CI 2.4–14.4 %).

Conclusions The morbidity rate was acceptable, but that of severe complications was high, suggesting that surgery for gastric cancer in elderly patients is risky and should be limited.

Introduction

Every year, more than 800,000 patients are newly diagnosed with gastric cancer, which is the second most common cause of cancer-related death in the world [1]. In Japan, 20 % of gastric cancer patients are >80 years old [2]. Therefore, it is not rare in clinical practice for gastric cancer patients over the age of 80 years to undergo gastrectomy. In addition, Phase III trials evaluating surgical treatment that are ongoing in Japan (JCOG0912: UMIN000003319 and JCOG1001:UMIN000003688) have age upper limits of 80 years. In the other words, it would be impossible to provide evidence about surgical treatment for patients >80 years old.

Complete tumor removal is essential to treat gastric cancer, and D2 gastrectomy is the standard surgical procedure in Japan. Morbidity and mortality after D2 surgery were 20.9 and 0.8 %, respectively, in a Japanese Phase III trial [3]. However, patients who entered into the clinical trials were relatively young and had no severe co-morbidities. No prospective data are available to confirm that D2 surgery is safe for elderly patients who are not eligible for inclusion in clinical trials.

Previously, many retrospective analyses have focused on morbidity and mortality in elderly gastric cancer patients [4–14]. Although some authors [6, 9, 13] reported that age was an independent factor that affects mortality and morbidity, others [4, 5, 7, 8, 10–12, 14] described gastrectomy for elderly patients as feasible and safe based

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on a low incidence of morbidity. Elderly cancer patients often have co-morbidities and age-related physiologic problems that may not increase the incidence but could increase the severity of morbidity after gastrectomy. However, no study has reported on the severity of complications. The safety of surgery should be evaluated not only by the overall morbidity rate but also by the rate of severe complications together with mortality.

Recently, Dindo [15] proposed a new classification for surgical complications, called the Clavien–Dindo classification, which categorizes morbidity from grade 1 to grade 5. It was validated in 2009 [16]. Our retrospective study aimed to assess the severity of postoperative complications after D2 or modified D2 gastrectomy in gastric cancer patients >80 years of age using the Clavien–Dindo classification.

Methods

Selection of patients

The patients were selected from the prospective database of the Kanagawa Cancer Center, Department of Gastrointestinal Surgery, Yokohama, Japan, according to the following criteria: (1) histologically proven gastric adenocarcinoma; (2) D2 or modified D2 gastrectomy for gastric cancer as a primary treatment between January 1990 and December 2009; (3) R0 or R1 resection achieved according to surgical and pathologic findings; (4) age $\geq$80 years.

Indication for surgery

Tumor progression was evaluated by physical examinations, endoscopy, upper gastrointestinal series, chest radiography, and abdominal computed tomography (CT). Surgery was considered when no metastasis to distant organs was apparent. Preoperative risks were assessed by activities of daily life, performance status, medical history, physical status, physical examinations, symptoms of chronic lung or heart disease, chest radiography, electrocardiography, pulmonary function test, and biochemical and hematologic tests. Four staff surgeons and at least one anesthesiologist evaluated the surgical indications. Surgery was selected when all five physicians agreed on the operability of the patients.

Surgical procedure

Extent of lymphadenectomy was according to the Japanese gastric cancer treatment guidelines 2010 [Japanese Gastric Cancer Guidelines (JGCG) version 3] [17]. In this study, D2 lymph node dissection included D2 defined by the JGCG version 3 and D2 with nodal dissection of nodes along the superior mesenteric vein. Thus, splenectomy was necessary for total gastrectomy with D2. On the other hand, modified D2 gastrectomy included D1+ or D1+ plus some nodal dissection which did not reach the definition of D2. Splenectomy was not performed in the modified D2 total gastrectomy.

In principle, D2 gastrectomy was adopted for T2–T4 disease, whereas modified D2 was used for T1 cancer. Because T1 gastric cancer rarely metastasizes to lymph nodes along the splenic or proper hepatic arteries, these lymph nodes are preserved with the modified D2. At present, this type of surgery has been regarded as standard treatment for T1 disease in Japan [18]. Spleen-preserving modified D2 gastrectomy was selected based on the physician's preference, the patient's co-morbidities, and the tumor status.

The depth of tumor and nodal involvement was determined by Japanese Classification of Gastric Carcinoma: 3rd English edition (JCGC 3rd English edition) [19].

Clavien–Dindo classification

The Clavien–Dindo classification categorizes surgical complications from grade 1 to grade 5 based on the invasiveness of the treatment required. Grade 1 requires no treatment; grade 2 needs medical therapy; grade 3a requires surgical, endoscopic, or radiologic intervention but not general anesthesia; grade 3b requires general anesthesia; grade 4 represents life-threatening complications that require intensive care; grade 5 represents the death of the patient.

In this study, we retrospectively determined complications ranging from grade 2 to 5 from patients' records during hospitalization and within 30 days after surgery. Grade 1 was not evaluated to exclude the possibility of description bias in patient records. Severe complications were defined as those graded as >3a. Mortality (grade 5) was defined as hospital death due to any cause after surgery.

This study was reviewed and approved by the institutional review board committee of Kanagawa Cancer Center.

Results

A total of 109 patients underwent D2 or modified D2 gastrectomy for gastric adenocarcinoma. Among them, 83 underwent R0 or R1 resection and were entered into the study. Table 1 summarizes the baseline characteristics of the patients. Among 83 patients, 82 (99 %) scored 0 or 1 for Eastern Cooperative Oncology Group (ECOG)

Table 1 Baseline characteristics of the patients

Characteristics	No. of patients	%
Age (range 80–88 years)		
80–84 years	70	84
≥85 years	13	16
Sex		
Male	49	59
Female	34	41
ECOG-PS		
0	72	87
1	10	12
2	1	1
ASA score		
2	66	80
3	17	20
Co-morbidities		
Heart disease	19	23
Cerebrovascular disease	10	12
Pulmonary disease	26	31
Diabetes mellitus	9	11
Liver disease	1	1
Hypoalbuminemia (<3.5 g/dl)	12	14
Anemia: hemoglobin (<10 g/dl)	12	14
Renal disfunction: (Ccr <30)	8	10
No. of co-morbidities[a]		
0	23	28
1	27	32
2	18	22
3	11	13
≥4	4	5

ECOG-PS Eastern Cooperative Oncology Group performance status, *ASA* American Society of Anesthesiologists, *Ccr* creatinine clearance

[a] Co-morbidities were applied to above eight co-morbidities

Table 2 Surgical procedure and pathologic findings

Parameter	No. of patients	%
Operative procedure		
Distal gastrectomy	65	78
Total gastrectomy	18	22
Extent of lymphadenectomy		
D2	45	54
Modified D2	38	46
Combined resection		
Spleen	4	5
Pancreas	1	1
Liver	1	1
Colon	1	1
Depth of invasion[a]		
T1a (m)	14	17
T1b (sm)	31	37
T2 (mp)	11	13
T3 (ss)	7	9
T4a (se)	16	19
T4b (si)	4	5
Lymph node metastasis[a]		
N0	55	66
N1 (1–2)	14	17
N2 (3–6)	5	6
N3a (7–14)	8	10
N3b (>15)	1	1
Stage[a]		
I	48	58
II	16	19
III	16	19
IV	3	4

[a] Tumor depth, nodal involvement, and staging classification were based on JCGA 3rd English edition

performance status, and 60 (72 %) patients had more than one co-morbidity. The most common co-morbidity was pulmonary disease, which included chronic obstructive pulmonary disease, chronic bronchitis, and interstitial pneumonitis requiring continuous drug therapy. Most patients had an American Society of Anesthesiologists (ASA) score of 2 in this series.

Surgical procedure and pathologic findings

More than half of the patients underwent D2 gastrectomy; splenectomy was performed in only 4 patients (5 %); and 18 (22 %) underwent total gastrectomy. In all, 48 patients (58 %) had early-stage disease (Table 2). Thus, 22 patients were candidates for D2 total gastrectomy including splenectomy.

Complications

A total of 18 perioperative complications were observed in 15 patients. Details of these complications are given in Table 3. The overall morbidity rate was 18 % [95 % confidence interval (CI) 9.7–26.2 %]: Anastomotic leakage occurred in 1 % and pancreas-related abscess in 2 %. Complications were classified as grade 2 in nine patients, grade 3a in one, grade 3b in four, grade 4 in one, and grade 5 in three. The rate of severe complications of grade ≥3a was 8.4 % (95 % CI 2.4–14.4 %), and that of severe complications of grade ≥3b was 7.2 % (95 % CI 1.6–12.8 %). There were no significant differences in the proportions of total or severe morbidities between modified D2 (20.0 % and 7.8 %, respectively) and D2 (16.0 % and 8.9 %, respectively). Death (grade 5) occurred in three