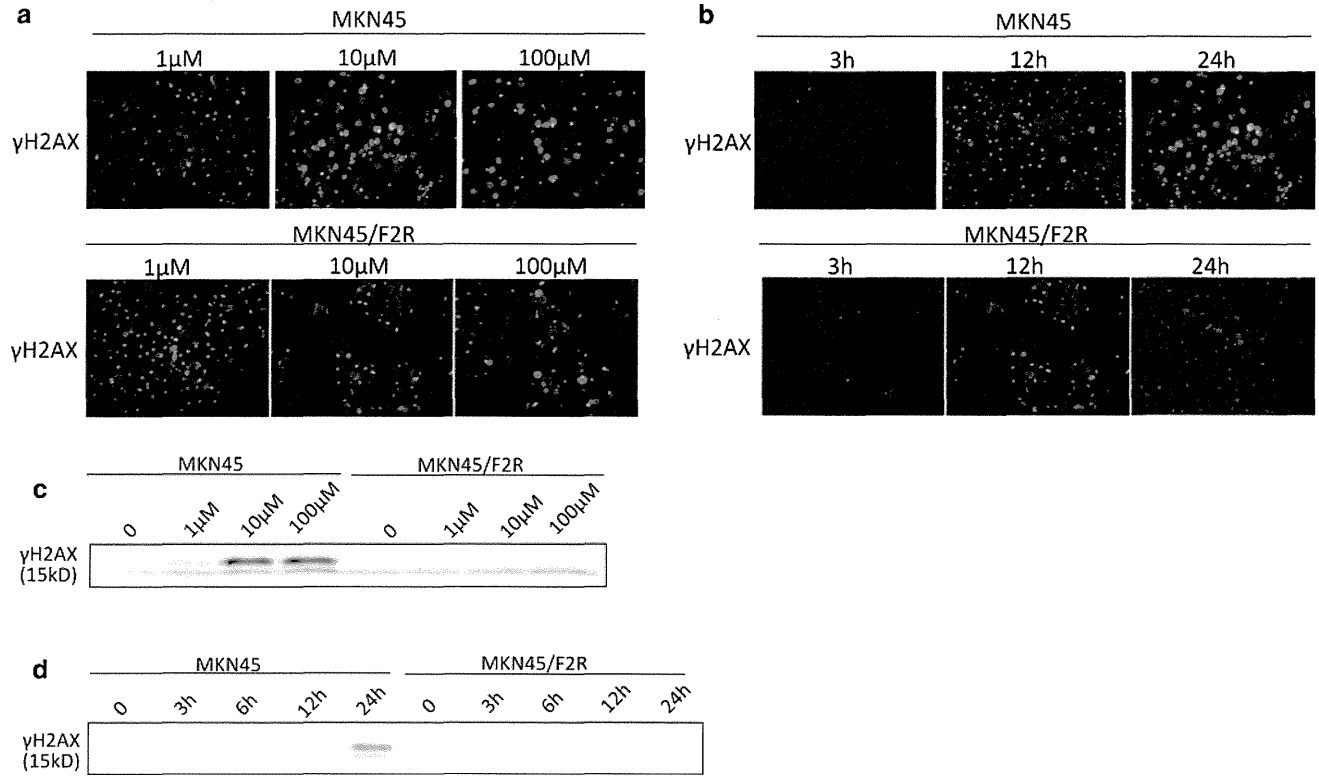

Fig. 3 The induction of double-strand breaks (DSBs) in MKN45 and MKN45/F2R cells after treatment with 5FU. An immunofluorescence analysis and a western blotting analysis for phosphor-H2AX, a DSB marker, were performed after treatment with the indicated concentrations of 5FU. **a** The results of the immunofluorescence analysis of MKN45 and MKN45/F2R cells treated with 5FU at concentrations of 1, 10, and 100 μM for 24 h. **b** The results of the immunofluorescence analysis of the MKN45 and MKN45/F2Rcells treated with 10 μM of 5FU for 3 h, 12 h, and 24 h. **c** The results of the western blotting analysis of MKN45 and MKN45/F2R cells treated with 5FU at concentrations of 1, 10, and 100 μM for 24 h. **d** The results of the western blotting analysis of MKN45 and MKN45/F2R cells treated with 10 μM of 5FU for 0, 3, 6,12, and 24 h

24 h compared to the control (Fig. 3c), and γH2AX was increased with 10 μM of 5FU in 24-h treatment compared with treatment for other periods (Fig. 3d).

MLH1 and PMS2 are linked to FANCJ after oxaliplatin treatment

The FANCJ/MutLα interaction is indispensable for ICL repair, and loss of FANCJ leads to failure of ICL repair [15]. To assess the interactions between these proteins and FANCJ after treatment in our cell lines, we performed co-immunoprecipitation studies.

After MKN45 cells were treated with 10 μM 5FU, 1 μM oxaliplatin,or both agents for 24 h, the cell lysates were immunoprecipitated with an anti-FANCJ antibody, and the presence of co-immunoprecipitated MLH1 and PMS2 was evaluated by a western blot analysis (Fig. 4). After the 5FU treatment, MLH1 and PMS2 were only minimally immunoprecipitated. However, after the oxaliplatin treatment, both MLH1 and PMS2 were immunoprecipitated to a greater extent than after the 5FU treatment, even though

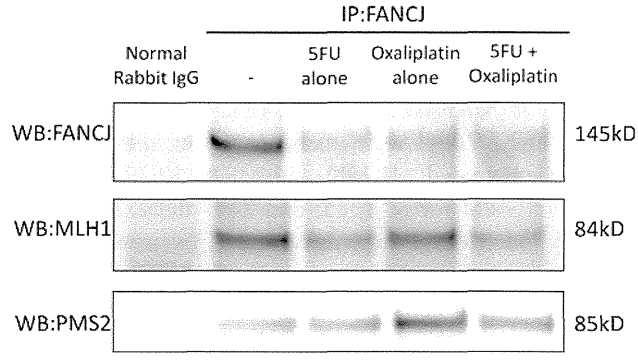

Fig. 4 Co-immunoprecipitation (*IP*) with an anti-FANCJ antibody. Co-immunoprecipitation of proteins with FANCJ after treatment of MKN45 cells with 10 μM 5FU and/or 1 μM oxaliplatin for 24 h. After oxaliplatin treatment, both MLH1 and PMS2 were immunoprecipitated to a greater extent than that after 5FU treatment alone, although the amount of FANCJ was decreased. *WB* Western blotting

the level of FANCJ decreased, suggesting that the amount of MutLα bound to FANCJ was increased after treatment with oxaliplatin in MKN45 cells.

FANCJ knockdown increases the sensitivity of MKN45 cells to oxaliplatin

The loss of FANCJ is thought to result in a failure of ICL repair [5], and we found that the FANCJ expression was decreased after 5FU treatment, as described above. Therefore, we hypothesized that the decrease in FANCJ caused by 5FU treatment contributes to the increase in the sensitivity of gastric cancer cells to oxaliplatin. To verify this hypothesis, siRNA directed against FANCJ was transfected into MKN45 and MKN45/F2R cells, and their sensitivity to oxaliplatin and 5FU was analyzed by the MTT assay. Before the sensitivity of the cells was analyzed, the mRNA and protein expression levels of FANCJ were evaluated to confirm that the FANCJ gene was knocked down. As shown in Fig. 5a, in the MKN45 cells transfected with the siRNA oligonucleotide against FANCJ, the expression of FANCJ was decreased to 15.3 % compared to that in the control cells. Similarly, the FANCJ expression in MKN45/FR2 cells was decreased to 25.1 % compared to that in control MKN45/F2R cells (Fig. 5b). Changes in the mRNA expression levels were also confirmed in these cells (data not shown).

We then performed MTT assays for cells treated with oxaliplatin and 5FU. As expected, the IC50 for oxaliplatin in the MKN45 cells after siRNA transfection decreased, to 0.075 μM from 0.177 μM (Fig. 5c; Table 2). On the other hand, the sensitivity of the MKN45 cells to 5FU was not

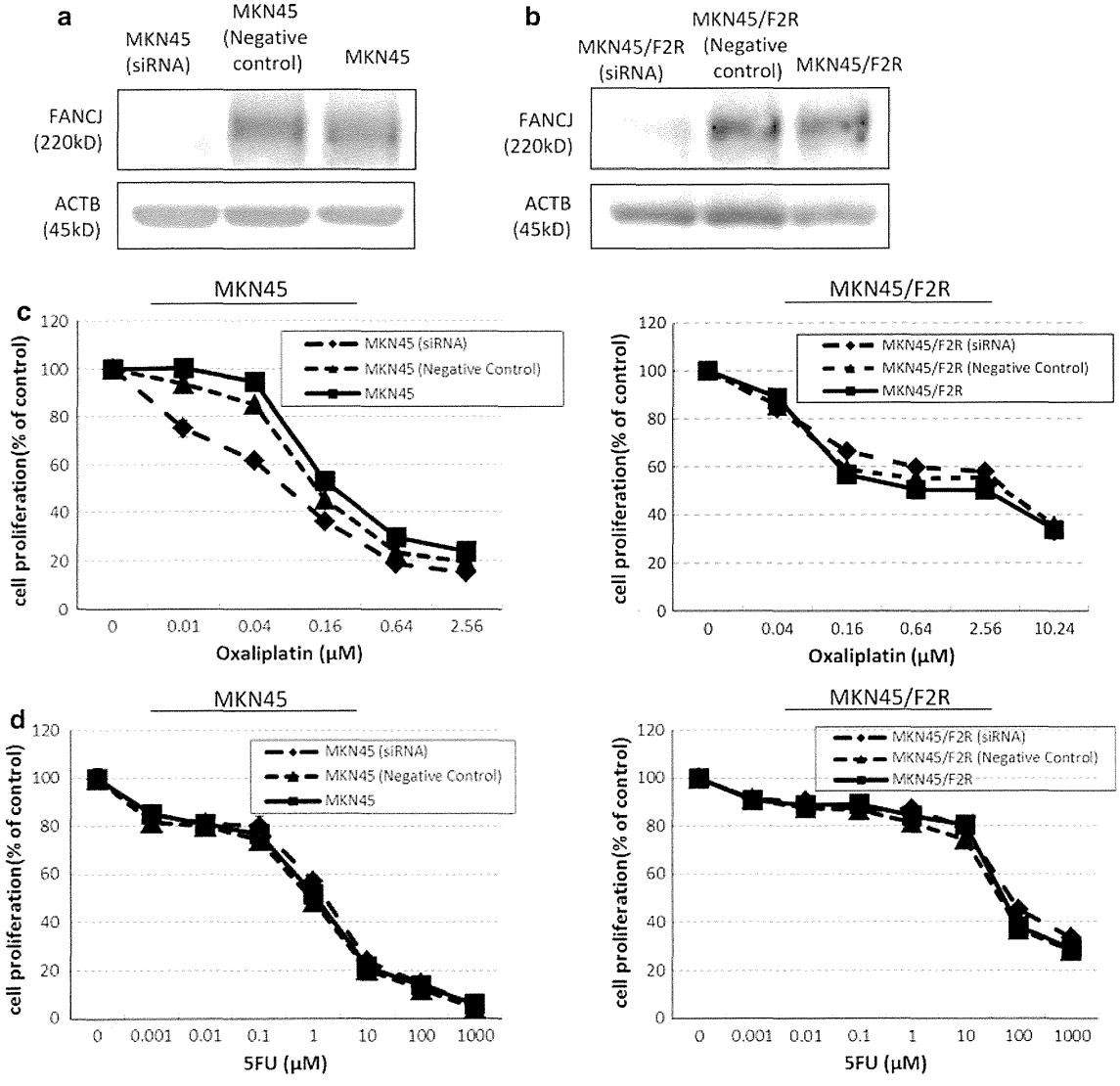

Fig. 5 The downregulation of FANCJ after transfection of cells with a small interfering (*si*) RNA oligonucleotide against FANCJ. An siRNA oligonucleotide against FANCJ was transfected into **a** MKN45 and **b** MKN45/F2R cells and the expression of FANCJ was evaluated. The in vitro sensitivity to **c** oxaliplatin or **d** 5FU after siRNA transfection demonstrated that the downregulation of FANCJ increased the sensitivity of MKN45 cells to oxaliplatin

Table 2 IC50 values for oxaliplatin and 5FU in MKN45 and MKN45/F2R cells after siRNA transfection

Cell line (treatment)	IC50 for oxaliplatin (average ± SE)	IC50 for 5FU (average ± SE)
MKN45 (no treatment)	0.177 ± 0.00992	1.14 ± 0.888
MKN45 (negative control)	0.135 ± 0.00175	0.882 ± 0.281
MKN45 (siRNA)	0.075 ± 0.0158*	1.65 ± 0.283
MKN45/F2R (no treatment)	2.58 ± 0.311	52.4 ± 8.35
MKN45/F2R (negative control)	3.75 ± 0.752	44.8 ± 6.02
MKN45/F2R (siRNA)	3.99 ± 0.854	72.0 ± 9.30

MKN45 and MKN45/F2R cells were transfected with a small interfering (si) RNA against FANCJ, and the IC50 values were calculated from the results of the MTT assay for oxaliplatin and/or 5FU. The IC50 for oxaliplatin in the MKN45 cells was significantly decreased after siRNA transfection. On the other hand, the IC50 for 5FU in the MKN45 cells was not altered. The IC50 for oxaliplatin and 5FU in the MKN45/F2R cells did not change after siRNA transfection

* $p < 0.05$ based on Student's t-test, compared with untreated MKN45 or MKN45/F2R cells (no treatment)

altered (Fig. 5d; Table 2). The sensitivity of MKN45/F2R cells to oxaliplatin and 5FU did not change after siRNA transfection. These results suggest that decreased FANCJ expression increased the sensitivity of MKN45 cells to oxaliplatin, but not to 5FU, while the sensitivity was not altered in 5FU-resistant MKN45/F2R gastric cancer cells.

Discussion

Oxaliplatin, a DACH-containing platinum agent, has a spectrum of activity and mechanisms of action and resistance that appear to be different from those of other platinum-containing compounds, notably cisplatin (CDDP) [22]. Moreover, its anticancer effects are optimized when it is administered in combination with other anticancer agents, such as 5-fluorouracil (5FU) [22], S-1 [23, 24], and capecitabine [25, 26] in gastric and colorectal cancers. There have been several reports about the relationship between the FA pathway and oxaliplatin. For example, it was demonstrated that FANCC- and FANCD2-mutant cells were more sensitive to oxaliplatin and CDDP than FANCA-mutant cells, and mono-ubiquitination of FANCD2, which is mediated by the FANCA- and FANCC-containing FA core complex, was not required for platinum resistance [27]. It was also shown that disruptions of FANCC and FANCG caused a 2-fold increase in the sensitivity of RKO cells to oxaliplatin [28].

With regard to the relationship between FANCJ and chemotherapy, Nakanishi et al. reported that there was a correlation between high expression of FANCJ and poor responsiveness of 5FU in colorectal cancer [29]. Our present study is the first to reveal the role of FANCJ in the synergism between 5FU and oxaliplatin. However, other reports about the synergistic effects of oxaliplatin or CDDP in combination with 5FU in vitro also exist. For example, Raymond et al. [10] reported that synergistic antiproliferative effects were observed when oxaliplatin was added to 5FU, and the synergistic effects of these combinations were maintained in the 5FU-resistant colon cancer cell line, HT29-5-FU. Scheithauer and Temsch [30] reported that the addition of CDDP to 5FU/leucovorin (LV) yielded synergistic growth inhibition in some human colon cancer cell lines. Our present study revealed that there were synergistic effects of oxaliplatin in combination with 5FU in the MKN45 gastric cancer cell line, and these effects were also observed with CDDP and 5FU (data not shown).

In our study, γH2AX was increased in MKN45 cells after 5FU treatment. In addition, although BRCA1 protein expression was induced by 5FU treatment, the expression of FANCJ was downregulated. This downregulation may have occurred because the FANCJ protein was bound to newly synthesized BRCA1 to repair the DSBs caused by 5FU treatment, and FANCJ may also have functioned via other mechanisms [31].

In contrast, in the MKN45/F2R 5FU-resistant cells, DSBs did not appear after 5FU treatment, and the expression levels of FANCJ and other proteins were not altered after 5FU treatment. These results confirmed that 5FU downregulated the FANCJ protein in sensitive cells, and this appears to be important for the activity of 5FU. In the present study, γH2AX was not detected after treatment with oxaliplatin to the same extent as it was with 5FU (data not shown), suggesting that the induction of DSBs was a phenomenon specifically related to 5FU treatment.

The interaction between FANCJ and MutLα (composed of MLH1 and PMS2) is essential for the ICL response [15]. The ICL is first sensed by MutSβ, but we examined the MutLα (MLH1-PMS2) complex because FANCJ directly binds to MutLα, but not to MutSα or MutSβ, and we considered that the interaction between FANCJ and MutLα was more directly related to the synergism between 5FU and oxaliplatin. As shown in Fig. 2, the expression levels of MLH1 and PMS2 were not altered after 5FU treatment, while there was decreased FANCJ because it was consumed to repair DSBs caused by 5FU treatment. This might have interfered with the repair of ICLs caused by oxaliplatin, thus resulting in the increased sensitivity to oxaliplatin. The involvement of MutSα or β should be examined in the future. A model for the potential involvement of these molecules is illustrated in Fig. 6.

Peng et al. [15] reported that, in the absence of the FANCJ protein, it was impossible to displace MutLα from recombination intermediates, and consequently, the MutLα

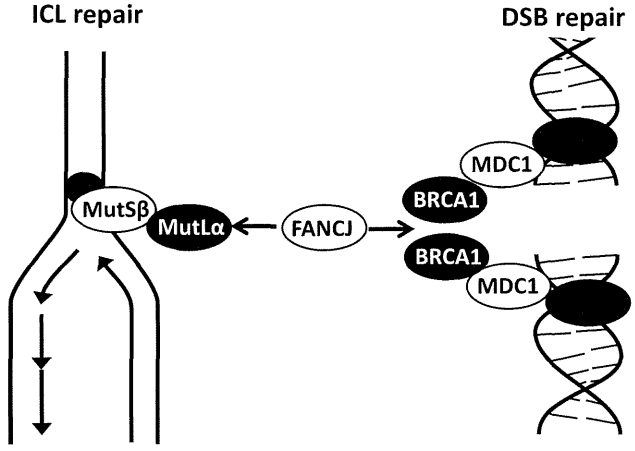

Fig. 6 A model of how FANCJ proteins function when cells are treated with 5FU and oxaliplatin. 5FU induces DSBs, while oxaliplatin induces ICLs. Both ICL repair and DSB repair require the FANCJ protein. Because there is a lack of FANCJ when cells are treated with both drugs, there is synergism between 5FU and oxaliplatin

complex remained stuck to DNA for a longer time period, delaying the exit from the G2/M arrest and enhancing ICL sensitivity [5]. In our study, the level of FANCJ in the MKN45 cells was decreased after 5FU treatment. As would be expected based on the report by Peng et al., the sensitivity of the MKN45 cells to oxaliplatin increased when FANCJ was knocked down by siRNA. We initially tried to force the expression of FANCJ in the cells by transfection, because we wanted to confirm whether the synergism between 5FU and oxaliplatin was reversed by FANCJ overexpression. However, there are various other molecules involved in the synergism, such as BRCA1, MLH1, and so on. This led us to examine the direct effects of FANCJ using an siRNA knockdown system. Our findings suggest that the decrease in FANCJ caused by 5FU treatment leads to an increase in the sensitivity to oxaliplatin, resulting in synergistic cytotoxic effects exerted by the combination of 5FU and oxaliplatin in MKN45 5FU-sensitive cells. In the MKN45/F2R cells, the synergistic effect of oxaliplatin and 5FU was not observed, partly because DSBs did not occur after 5FU treatment in these cells.

In conclusion, the present study provides the first evidence of the role of FANCJ in the synergism between 5FU and oxaliplatin, and can be regarded as providing a rationale for using a combination of fluoropyrimidine and platinum agents for the treatment of gastric carcinomas [22].

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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

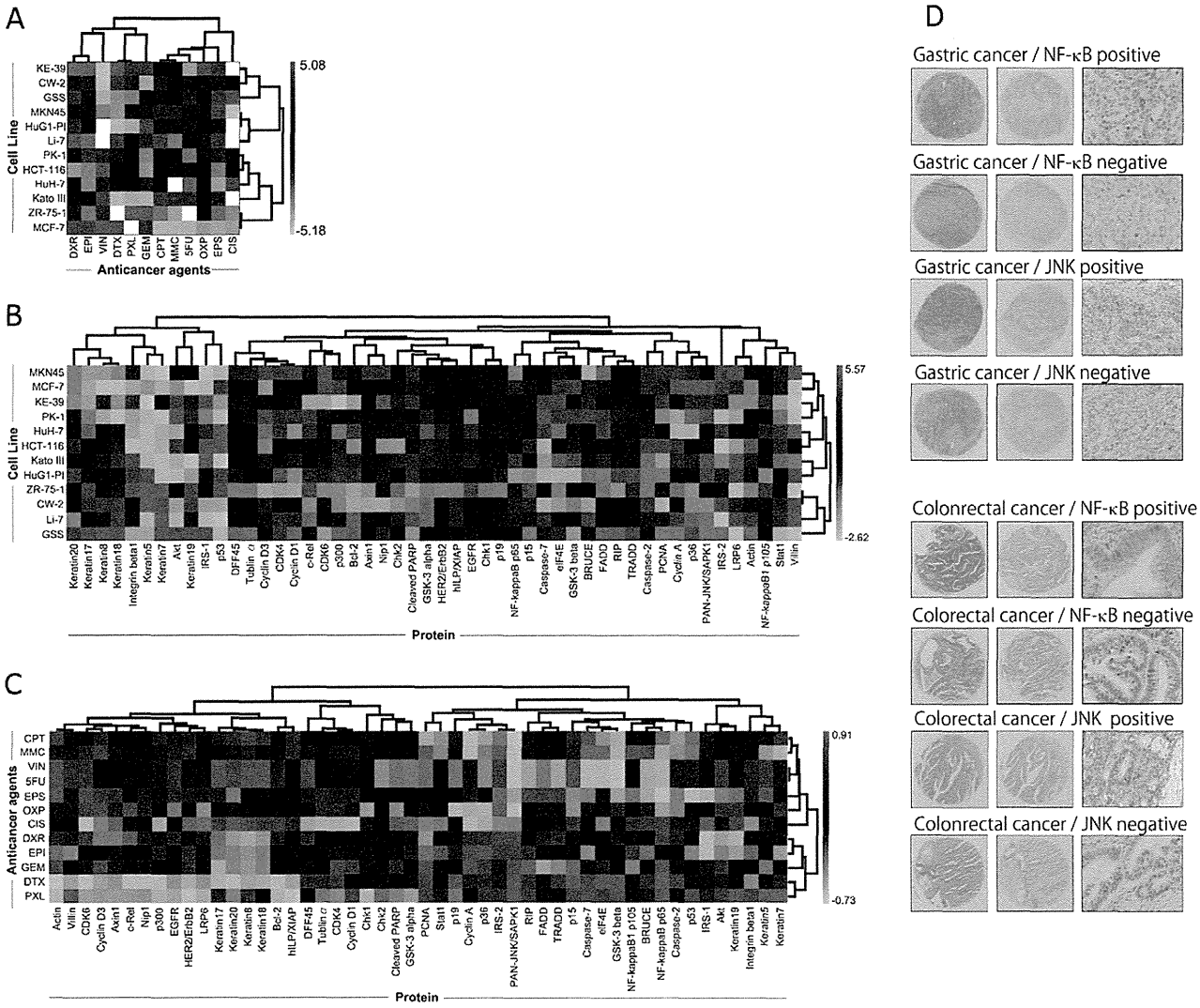

Figure 1. Hierarchical clustering of three different matrices and results of immunohistochemical examinations of candidate markers. (A) Based on a chemosensitivity assay of a cancer cell line panel, the A (activity) × C (cells) = AC matrix was created. (B) Quantitative protein expression data of each 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

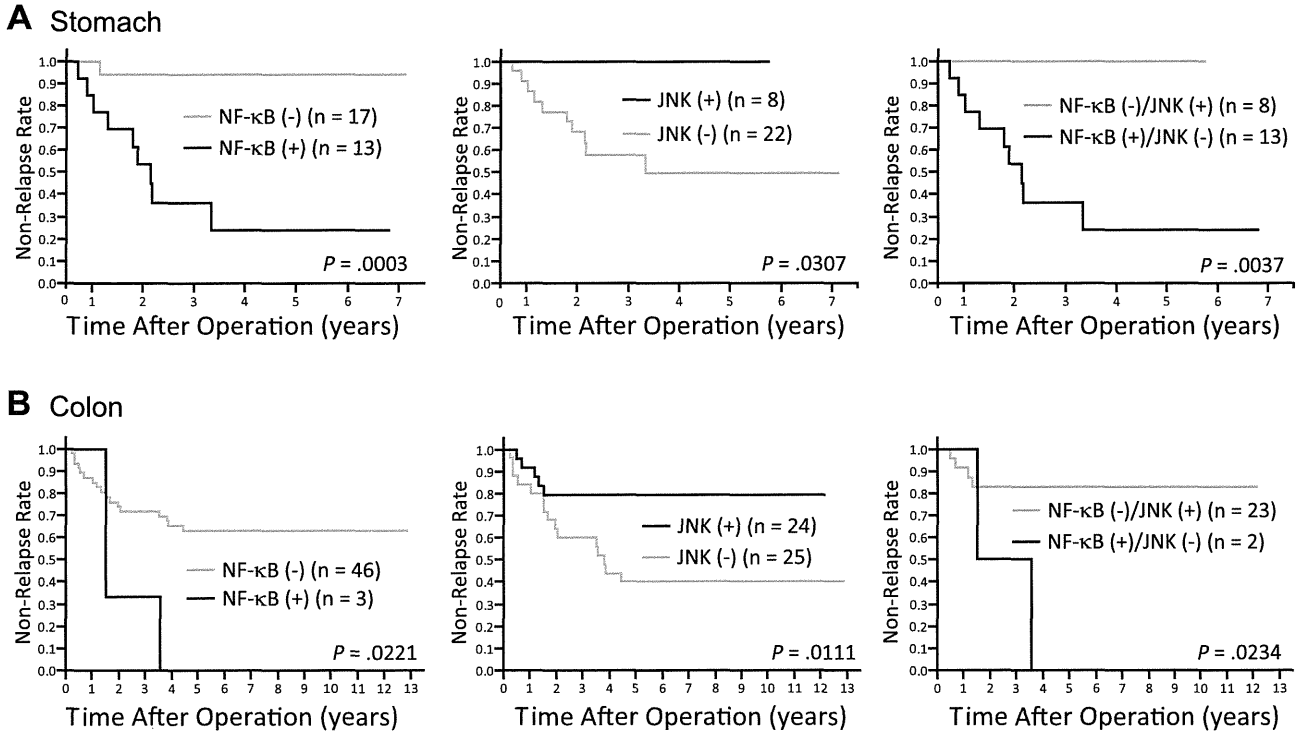

Figure 2. Time-to-relapse (TTR) rates on the basis of NF-κB and JNK protein expression in gastric and colon carcinomas.
doi:10.1371/journal.pone.0043236.g002

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].

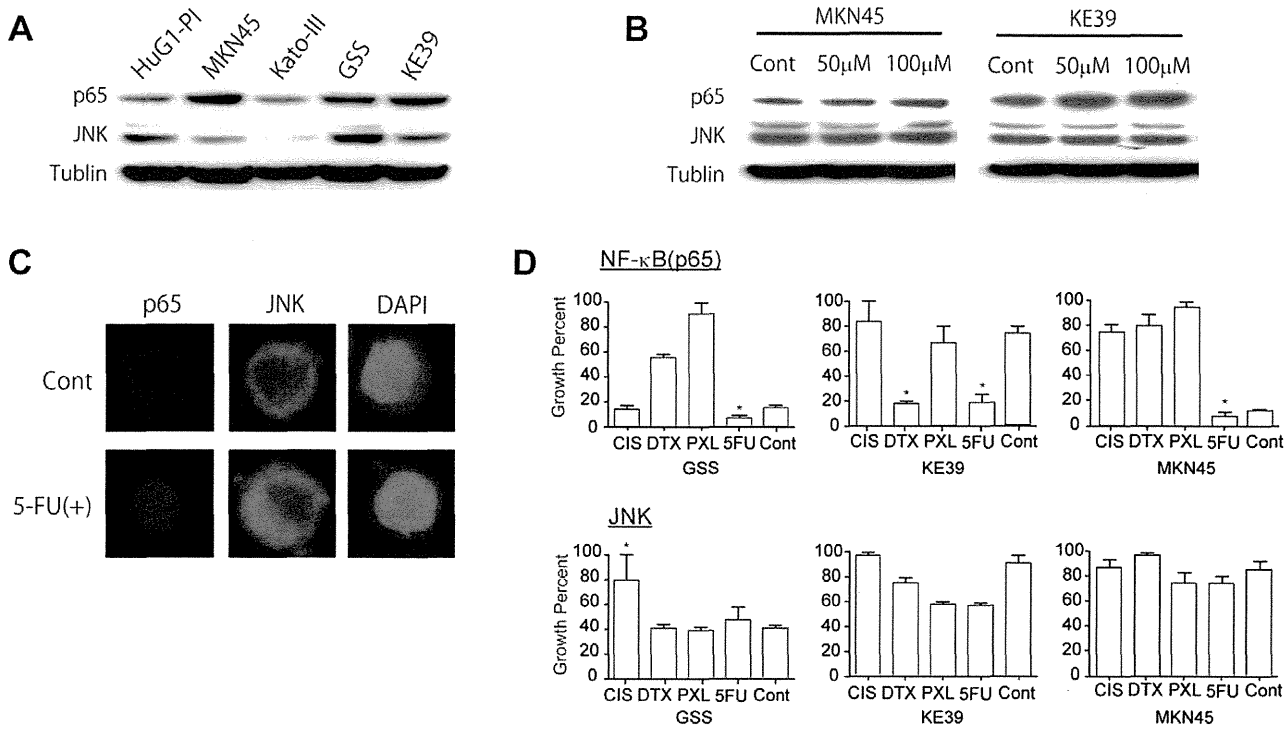

Figure 3. Induction of biomarkers by 5-FU treatment. (A) Baseline protein expression of NF-κB and JNK in five gastric cancer cell lines. Tublin was used as a loading control. (B) Induction of candidate biomarkers in response to 5-FU treatment in different concentrations in MKN45 and KE39. (C) Examination 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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ORIGINAL ARTICLE

Ultrasonic Scalpel for Gastric Cancer Surgery: a Prospective Randomized Study

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Abstract
Background The aim of the study was to evaluate the potential advantages of the ultrasonic scalpel compared with the conventional technique in gastric cancer surgery.
Methods Patients with resectable adenocarcinoma of the stomach were randomly assigned to ultrasonic scalpel or conventional technique. We used the HARMONIC FOCUS® (Ethicon Endo-Surgery, Inc.) as ultrasonic scalpel.
Results Between February 2010 and December 2010, 60 patients with resectable gastric cancer were enrolled into the study. Operative time was significantly shorter with the ultrasonic arm than with the conventional arm (median 238.5 vs. 300.5 min; $P=0.0004$). Blood loss was also significantly lower in the ultrasonic arm than in the conventional arm (median 351.0 vs. 569.5 ml; $P=0.016$). Clavien–Dindo grades of postoperative complications were similar in the two groups. From a questionnaire survey of operators, the ultrasonic scalpel significantly reduced the stress of lymph node dissection (3.67 vs. 2.87; $P=0.0006$). However, in assisting surgeons, the contributions to surgery, study, and technical improvement of the ultrasonic group were lower than in the conventional group.
Conclusions This study shows that the ultrasonic scalpel is a reliable and safe tool for open gastric cancer surgery.

Keywords HARMONIC FOCUS · Harmonic scalpel · Lymph node dissection · Gastrectomy · Training

Introduction

Gastric cancer is still one of the most common cancers globally; 876,000 new cases were anticipated worldwide in the year 2000.[1] In Japan, 110,323 new cases were anticipated in the year 2003 and the 5-year survival rate of gastric cancer diagnosed from 1993 to 1996 was 54.4 %.[2,3] Currently, gastrectomy with lymph node dissection remains the mainstay of curative treatment.[4,5] The operation requires exhaustive hemostasis with a dry operative field for high-quality lymph node dissection and to avoid inadvertent damage to important structures such as the pancreas. The conventional technique for hemostasis during gastrectomy relies on knot-tying and electrocoagulation.

In recent years, new hemostatic tools have been developed with the advent of laparoscopic surgery. Ultrasonic surgical instruments have been used in head and neck and abdominal surgeries, such as hysterectomy, colectomy, and thyroid surgery.[6–8] Several study groups have published data comparing these ultrasonic surgical devices to conventional surgery within these operations.[9–11] Ultrasonic surgical devices provide consistent advantages in terms of

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operative time and blood loss.[9–11] However, there are no available data based on prospective randomized studies showing the efficacy, safety, advantages, or disadvantages of their use compared with traditional dissection in gastric cancer surgery. The aim of the present randomized study was to evaluate the potential advantages of the ultrasonic scalpel compared with the conventional technique in gastric cancer surgery.

Patients and Methods

Study Design and Inclusion Criteria

The study was conducted as a prospective randomized trial at the Gastrointestinal Surgical Division of Kansai Medical University Hirakata Hospital in Osaka, Japan. Patients with resectable adenocarcinoma of the stomach were randomly assigned to ultrasonic scalpel or conventional technique (knot-tying and electrocoagulation). We used the HARMONIC FOCUS® (Ethicon Endo-Surgery, Inc.) as an ultrasonic scalpel. This product consists of ultrasonic curved shears, fashioned for open surgery and enabling dissection, coagulation, and cutting. It transects and seals vessels ≤5 mm as well as lymphatics.

Study inclusion criteria were as follows: age ≥20 years; American Society of Anesthesiologists (ASA) performance status 1 to 3; histologically proven adenocarcinoma of the stomach; no evidence of distant metastases or disease considered unresectable by computed tomography and staging laparoscopy for T3–4 tumor; no prior upper abdominal surgery; no uncontrolled infections, diabetes, or cardiac disease; and adequate renal function. The protocol was reviewed and approved by the institutional ethics committee (www.umin.ac.jp/ctr/index/htm/, protocol UMIN000003169). All patients provided written informed consent.

Randomization and Surgery

Patients were randomly assigned into two groups: ultrasonic group, in which the ultrasonic scalpel was used, and a conventional group, in which the conventional technique was used for dissection. For patients in the ultrasonic group, we used the ultrasonic scalpel, electrocautery (monopolar), and ligation with silk thread. We used the ultrasonic scalpel for ≤5 mm vessels and lymphatics. Electrocautery was used for dissection of avascular planes, minute vessels, and lymphatics. For patients in the conventional group, we used only electrocautery (monopolar) and ligation with silk thread. Randomization was performed before surgery stratified by body mass index (BMI, <25 or ≥25), gender, primary tumor extension (cT1 or T2–4), and presence of preoperative chemotherapy.

Surgeries were performed by four or five individuals, including one operator, one instructive assistant surgeon, one or two assistant surgeons, and one scrub nurse. Operators were five surgical residents (one surgeon, 2 years in surgical practice; three surgeons, 4 years in surgical practice; and one surgeon, 7 years in surgical practice), five junior surgeons (three surgeons, 12 years in surgical practice; two surgeons, 13 years in surgical practice), or two senior surgeons (one surgeon with 15 years in surgical practice and one surgeon with 18 years in surgical practice). Instructive assistant surgeons were supervising surgeon (one surgeon with over 38 years in surgical practice) or senior surgeons. Assistant surgeons were surgical residents or junior surgeons. Scrub nurses of gastric cancer surgery had less than 2 years in practice. Ultrasonic scalpels (Harmonic Ace, Ethicon Endo-Surgery, Inc., or SonoSurg, Olympus Corporation) had been used in our hospital for several years.

Gastrectomies with lymph node dissections were performed according to the Guidelines for Diagnosis and Treatment of Carcinoma of the Stomach.[12] Drainage was applied according to the surgeon's judgment. When drainage was applied, one or two closed suction drains were left in the resection area of lymph nodes around the pancreas. The drains were usually removed after checking drainage volume and amylase (AMY) levels on the fourth postoperative day. All other aspects of patient care followed our in-house-established protocol.

End Points, Assessments, and Sample Size

This study focused on operative time, blood loss, the number of threads, the number of gauzes, and surgery-related complications. We also focused on responses of operative staff to the ultrasonic scalpel.

Specimens were classified according to the Second English Edition Japanese Classification of Gastric Carcinoma.[13] Operative complications were recorded using the Clavien–Dindo classification.[14] Blood loss was measured from the increase in the weight of the bloodied gauze or blood vacuum volume.

At the end of each surgery, we conducted a questionnaire survey on the operator, assisting surgeons, and scrub nurse. The questionnaire comprised 11 statements (two specifically for the operator, three specifically for the assisting surgeons and scrub nurse) that had to be rated on a five-point Likert scale, where 1 = totally disagree, 2 = disagree, 3 = neutral, 4 = agree, and 5 = totally agree. We also asked which approach they would like at the next surgery.

Because the ultrasonic scalpel is a single-patient-use instrument and expensive, the number of patients recruited to the study was determined by the limited number of instruments available at the time according to our budget from Kansai Medical University Hirakata Hospital. We set the sample size as 60 patients in both groups.

Costs

Calculations of costs were made for direct costs for the two procedures, that is, different equipment and operation time. Thereby, market prices for ligatures and gauzes, as well as for the ultrasonic scalpel, were used. Specifically, the cost for the HARMONIC FOCUS was US$1,067.3 (US$1= 78.7 JPY; June 6, 2012). The costs for ligatures and gauzes were US$2.9 per one pack (10 ligatures) and US$13.3 per one pack (30 sheets), respectively. The cost of operating room time depends on many factors. Physician and resource costs vary from country to country. The variable cost also depends in large part on how the operative room staff is paid (e.g., hourly or salaried). We used a ballpark number as operative room time cost at approximately US$15 per operative minute.[15]

Statistical Analysis

Statistical analysis was performed for all randomly assigned patients on an intent-to-treat basis. All data analyses were performed using JMP 9.0.0 software (SAS Inc., Cary, NC, USA), and comparisons between different groups were performed by the Wilcoxon test, the Chi-square test, and the means/ANOVA/pooled t test. The value for P was determined with a 95 % confidence interval (CI).

Results

Patients and Surgical Procedures

Between February 2010 and December 2010, 60 patients with resectable gastric cancer were enrolled into the study. Baseline characteristics were evenly distributed in terms of gender, age, BMI, PS (ASA), presence of preoperative chemotherapy, and cT stage (Table 1). Apart from a slight imbalance in operator status (40.0 % resident in the ultrasonic arm vs. 56.7 % in the conventional arm), surgical procedures were similar (Table 1).

Surgical Outcomes

Surgical outcomes are summarized in Table 2. Operative time was significantly shorter with the ultrasonic scalpel than with the conventional technique (median 238.5 vs. 300.5 min; P= 0.0004). Blood loss was also significantly lower in the ultrasonic arm than in the conventional arm (median 351.0 vs. 569.5 ml; P=0.016). These differences were seen even if we stratified them by operator (surgical resident or surgical staff). While blood loss was different, clinically significant blood loss (need for transfusions) was the same. When the ultrasonic scalpel was used, significantly fewer threads and gauzes were required to achieve hemostasis. The numbers of retrieved lymph nodes were similar in the two groups. Closed suction drains were used for 45 patients (19 in the ultrasonic vs. 26 in the conventional). Drainage volume and AMY level were similar in the two groups.

There were no complications documented during surgery. There were also no serious postoperative complications. Clavien–Dindo grades of postoperative complications were similar in the two groups. The most common complications were pancreatic fistula (n=4; two in the ultrasonic group vs. two in the conventional group) and gastrointestinal paresis (n=3; two in the ultrasonic group vs. one in the conventional group). The other complications were leakage of esophagojejunostomy (n=1; conventional group), wound infection (n=1; ultrasonic group), and cystitis (n=1; conventional group). One patient in the ultrasonic group underwent laparoscopic cholecystectomy for postoperative acute cholecystitis. The postoperative hospital stay and cost did not differ in the two groups (Tables 2 and 3).

Questionnaire to Operators, Assisting Surgeons, and Scrub Nurses

Questionnaire outcomes are summarized in Table 4. For this study, 21 surgeons and 20 scrub nurses worked during operations with the ultrasonic scalpel or the conventional technique. Second assisting surgeons participated in 33 of 60 cases (15 in the ultrasonic vs. 18 in the conventional).

In the questionnaire to operators, there was no difference in terms of the quality of lymph node dissection between the groups. However, the ultrasonic scalpel significantly reduced the stress of lymph node dissection (3.67 vs. 2.87; P=0.0006). This difference was seen even if we stratified it by operator (surgical resident or surgical staff). There was no difference in terms of the contributions to surgery, study, and technical improvement as shown by the results from the questionnaire to scrub nurses. However, in assisting surgeons, the contributions to surgery, study, and technical improvement in the ultrasonic group were lower than in the conventional group.

For the question of, "Which approach would you like to use at the next surgery," 36 (60.0 %) of 60 operators and 34 (56.7 %) of 60 scrub nurses answered the ultrasonic scalpel (Table 5). However, only 30 (50.0 %) of 60 first assisting surgeons and 13 (39.3 %) of 33 second assisting surgeons answered the ultrasonic scalpel.

Discussion

Traditionally, hemostasis during gastrectomy is obtained by time-consuming electrocautery and ligation by a knot-tying technique. In recent years, ultrasonic surgical instruments have

Table 1 Patient characteristics and surgical procedures

		Ultrasonic		Conventional		*P* value
		Cases	%	Cases	%	
Gender	Male	21	70.0	22	73.3	0.77
	Female	9	30.0	8	26.7	
Age	Median (range)	64	(41–79)	67	(45–92)	0.57
BMI	Median (range)	22.4	(17.1–26.8)	23.3	(16.8–29.6)	0.22
		Cases	%	Cases	%	
PS (ASA)	1	12	40.0	8	26.7	0.30
	2	18	60.0	21	70.0	
	3	0	0.0	1	3.3	
Preoperative chemotherapy	None	27	90.0	27	90.0	0.45
	S-1/CDDP	3	10.0	2	6.7	
	Docetaxel/S-1/CDDP	0	0.0	1	3.3	
cT	cT1	12	40.0	8	26.7	0.27
	cT2-4	18	60.0	22	73.3	
Operator	Resident	12	40.0	17	56.7	0.41
	Junior surgeon	16	53.3	11	36.7	
	Senior surgeon	2	6.7	2	6.7	
	Surgical practice years, mean (95 %CI)	9.6	(8.2–10.9)	9.2	(7.9–10.6)	0.73
Gastrectomy	Total	7	23.3	11	36.7	0.52
	Distal	15	50.0	13	43.3	
	PPG	6	20.0	3	10.0	
	Proximal	2	6.7	3	10.0	
Node dissection	D0	1	3.3	0	0.0	0.31
	D1	4	13.3	1	3.3	
	D1+	6	20.0	7	23.3	
	D2	19	63.3	22	73.3	
Combined resection	None	24	80.0	24	80.0	0.63
	Gallbladder	3	10.0	1	3.3	
	Spleen	2	6.7	4	13.3	
	Spleen pancreatic tail	1	3.3	1	3.3	

CDDP cis-diamminedichloroplatinum(II)), *PPG* pylorus-preserving gastrectomy, *cT* clinical T stage

been developed with the advent of laparoscopic surgery. Many gastric surgeons have gotten used to these new hemostatic tools and ultrasonic scalpel came to be used in open gastrectomy.[16] Ultrasonic technology uses high-frequency mechanical energy to cut and coagulate tissue simultaneously. Sound is generated and transmitted to an active blade. This causes the instrument to vibrate at 55,500 times a second, which results in collagen molecules within the tissue becoming denatured and forming a coagulum. It transects and seals vessels ≤5 mm as well as lymphatics. Among the HARMONIC® Shears, the HARMONIC ACE® is the instrument most frequently used during both laparoscopic and open gastrectomy, but some surgeons consider this tool to be large and cumbersome, especially for fine grasping and dissection capabilities.

The design of ultrasonic scalpel shears involved a complete overhaul, reproducing the familiar "Kelly clamp" in shape, with very thin and delicate tips. The new tool allows the surgeon to dissect, coagulate, and cut vessels easily in narrow spaces. Some studies reported the safety and efficacy of the ultrasonic scalpel in head and neck surgery, and particularly during thyroidectomy, in terms of operative time and blood loss.[17–19] The ultrasonic scalpel might be useful for open gastrectomy with lymph node dissection. This study is limited in that it is based on experiences at a single institution and has an explorative design. However, to our knowledge, no randomized controlled studies have been reported comparing the use of this new device with conventional electrocautery and knot-tying techniques during gastrectomy.

In our study, we found a significant reduction of operative time in the ultrasonic group (238.5 vs. 300.5 min). This difference was seen in the groups stratified by operator (surgical resident or surgical staff). The small number of ligations seemed to reduce the operative time (5 vs. 11 packs of threads; one pack, 10 threads). We might also assume that

Table 2 Surgical outcomes

	Ultrasonic (n=30)		Conventional (n=30)		P value
	Median	95 %CI	Median	95 %CI	
Operation time (min)	238.5	[225.5–270.8]	300.5	[287.6–332.9]	0.0004
Surgical resident	216.5	[206.5–291.9]	315.0	[289.7–361.5]	0.0069
Surgical staff	242.5	[223.2–271.8]	293.0	[261.7–318.8]	0.0043
Blood loss (ml)	351.0	[263.1–722.3]	569.5	[577.2–1,036.3]	0.016
Surgical resident	288.5	[146.8–848.5]	643.0	[520.5–1,110.1]	0.011
Surgical staff	233.3	[161.6–817.2]	304.0	[409.9–1,181.2]	0.412
Threads[a] (pack)	5	[4–8]	11	[10–15]	<0.0001
Gauzes (sheet)	41	[38–53]	60	[53–68]	0.015
	Cases	%	Cases	%	
Blood transfusion	6	20.0	8	26.7	0.54
Retrieved lymph nodes	26.5	[22.6–32.9]	31.0	[24.9–35.3]	0.53
Drain	19	63.3	26	86.7	
	Median	95 %CI	Median	95 %CI	
Volume on POD4 (ml)	48.0	[39.6–108.8]	40.5	[34.7–93.9]	0.66
Drain AMY on POD4 (U/L)	132.0	[81.8–872]	107.5	[12.6–688.7]	0.63
Postoperative complication (Clavien–Dindo Classification)					
	Cases	%	Cases	%	0.37
None	24	80.0	25	83.3	
Grade I	3	10.0	1	3.3	
Grade II	2	6.7	4	10.0	
Grade III	1	3.3	0	1.67	
Grade IV	0	0.0	0	0	
	Median	95 %CI	Median	95 %CI	
Postoperative hospital stay (days)	12	[11.4–14.9]	12.5	[12.9–16.3]	0.24

There are 10 threads in one pack

POD postoperative day

[a]In the conventional arm, data were missing for one patient

the good hemostasis avoids the use of gauze, which is time-consuming (41 vs. 60 sheets).

The safety and efficacy of the ultrasonic scalpel were proved by the absence of postoperative bleeding, by the similarly low incidence of pancreatic fistula, and by the significantly lower intraoperative blood loss. Drainage volume and AMY level were also similar in the two groups.

The use of a single-patient-use ultrasonic instrument is undoubtedly more expensive than the conventional technique. However, the described improvement with the ultrasonic scalpel may confer an overall cost benefit compared with the conventional surgery by decreasing operating room time cost.

Apart from the previously discussed benefit of the ultrasonic scalpel for gastric cancer surgery, there is considerable interest in the impressions of this instrument among operative staff. In the questionnaire to operators, the ultrasonic scalpel was described as significantly reducing the stress of lymph node dissection. However, in assisting surgeons, the contributions to surgery, study, and technical improvement of the ultrasonic group were lower than in the conventional group. All procedures in this study were performed by four

Table 3 Cost analysis

	Ultrasonic (n=30)		Conventional (n=29)		P value
	Mean US$	95 %CI	Mean US$	95 %CI	
Ultrasonic scalpel	1,067.3		0		
Ligature[a]	17.4	[10.5–24.3]	37.0	[30.0–43.9]	0.0002
Gauze	23.5	[20.3–26.7]	30.7	[26.9–33.6]	0.0043
Operating room	3,722.5	[3,380.9–4,064.1]	4,632.4	[4,285.0–4,979.9]	0.0004
Total	4,830.7	[4,485.4–5,176.0]	4,699.7	[4,348.5–5,050.9]	0.596

[a]In the conventional arm, the data of ligature were missing for one patient. The patient was excluded from the cost analysis

Table 4 Questionnaires to operator, assisting surgeon, and scrub nurse

		Ultrasonic	Conventional	*P* value
Questionnaire to operator (number of surgeons)		30 (10)	30 (10)	
Was the lymph node dissection high quality?	Mean	3.76	3.86	0.61
	(95 %CI)	(3.49–4.04)	(3.59–4.14)	
Did you perform the lymph node dissection without feeling stress?	Mean	3.67	2.87	0.0006
	(95 %CI)	(3.36–3.98)	(2.56–3.18)	
Surgical resident	Mean	3.83	2.82	0.003
	(95 %CI)	(3.34–4.32)	(2.42–3.23)	
Surgical staff	Mean	3.56	2.92	0.0627
	(95 %CI)	(3.12–3.99)	(2.41–3.43)	
Questionnaire to 1st assisting surgeon (number of surgeons)		30 (10)	30 (8)	
Did you contribute to the surgery?	Mean	3.46	3.90	0.024
	(95 %CI)	(3.20–3.73)	(3.64–4.16)	
Did you learn anything?	Mean	4.03	3.97	0.79
	(95 %CI)	(3.69–4.38)	(3.62–4.31)	
Did your technique improve?	Mean	3.27	3.40	0.47
	(95 %CI)	(3.08–3.53)	(3.14–3.66)	
Questionnaire to 2nd assisting surgeon (number of surgeons)		15 (8)	18 (12)	
Did you contribute to the surgery?	Mean	2.6	3.06	0.10
	(95 %CI)	(2.19–3.00)	(2.68–3.43)	
Did you learn anything?	Mean	3.27	4.00	0.0028
	(95 %CI)	(2.93–3.61)	(3.69–4.31)	
Did your technique improve?	Mean	3.07	3.56	0.087
	(95 %CI)	(2.65–3.48)	(3.18–3.94)	
Questionnaires to scrub nurse (number of scrub nurses)		30 (17)	30 (17)	
Did you contribute to the surgery?	Mean	3.63	3.53	0.65
	(95 %CI)	(3.32–3.95)	(3.22–3.85)	
Did you learn anything?	Mean	4.13	4.03	0.55
	(95 %CI)	(3.89–4.37)	(3.79–4.27)	
Did your technique improve?	Mean	3.60	3.70	0.58
	(95 %CI)	(3.35–3.85)	(3.45–3.95)	

or five individuals, including one operator, one instructive assistant surgeon, one or two assistants, and one scrub nurse. The limited use of ligation and gauze reduces the practice opportunities of young surgeons who work as assistants. Surgical expertise requires repeated practice over many years. The ultrasonic scalpel would lead to a reduction in human resources and, theoretically, a reduction in global costs. However, it would not provide good practice opportunities to young surgeons.

In conclusion, this study shows that the ultrasonic scalpel is a reliable and safe tool for open gastric cancer surgery. Its use is associated with a shorter operative time and less blood loss. Cost remains a major concern; however, a shorter operative time, less blood loss, less stress on the operator,

Table 5 Questionnaire "Which approach would you like to use at the next surgery?"

No. of questionnaires (no. of people)		Ultrasonic		Either		Conventional	
		n	%	*n*	%	*n*	%
Operator	60 (12)	36	60.0	20	33.3	4	6.7
1st assisting surgeon	60 (10)	30	50.0	28	46.7	2	3.3
2nd assisting surgeon	33 (11)	13	39.3	14	42.4	6	18.2
Scrub nurse	60 (20)	34	56.7	21	35.0	5	8.3