

autoclaving (121°C) for 15 min in 0.01 mol/L citrate-buffered saline (pH 6.0) for antigen retrieval. Endogenous peroxidase activity was blocked by incubation with 0.3% H<sub>2</sub>O<sub>2</sub> for 10 min. The sections were incubated with mouse monoclonal antibodies against BUBR1 (Clone 9, 1:100; BD Transduction Laboratories, San Jose, CA, USA) at 4°C overnight. Streptavidin-biotin complex and horseradish peroxidase were applied, and the reaction products were visualized using the Histofine SAB-PO (M) immunohistochemical staining kit (Nichirei, Tokyo, Japan), according to the manufacturer's instructions. Two blinded observers (K.A. and Y.Z.) independently examined immunostained sections. The intensity of cytoplasmic staining of BUBR1 was scored on a three-point scale in comparison to the staining of lymph follicles which were equally stained in all 181 specimens. Weaker staining was scored 0, and similar and stronger than the follicle were scored 1 and 2, respectively.

**Analysis for DNA ploidy.** Nuclear DNA content was measured by using laser scanning cytometry (LSC; CompuCyte, Westwood, MA, USA) as described previously.<sup>(23,24)</sup> The same paraffin-embedded blocks that were used for immunohistochemical staining were used for this analysis. A DNA content histogram was generated and DNA ploidy was determined. DNA index (DI) was calculated according to previously published principles.<sup>(25,26)</sup> For every case, the nuclei were observed after each scan to exclude debris and attached nuclei from the analysis. The DI of G0/G1-phase lymphocyte or fibroblasts were used as a reference of DI = 1.0. Tumors with a DI < 1.2 were defined as diploid; DI ≥ 1.2 or multi-indexed samples were defined as aneuploid.

**High-resolution fluorescent microsatellite analysis (HRFMA) for MSI.** HRFMA has been described in detail elsewhere.<sup>(27)</sup> Briefly, genomic DNA isolated from cancerous and corresponding noncancerous tissue specimens was used to amplify microsatellite loci by polymerase chain reaction (PCR) using primer sets labeled with a fluorescent compound, ROX (6-carboxy-x-rhodamine) or HEX (6-carboxy-20,40,70,4,7-hexachlorofluorescein). The fluorescently labeled PCR products were mixed, denatured, and loaded onto an ABI 310 sequencer (Applied Biosystems, Foster City, CA, USA) for fragment analysis. The data were processed using the GeneScan software package (Applied Biosystems). An alternation in the length of a microsatellite PCR fragment from cancerous tissues was defined as MSI positive. According to the guidelines established by the National Cancer Institute (NCI), MSI was defined by the frequency of positive findings of five reference markers: D2S123, D5S107, D10S197, D11S904, D13S175.<sup>(28)</sup> MSI status was classified as follows: microsatellite instability high (MSI-H), >30% of loci demonstrate MSI; microsatellite instability low, ≤30% of loci demonstrate MSI; and microsatellite stability, no positive MSI detected in any of the loci. MSI-H was labeled 'MSI (+)' and the rest 'MSI (-)'.

**TP53 gene mutation analysis.** The TP53 gene exon 5 to exon 9 including exon-intron junctions were amplified by PCR using 953 primers (Nippon Gene, Tokyo, Japan) and *Ex Taq* DNA polymerase with 3' exonuclease activity (Takara Bio, Tokyo, Japan). The PCR products were purified and used as templates for cycle-sequencing reactions with the Big Dye Terminator Cycle Sequencing Kit version 1 (Applied Biosystems). Mutations found in a PCR product were verified by reverse sequencing and reconfirmed in two independently amplified PCR products.

**Gastric cancer cell lines and cell culture.** Human gastric cancer cell lines MKN-28 (JCRB0253), MKN-74 (JCRB0255), SNU-1 (CRL-5971), SNU-16 (CRL-5974), NUGC-4 (JCRB0834), KATO III (JCRB0611), NCI-N87 (CRL-5822), and human fibroblast MRC-5 (CCL-171) were grown in monolayer cultures in RPMI-1640 supplemented with fetal bovine serum (10% v/v) and glutamine (2 mM) at 37°C in a 5% CO<sub>2</sub> environment. MKN-

28, MKN-74, NUGC-4, and KATO III were obtained from the Japanese Cancer Research Resource Bank (JCRB). SNU-1, SNU-16, NCI-N87, and MRC-5 were obtained from ATCC (Manassas, VA, USA). In this experiment, cell lines were used between passage 3 and passage 6.

**Analysis for DNA ploidy in gastric cancer cell lines.** Each of the cell lines were incubated in four-well chamber slide glasses. MRC-5 was grown in one of the chambers as a normal control. When they reached 80% confluence, generally after 3 days, the media were changed to serum-free medium for serum starvation.<sup>(29)</sup> After 48 h of serum starvation, the slides were fixed in 70% ethanol and stained with propidium iodide (PI) (25 µg/mL) including RNaseA (10 mg/mL). DNA ploidy was analyzed in LSC. The DNA index of the G0/G1 phase of MRC-5 (normal human fibroblast cell line) was used as reference of DI = 1.0. Cell lines with a DI < 1.2 were defined as diploid; DI ≥ 1.2 or multi-indexed cell lines were defined as aneuploid.

**Quantification of the mRNA of gastric cancer cell lines using real-time quantitative RT-PCR.** The pellets of each of the gastric cancer cell lines were homogenized. Total RNA was isolated using an RNeasy mini kit (Qiagen, Chatsworth, CA, USA) according to the manufacturer's instructions. cDNA was synthesized with random hexamer primers and Superscript III reverse transcriptase according to manufacturer's instructions and the product was used for further analysis. BUBR1 transcription was quantified using the LightCycler (Roche Molecular Biochemicals, Mannheim, Germany) PCR protocol, in which fluorescence emission is attributable to binding of SYBR Green I dye to amplified products and can be detected and measured essentially. The relative quantitation value is expressed as 2-Ct, where Ct is the difference between the mean Ct value of triplicates of the sample and of the endogenous β-actin control. The primer sequences for Real-time RT-PCR were as follows: BUBR1, 5'-CTCGTGGCAATACAGCTTCA-3' (forward) and 5'-CTGGTCAATAGCTCGGCTTC-3' (reverse);<sup>(30)</sup> Ki-67, 5'-ACTTGCTCTTAATACGCC-3' (forward) and 5'-TTACTACATCTGCCATGA-3' (reverse);<sup>(31)</sup> and β-actin, 5'-CCACGAAATACCTTCAAC-3' (forward) and 5'-GATCTTCATTGTCTGGG-3' (reverse).

**BUBR1 transfection to MKN-28 and SNU-1.** Gastric cancer cell lines MKN-28 and SNU-1 were transfected with pcDNA3.1 + BUBR1 or pcDNA3.1 + vector alone using FuGene HD (Roche Diagnostics, Indianapolis, IN, USA) as a transfection vehicle. The transfectants (MKN-28 Mock, MKN-28 BUBR1, SNU-1 Mock, and SNU-1 BUBR1) were selected by addition of G418 (200 µg/mL) to the culture medium.

**Western blotting.** Cells were lysed in a sodium dodecyl sulfate (SDS)-sample buffer containing 10% glycerol, 5% β-mercaptoethanol, 2.3% SDS, and 62.5 mM Tris-HCl (pH 6.8). Ten µg of protein was analysed by SDS-PAGE followed by Western blotting. The protein blots were first probed with a mouse anti-BUBR1 (Clone 9, 1:1000; BD Transduction Laboratories) antibody or a mouse anti-β-actin (Clone AC-15, 1:2000; Sigma-Aldrich, St. Louis, MO, USA) antibody, and then with a goat-antimouse IgG conjugated with horseradish peroxidase. The specific signals on the blot were detected by the enhanced chemiluminescent method.

**Growth curve assay.** MKN-28 Mock and MKN-28 BUBR1 were plated in triplicate at a density of 1 × 10<sup>5</sup> cells per dish in 6-cm culture dishes. At the indicated time points after plating, cells were trypsinized, stained with Trypan blue, and the number of viable cells was directly scored by using the hemocytometer.

**Statistical analysis.** The statistical analysis was performed by using the JMP 6.0 statistical software package (SAS Institute, Cary, NC, USA). The Student's *t*-test, the  $\chi^2$ -test, Fisher's exact test, and ANOVA one-way test were used where appropriate. The Kaplan-Meier analysis was used for overall survival.

## Results

**DNA ploidy in gastric cancer.** The DNA ploidy patterns of 116 gastric cancer patients were analyzed by LSC. Of those, 70 (60.3%) showed DNA aneuploidy, which was consistent with a previous report.<sup>(32)</sup>

As is shown in Table 1, DNA aneuploid tumors were significantly correlated with differentiated tumors ( $P < 0.01$ ). This result was consistent with a previous report on DNA ploidy in gastric cancer.<sup>(33)</sup> In this study, we defined papillary and tubular adenocarcinoma as differentiated tumors and poorly differentiated adenocarcinoma and signet-ring cell carcinoma as undifferentiated tumors.<sup>(20)</sup> Mucinous carcinoma was included in undifferentiated tumors. Also, DNA aneuploid tumors had more positive vascular involvement than diploid tumors ( $P < 0.05$ ). *TP53* gene mutation and MSI statuses were also analyzed in the same gastric cancer samples. *TP53* gene mutation was analyzed in 84 patients and 20 (23.8%) had mutations. MSI status was analyzed in 116 patients and 12 (10.3%) were MSI (+). DNA aneuploidy significantly correlated with *TP53* gene mutation ( $P < 0.05$ ), whereas MSI (+), a marker for microsatellite stability, was reversely correlated ( $P < 0.05$ ; Table 2).

**High expression of BUBR1 in gastric cancer correlated with malignant features in gastric cancer.** Abnormal spindle assembly checkpoint is suggested to be one of the major causes for DNA aneuploidy.<sup>(34)</sup> BUBR1 is one of the factors in this checkpoint and its high expression is predicted to correlate with DNA aneuploidy.<sup>(18,19)</sup> Therefore, BUBR1 expression was investigated by immunohistochemistry in 181 gastric cancer samples. The BUBR1 was mainly expressed in the cytoplasm in the tumor cells (Fig. 1). In the normal gastric epithelium, BUBR1 was expressed only in the mitosis (Fig. 1a, arrow pointed cells). This was seen in all 181 cases. These cells whose intensity of staining was similar to the lymph follicle were scored 1

**Table 1. DNA ploidy and clinicopathological factors in gastric cancer**

Factors	DNA ploidy		P-values
	Diploidy (n = 46)	DNA aneuploidy (n = 70)	
Age (mean ± SD)	61.9 ± 13.9	65.8 ± 11.3	0.116
Gender			
Male	30 (65.2)	49 (70.0)	0.589
Female	16 (34.8)	21 (30.0)	
Differentiation			
Differentiated	14 (30.4)	41 (59.4)	0.002*
Undifferentiated	32 (69.6)	28 (40.6)	
Depth of invasion			
M, SM	7 (15.2)	8 (11.4)	0.554
MP, S5, SE, S1	39 (84.8)	62 (88.6)	
Vascular involvement			
Negative	34 (73.9)	37 (53.6)	0.026**
Positive	12 (26.1)	32 (46.4)	
Lymph node metastasis			
Negative	15 (32.6)	14 (20.0)	0.127
Positive	31 (67.4)	56 (80.0)	
Liver metastasis			
Negative	45 (97.8)	67 (95.7)	0.53
Positive	1 (2.2)	3 (4.3)	
Stage			
I	13 (28.3)	12 (17.1)	0.351
II	7 (15.2)	13 (18.6)	
III	7 (15.2)	18 (25.7)	
IV	19 (41.3)	27 (38.6)	

The values in parentheses are expressed in %. \* $P < 0.01$ , \*\* $P < 0.05$ . M, mucosa; MP, muscularis propria; SE, penetration of serosa; S1, invasion of adjacent structures; SM, submucosa; S5, subserosa.

**Table 2. DNA ploidy and genetic factors in gastric cancer**

Factors	DNA ploidy		P-values
	Diploidy	Aneuploidy	
<i>TP53</i> status			
Wild	27 (90.0)	37 (68.5)	0.019*
Mutation	3 (10.0)	17 (31.5)	
MSI status			
MSI (-)	38 (82.6)	66 (94.3)	0.045*
MSI (+)	8 (17.4)	4 (5.7)	

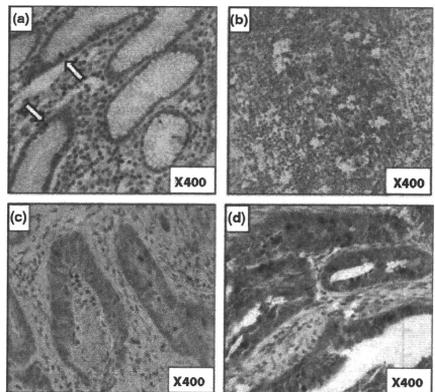
The values in parentheses are expressed in %. \* $P < 0.05$ . MSI, microsatellite instability.

(Fig. 1b); this was used as the inner control. Based on BUBR1 expression in the normal gastric epithelium which was scored 1, BUBR1 was considered to be highly expressed in gastric cancer when score 2 expression was seen (Fig. 1d), and the others as low expression (Fig. 1c).

BUBR1 score 2 expression was observed in 91 (50.3%) of the 181 patients.

Table 3 shows the relationship between the clinicopathological features and expression level of BUBR1 in patients with gastric cancer. The tumors with BUBR1 score 2 expression showed deeper invasion than those with a lower BUBR1 expression level ( $P < 0.01$ ). Furthermore, the BUBR1 highly expressing tumors had more lymph node and liver metastases ( $P < 0.01$  and  $P < 0.05$ , respectively). No difference was seen between BUBR1 score 0 expression and score 1 expression in gastric cancer.

**DNA aneuploidy and BUBR1 expression had a significant relationship in gastric cancer.** The relationship between the DNA ploidy pattern and BUBR1 expression level in gastric cancers was analyzed. The result is shown in the lower part of



**Fig. 1.** Immunohistochemical staining of BUBR1 in gastric cancer. (a) BUBR1 staining in normal epithelium. Arrows show the mitotic cells whose BUBR1 staining is scored 1 (magnification,  $\times 400$ ). (b) BUBR1 staining in lymph follicle. BUBR1 score 1 expression was based on this intensity of BUBR1 staining (magnification,  $\times 400$ ). (c) BUBR1 staining in gastric cancer cells. This shows the BUBR1 score 1 expression (magnification,  $\times 400$ ). (d) BUBR1 staining in gastric cancer cells. This shows the BUBR1 score 2 expression, which indicates high expression of BUBR1 (magnification,  $\times 400$ ).

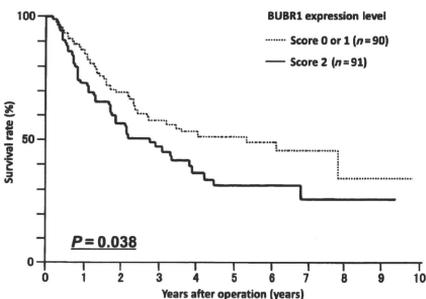
**Table 3. BUBR1 expression and clinicopathological factors in gastric cancer**

Factors	BUBR1 expression level		P-values
	Score 0,1 (n = 90)	Score 2 (n = 91)	
Age (mean ± SD)	62.5 ± 13.1	65.8 ± 10.5	0.053
Gender			
Male	60 (66.7)	61 (67.0)	0.958
Female	30 (33.3)	30 (33.0)	
Differentiation			
Differentiated	35 (38.9)	44 (48.9)	0.176
Undifferentiated	55 (61.1)	46 (51.1)	
Depth of invasion			
M, SM	17 (18.9)	5 (5.5)	0.004*
MP, S5, SE, S1	73 (81.1)	86 (94.5)	
Vascular involvement			
Negative	53 (58.9)	49 (54.4)	0.547
Positive	37 (41.1)	41 (45.6)	
Lymph node metastasis			
Negative	38 (42.2)	20 (22.0)	0.002*
Positive	52 (57.8)	71 (78.0)	
Liver metastasis			
Negative	89 (98.9)	85 (93.4)	0.044**
Positive	1 (1.1)	6 (6.6)	
Stage			
I	27 (30.0)	16 (17.6)	0.246
II	18 (20.0)	20 (22.0)	
III	20 (22.2)	22 (24.2)	
IV	25 (27.8)	33 (36.3)	
DNA ploidy			
Diploidy	28 (60.9)	18 (39.1)	0.012**
Aneuploidy	26 (39.1)	44 (62.9)	

The values in parentheses are expressed in %. \* $P < 0.01$ , \*\* $P < 0.05$ . M, mucosa; MP, muscularis propria; SE, penetration of serosa; S1, invasion of adjacent structures; SM, submucosa; S5, subserosa.

Table 3. Tumors with BUBR1 score 2 expression showed a significant correlation with DNA aneuploidy ( $P < 0.05$ ). Not only *TP53* gene mutation but also high BUBR1 expression seemed to be an important factor for DNA aneuploidy in gastric cancer.

High expression of BUBR1 correlated with poor survival. Figure 2 shows the survival curve for patients according to expression levels of BUBR1. Patients with BUBR1 score 2 expression tumors had a significantly lower survival rate



**Fig. 2.** Survival curve for patients with gastric cancer in relation to BUBR1 expression. Patients with BUBR1 score 2 tumors (n = 91) had a poorer survival rate compared to those with BUBR1 score 0 or 1 tumors (n = 90,  $P = 0.038$ ).

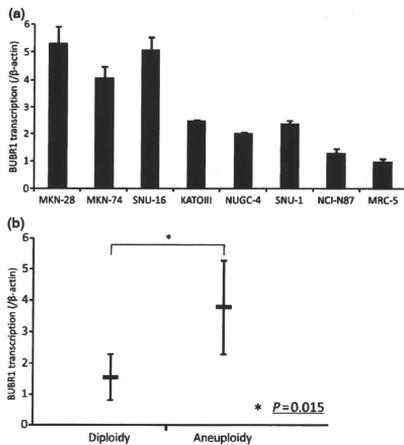
compared to patients with score 0 or 1 tumors ( $P < 0.05$ ). A multivariate Cox regression analysis showed that the BUBR1 expression level was not an independent prognostic factor. This may be because in tumors with BUBR1 score 2 expression, there was a significant relationship between tumor depth and lymph node metastasis, which were strong independent prognostic factors (data not shown).

**High expression of BUBR1 correlated with DNA aneuploidy in gastric cancer cell lines.** Next we examined the relationship between DNA ploidy and BUBR1 expression in gastric cancer cell lines. First, the DNA ploidy patterns of gastric cancer cell lines were analyzed. Growing cells on four-well chamber slide glass made it easier to compare the DNA index of gastric cancer cell lines with that of MRC-5 which was used as a normal control. LSC demonstrated that the gastric cancer cell lines MKN-28, MKN-74, NUGC-4, KATO III, and SNU-16 were aneuploid cell lines. SNU-1 and NCI-N87 (and also MRC-5) were diploid cell lines. The DNA ploidy patterns of KATO III, SNU-16, SNU-1, and NCI-N87 were the same as those shown in the ATCC cell bank information (<http://www.atcc.org/>).

The expression levels of BUBR1 in these cell lines were examined by real-time quantitative RT-PCR (Fig. 3a). High expression of BUBR1 was seen in MKN-28, MKN-74, and SNU-16 which were all aneuploid cell lines. A statistical analysis showed that aneuploid gastric cancer cell lines had significantly higher expression of BUBR1 compared to diploid cell lines (Fig. 3b).

**Enforced expression of BUBR1 in gastric cancer cell lines led to a change in DNA ploidy pattern and high proliferation activity.** Then we investigated whether or not high expression of BUBR1 would be a cause for changes to the DNA ploidy pattern.

We transfected gastric cancer cell line MKN-28 and diploid gastric cancer cell line SNU-1 with pcDNA3.1(+)-BUBR1 and an empty vector as a mock. As is shown in Figure 4a, the transfection was successful.



**Fig. 3.** DNA ploidy and BUBR1 expression in cell lines. (a) BUBR1 expression in cell lines. BUBR1 expression was determined by real-time RT-PCR. The bar indicates the SD. (b) Statistical analysis in BUBR1 expression and DNA ploidy in cell lines. DNA aneuploid cell lines had significantly higher BUBR1 expression levels ( $P = 0.015$ ).

The DNA ploidy patterns of these cells were analyzed with LSC. The DNA indexes of the mock cells were referred as 1.0. As seen in Figure 4b, the DNA indexes of MKN-28 BUBR1 and SNU-1 BUBR1 were increased significantly compared to the mock cells. This result meant that the DNA ploidy pattern had changed in MKN-28 BUBR1 and SNU-1 BUBR1.

We also counted the cell numbers of MKN-28 BUBR1 and MKN-28 Mock to investigate the growth activity. As is shown in Figure 5a, MKN-28 BUBR1 had higher growth activity compared to MKN-28 Mock. Also, in MKN-28 BUBR1 and SNU-1 BUBR1 the expression of Ki-67 was higher than in the mock cells (Fig. 5b).

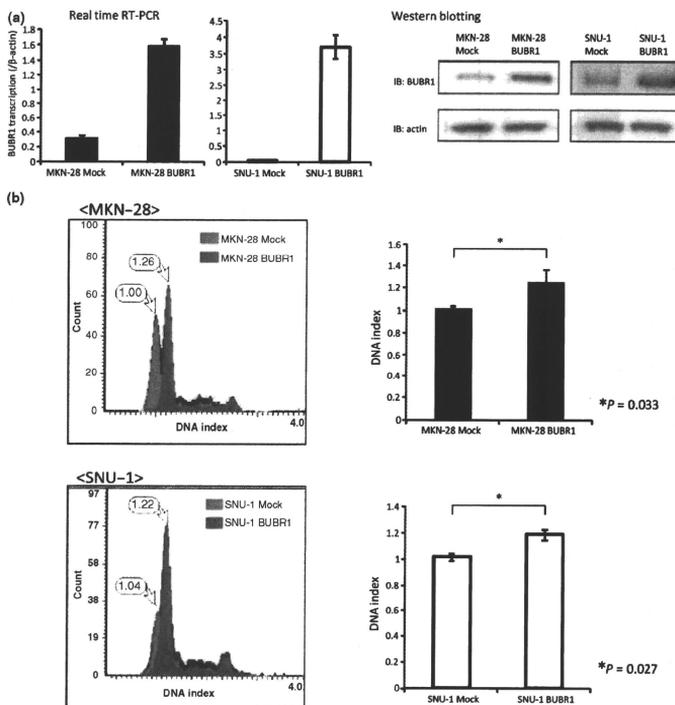
## Discussion

In the current study, genomic instability was associated with DNA aneuploidy; and the chromosomal instability pathway seemed to be important in gastric carcinogenesis. Chromosomal

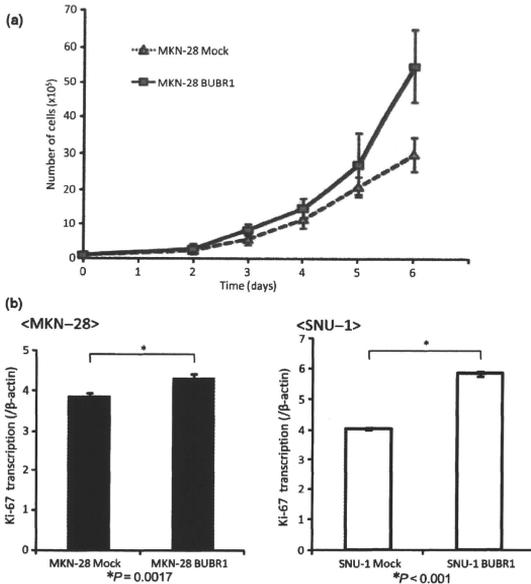
instability and microsatellite instability pathways are reported to be exclusive in colorectal cancers.<sup>(2)</sup> However, in gastric cancer, both DNA aneuploidy and MSI (+) were observed in four (3.4%) tumors and both DNA diploidy and MSI (-) were observed in 38 (32.8%) tumors in this study. It seems that chromosomal instability and microsatellite instability pathways are not always mutually exclusive in gastric cancer, and additional mechanisms, such as the CpG island methylator phenotype (CIMP), may contribute to gastric carcinogenesis.<sup>(35)</sup>

It is well known that mutation in the *TP53* gene contributes to DNA aneuploidy. In our study, DNA aneuploidy and *TP53* gene mutation were significantly correlated. However, the frequency of the *TP53* gene mutation (23.8%) was less than DNA aneuploidy (60.3%). Thus it seemed that the mutation in the *TP53* gene did not explain all the DNA aneuploidy in gastric cancers.<sup>(3)</sup>

This study revealed that, by immunohistochemical staining, high expression of BUBR1, a key molecule in the spindle assembly checkpoint, had a significant correlation with DNA



**Fig. 4.** Enforced expression of BUBR1 in gastric cancer cell lines led to a change in DNA ploidy pattern. (a) Establishing BUBR1 highly expressed gastric cancer cell line MKN-28 and SNU-1. The expression levels of BUBR1 in the mock cells and BUBR1-enforced cells were investigated by real-time RT-PCR and Western blotting. High expression of BUBR1 can be seen in BUBR1-enforced cells at both the mRNA and protein level. (b) DNA ploidy pattern change in BUBR1 highly expressed cells. The DNA ploidy patterns were investigated by laser scanning cytometry. The DNA indexes of the mock cells were determined as 1.0. The change in DNA ploidy pattern can be seen in MKN-28 BUBR1 and SNU-1 BUBR1. Also the DNA index was significantly changed in MKN-28 BUBR1 and SNU-1 BUBR1 compared to the mock cells (right panel,  $P = 0.033$  and  $P = 0.027$ , respectively).



**Fig. 5.** High expression of BUBR1 had high proliferation activity in gastric cancer cell lines. (a) The growth curve of MKN-28 Mock and MKN-28 BUBR1. MKN-28 BUBR1 had higher growth activity. (b) Ki-67 expression in mock cells and BUBR1 highly expressed cells. Ki-67 expression was determined by real-time RT-PCR. Higher expression of Ki-67 was seen in BUBR1 highly expressed cells.

aneuploidy in gastric cancer. This result was consistent with the study on bladder cancer by Yamamoto *et al.*<sup>(19)</sup> Also the gastric cancer cell lines which showed DNA aneuploidy had higher BUBR1 expression in comparison to DNA diploid cell lines. High expression of BUBR1 may be an important factor for DNA aneuploidy and may contribute to chromosomal instability.

In this study, we also found that the enforced expression of BUBR1 in the gastric cancer cell line MKN-28 and the diploid gastric cancer cell line SNU-1 contributed to the change to the DNA ploidy pattern compared with mock cells. The mechanism for changes to the DNA ploidy pattern in the BUBR1 highly expressed cell line is unclear. It is known that BUBR1 forms a complex with Bub3, Mad2, and Cdc20 at the spindle assembly checkpoint and thus inhibits Cdc20 activity.<sup>(36)</sup> In gastric cancer cells with high BUBR1 expression, the formation of this complex might be compromised and the spindle assembly checkpoint may be overridden, which may result in DNA aneuploidy.

When BUBR1 is depleted in human cells, it results in chromosome misalignment,<sup>(37)</sup> and its suppression is invariably lethal due to massive chromosomal loss.<sup>(38)</sup> It is reported that reduction in BUBR1 levels in *Apc*<sup>Min/+</sup> mice, which carry one mutant *Apc* allele encoding a truncated protein that causes intestinal neoplasias, resulted in a 10-fold increase in the number of tumors that developed by the same age, and these tumors were also of a higher grade.<sup>(39)</sup> Moreover, the overexpression of Mad2, another molecule at the spindle assembly checkpoint, caused DNA aneuploidy and carcinogenesis in mice.<sup>(40)</sup> An abnormal expression level in spindle assembly checkpoint molecules, including BUBR1, seems to be important for carcinogenesis.

On the other hand, we also found that high expression of BUBR1 correlated with malignant features and liver metastasis

in gastric cancer. And those tumors demonstrating high BUBR1 expression were associated with a poorer survival rate compared to the tumors with low expression of BUBR1. These results might reflect the fact that BUBR1 high-expressed tumors have a high proliferation activity. In fact, the current study showed that with enforced expression of BUBR1 in MKN-28 gastric cancer cell line, the growth of the cells increased. And in both MKN-28 BUBR1 and SNU-1 BUBR1, the expression of Ki-67 increased significantly. It has been reported that enforced expression of BUBR1 in HeLa cells increased the mitotic index.<sup>(10)</sup> Also, Grabsh *et al.*<sup>(41)</sup> reported that BUBR1 high expression had a significant relationship with Ki-67 expression in gastric cancer clinical samples, which was a similar result to our study in gastric cancer cell lines.

Taken together, the high expression of BUBR1 may result in the proliferation of tumor cells and DNA aneuploidy.

A high expression of BUBR1 may correlate with DNA aneuploidy in gastric cancer. However, a question remains. As malignancies which show DNA aneuploidy have aberrant chromosome numbers, high expression of BUBR1 may be an effect of the DNA aneuploidy. In our study, we only observed the DNA ploidy pattern change in MKN-28 and SNU-1 gastric cancer cells. Further studies on BUBR1 and other factors in the spindle assembly checkpoint as a cause or effect of chromosomal instability are necessary.

Association between BUBR1 expression and taxane sensitivity was reported in breast and esophageal cancer.<sup>(42,43)</sup> Our succinic dehydrogenase inhibition (SDI) test data of gastric cancer specimens, however, did not indicate significant association between BUBR1 expression and taxane sensitivity (data not shown). Therefore, we may not be able to predict the therapeutic

effect of taxane from the expression level of BUBR1 in gastric cancer. Recently, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) has attracted interest as an anticancer treatment when used in conjunction with standard chemotherapy, such as the taxanes. Kim *et al.* reported that adding TRAIL to the taxanes accentuates the degradation of BUBR1 and induces cancer cell death.<sup>(44)</sup> Using TRAIL with the taxanes in BUBR1 high-expressed gastric cancers might therefore be a successful treatment.

In conclusion, the high expression of BUBR1 was associated with DNA aneuploidy and was also correlated with tumor invasion

and metastasis in gastric cancer. The elucidation of the spindle assembly checkpoint may therefore help to clarify the mechanism for DNA aneuploidy and progression in gastric cancer.

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## Original Article

# Impact of Perioperative Peripheral Blood Values on Postoperative Complications After Esophageal Surgery

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### Abstract

**Purpose.** Prediction of the postoperative course of esophagectomy is an important part of the strict perioperative management of patients undergoing surgery for esophageal cancer.

**Methods.** To evaluate their clinical importance, peripheral blood values, including white blood cell count (WBC), lymphocyte count, and the levels of total protein, transferrin, factor XIII, D-dimer, fibrin, and fibrinogen degradation products (FDP) were measured before and after esophagectomy for esophageal cancer in 24 patients.

**Results.** The preoperative WBC and the pre- and postoperative lymphocyte count were decreased remarkably in patients who received preoperative chemoradiotherapy. The values of perioperative serum transferrin were significantly lower in patients with postoperative pneumonia than in those without. The activity of plasma factor XIII was suppressed on postoperative day (POD) 7 in patients with pneumonia and on POD 14 in patients with leakage.

**Conclusions.** These results suggest that patients who receive preoperative chemoradiotherapy are potentially immunosuppressed, the preoperative serum transferrin level is a possible predictive marker of postoperative pneumonia, and suppression of factor XIII activity is related to anastomotic insufficiency.

**Key words** Esophagectomy · Preoperative therapy · Pneumonia · Leakage · Transferrin · Factor XIII

### Introduction

Surgery remains the standard therapy for esophageal cancer because it provides the best chance of cure.<sup>1</sup> However, resection and reconstruction of the esophagus is one of the most invasive treatments for cancers of the digestive organs. Although the surgical techniques and perioperative management of esophageal surgery have improved remarkably, the morbidity and mortality rates are still high in comparison with those associated with other procedures.<sup>2-5</sup> Esophageal cancer usually occurs in older patients who often have coexisting diseases, particularly of the heart and lungs, and these comorbidities can impair the tolerance of patients to the invasiveness of an esophagectomy. Analyses of the preoperative risk factors of complications have been carried out,<sup>6</sup> however, clinically practical methods of assessing operative risks are still required. The present study prospectively evaluates the perioperative peripheral blood values of measures of immunity, nutrition, and wound healing, to predict the postoperative clinical course, focusing on preoperative treatment and postoperative pneumonia and leakage. Our data clarified the relationship between the various values and the postoperative clinical course and the likelihood of postoperative complications after esophagectomy.

### Patients and Methods

#### Patients

The subjects of this study were 24 patients who underwent esophageal resection and reconstruction for thoracic esophageal cancer in the Department of Surgery and Science, Graduate School of Medical Sciences, Kyushu University, between 2007 and 2008. The median age was 62 years (range, 47–78 years) and there were 21 men and 3 women. All cases were in a World Health

Organization (WHO) performance status of either 0 or 1. Squamous cell carcinoma was diagnosed histologically in all the patients. Eight of the patients received preoperative chemoradiotherapy, three of whom received definitive chemoradiotherapy with more than 50 Gy of radiation.

#### Treatment Procedures

Thoracic esophageal cancer was treated with either subtotal esophagectomy with an anterolateral right thoracotomy and cervical anastomosis, or distal esophagectomy with an anterolateral right thoracotomy, laparotomy, and intrathoracic anastomosis. Patients with carcinoma of the upper- or mid-thoracic esophagus or advanced carcinoma of the lower thoracic esophagus, who were in good health otherwise, generally underwent subtotal esophagectomy via a cervico-right thoracoabdominal approach. To adjust the background of the surgical procedures, only patients who underwent subtotal esophagectomy with an anterolateral right thoracotomy and cervical anastomosis were included in this study. Reconstruction was accomplished using a pulled-up gastric tube in all patients. Three-field and two-field lymph node dissections were performed in 18 (75.0%) and 6 (25.0%) patients, respectively. Five patients with locally advanced cancer received preoperative chemoradiotherapy, consisting of 35–42 Gy of radiation and cisplatin plus 5-fluorouracil. Three patients received definitive chemoradiotherapy, consisting of more than 50 Gy of radiation and cisplatin plus 5-fluorouracil.

#### Measurement of Peripheral Blood Values

The blood was analyzed within 1 week before surgery, and then on postoperative day (POD) 7 and 14. We measured the white blood cell count (WBC), the lymphocyte count, and the levels of total protein, transferrin, factor XIII, D-dimer, fibrin, and fibrinogen degradation products (FDP). The normal ranges of each measurement are as follows: WBC, 3500–9000/ $\mu$ l; lymphocytes, 1800–5300/ $\mu$ l; total protein, 6.7–8.3 g/dl; transferrin, 190–300 mg/dl; factor XIII, 55%–145%; D-dimer,  $\geq 1 \mu$ g/dl; FDP,  $> 5 \mu$ g/dl.

#### Statistical Analysis

The clinicopathological factors and the various peripheral blood values were compared in relation to the preoperative treatment, postoperative pneumonia (defined as an infiltration shadow on chest X-rays and a positive bacterial culture of sputum), and cervical anastomotic leakage (confirmed by postoperative esophagogram or saliva discharge through the fistula). Significant differences were established using Fisher's exact test in the

distribution frequencies of the data in Tables 1–3 except for age. The Mann-Whitney *U*-test was applied for differences in age, the operative time, blood loss, and the values of these parameters between the groups. A *P*

**Table 1.** Clinicopathological features according to preoperative treatment

Factor	Preoperative treatment	
	No ( <i>n</i> = 16)	Yes ( <i>n</i> = 8)
Sex		
Male	14 (87.5)	7 (87.5)
Female	2 (12.5)	1 (12.5)
Age (mean $\pm$ SD)	62.2 $\pm$ 6.8	61.5 $\pm$ 10.1
pStage		
0, I, II	10 (62.5)	5 (62.5)
III, IV	6 (37.5)	3 (37.5)
Postoperative pneumonia		
(–)	12 (75.0)	6 (75.0)
(+)	4 (25.0)	2 (25.0)
Postoperative leakage		
(–)	12 (75.0)	6 (75.0)
(+)	4 (25.0)	2 (25.0)

No significant difference was observed between the two groups. The numbers in parentheses are percentages

**Table 2.** Clinicopathological features and the clinical development of postoperative pneumonia

Factor	Postoperative pneumonia	
	No ( <i>n</i> = 18)	Yes ( <i>n</i> = 6)
Sex		
Male	15 (83.3)	6 (100.0)
Female	3 (16.7)	0 (0)
Age (mean $\pm$ SD)*	60.0 $\pm$ 7.7	67.8 $\pm$ 4.9
pStage		
0, I, II	12 (66.7)	3 (50.0)
III, IV	6 (33.3)	3 (50.0)
Postoperative leakage		
(–)	15 (83.3)	3 (50.0)
(+)	3 (16.7)	3 (50.0)

\*The ages of the patients in the two groups differed significantly (*P* < 0.05). The numbers in parentheses are percentages

**Table 3.** Clinicopathological features and the clinical development of postoperative leakage

Factor	Postoperative leakage	
	No ( <i>n</i> = 18)	Yes ( <i>n</i> = 6)
Sex		
Male	15 (83.3)	6 (100.0)
Female	3 (16.7)	0 (0)
Age (mean $\pm$ SD)	61.8 $\pm$ 7.9	62.5 $\pm$ 8.8
pStage		
0, I, II	10 (55.6)	5 (83.3)
III, IV	8 (44.4)	1 (16.7)

No significant difference was observed between the two groups. The numbers in parentheses are percentages

value of less than 0.05 was considered significant. Data were analyzed using the StatView software package (Abacus Concepts, Berkeley, CA, USA).

## Results

### Patient Characteristics According to Preoperative Treatment and the Postoperative Complications of Pneumonia and Anastomotic Leakage

There were no significant differences between the two groups in sex, mean age, pathological stage of esophageal cancer, or in the incidences of postoperative pneumonia and anastomotic leakage (Tables 1–3). The mean age of the patients who suffered from postoperative pneumonia was significantly higher than that of those who did not (67.8 vs 60.0 years old, respectively;  $P < 0.05$ ). There were no significant differences between the groups in any of the factors related to postoperative leakage. The extent of lymph node dissection, the operative time, and the blood loss had no association with any of the preoperative treatments, postoperative pneumonia, or anastomotic leakage.

### Relationship Between Peripheral Blood Values and Preoperative Treatment

We compared the peripheral blood values between the preoperative treatment group and the no treatment group (Fig. 1). The preoperative WBC and lymphocyte count, and the lymphocyte count on PODs 7 and 14 were all significantly lower in the patients who received preoperative chemoradiation therapy (4325/ $\mu$ l vs 5622/

$\mu$ l, 759/ $\mu$ l vs 1745/ $\mu$ l, 582/ $\mu$ l vs 1294/ $\mu$ l, 548/ $\mu$ l vs 1367/ $\mu$ l, respectively; all  $P < 0.05$ ). There were no significant differences in transferrin, factor XIII, D-dimer, and FDP.

### Peripheral Blood Values in Relation to Postoperative Pneumonia

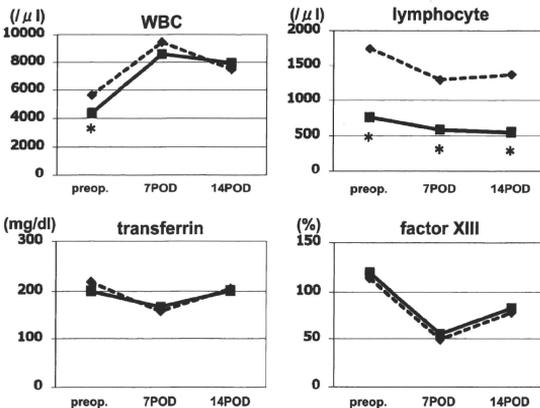
The nutrition markers were significantly lower at some points in the patients who suffered pneumonia than in those who did not (Fig. 2) (total protein on POD 7, 5.5 g/dl vs 6.1 g/dl; preoperative transferrin, 181 mg/dl vs 224 mg/dl; transferrin on POD 7, 112 mg/dl vs 177 mg/dl; transferrin on POD 14, 157 mg/dl vs 220 mg/dl; all  $P < 0.05$ ). The activity of plasma factor XIII on POD 7 in the pneumonia group was significantly lower than that in the group without pneumonia (35.7% vs 55.9%;  $P < 0.05$ ). There were no significant differences in WBC, lymphocyte, D-dimer, and FDP.

### Peripheral Blood Values in Relation to Postoperative Leakage

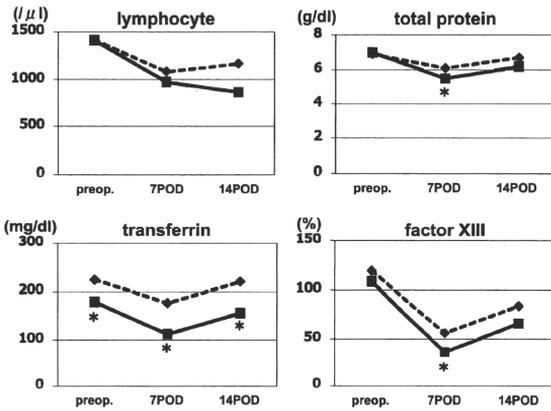
There was no significant difference in plasma factor XIII activity on POD 14 between the two groups (Fig. 3) (63% in the leakage group vs 85% in the no leakage group;  $P < 0.05$ ). There were also no significant differences in WBC, lymphocyte, total protein, transferrin, D-dimer, and FDP.

## Discussion

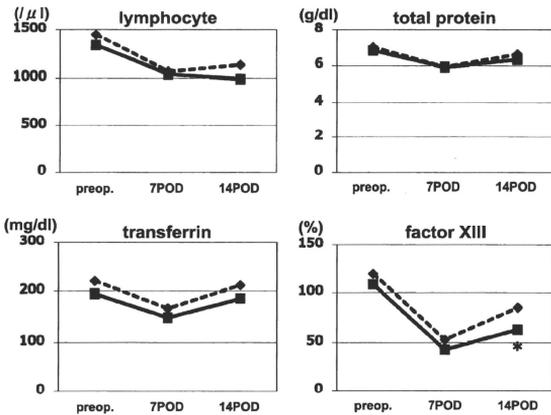
Mortality rates after esophageal surgery range from 2% to 25%,<sup>7</sup> and are primarily determined by the develop-



**Fig. 1.** Differences in peripheral blood values according to preoperative treatment. *Diamonds* (group A), patients who did not receive preoperative treatment ( $n = 16$ ); *squares* (group B), patients who received preoperative treatment ( $n = 8$ ). \* $P < 0.05$



**Fig. 2.** Differences in peripheral blood values according to the development of postoperative pneumonia. *Diamonds* (group C), patients without postoperative pneumonia ( $n = 18$ ); *squares* (group D), patients with postoperative pneumonia ( $n = 6$ ). \* $P < 0.05$



**Fig. 3.** Differences in peripheral blood values according to postoperative leakage. *Diamonds* (group E), patients without postoperative leakage ( $n = 18$ ); *squares* (group F), patients with postoperative leakage ( $n = 6$ ). \* $P < 0.05$

ment of pulmonary complications and anastomotic leakage.<sup>8-10</sup> Various risk analyses have also revealed impaired heart, liver, and respiratory systems, in addition to the patients' age and general condition as independent predictors of morbidity and mortality.<sup>7,11-13</sup> However, no perioperative marker has been established to predict the postoperative course. We evaluated the blood cell counts as an inflammatory marker, the total protein, and transferrin as nutrition markers, and factor XIII, D-dimer, and FDP as coagulation markers. These

markers are considered to be representative of the perioperative condition of patients with esophageal cancer.

The preoperative WBC and the pre- and postoperative lymphocyte counts were significantly lower in the patients who received preoperative treatment; however, the factors associated with the clinical benefit of preoperative chemoradiotherapy and its adverse effects on the development of postoperative complications are still unclear. According to some reports, preoperative chemoradiotherapy resulted in an increase of the inci-

dence of postoperative complications after esophagectomy,<sup>14-16</sup> whereas according to others, the incidences were similar with and without preoperative chemoradiotherapy.<sup>17-19</sup> An analysis of 1000 consecutive patients with esophageal cancer<sup>3</sup> revealed preoperative treatment as an independent factor associated with the postoperative development of pulmonary complications. Multiple immunologic values examined in patients with esophageal cancer revealed that preoperative treatment induced significant reductions in the phytohemagglutinin (PHA) response and natural killer (NK) activity.<sup>20</sup> Preoperative treatment did not affect the incidence of postoperative complications in the current series, probably because of the small number of patients evaluated. Nevertheless, our data indicate that patients who receive preoperative chemoradiotherapy are in a potentially immunosuppressed state and they should therefore be managed carefully.

Most operative deaths are caused by severe pulmonary complications,<sup>3,21,22</sup> so their prevention is of the utmost importance for decreasing the incidence of postoperative deaths. Serum transferrin is associated with overall protein synthesis. Protein synthesis is suppressed after esophageal surgery because of the host response to stress.<sup>23</sup> In this study, the pre- and postoperative levels of serum transferrin were significantly lower in the patients who suffered postoperative pneumonia than in those who did not. These data suggest that the preoperative level of serum transferrin could be a predictor of postoperative pneumonia. Since the serum transferrin level is a marker reflecting the nutritional status of the acute phase, this also suggests that preoperative enteral immunonutrition<sup>23</sup> may promote the patient's postoperative nutritional status and prevent the development of pneumonia. To evaluate the importance of the changes in transferrin values during the clinical course, the preoperative values were set as 100%, and then the values on PODs 7 and 14 were compared. Although both values tended to be lower in the pneumonia group patients than in the patients without pneumonia (data not shown), the differences were not significant. We think that depression of the absolute value of transferrin is a truer indicator of the development of postoperative pneumonia.

Esophagogastric anastomotic leakage is the most feared surgical complication of esophageal resection and reconstruction. Traditionally, symptomatic postoperative esophageal fistula is repaired surgically, and if this is unsuccessful, esophageal diversion is done, which prolongs hospitalization and delays oral hydration and nutrition. Coagulation factor XIII was first described by Laki and Lóránd in 1948 as a "fibrin-stabilizing factor."<sup>24</sup> Factor XIII is the last enzyme (plasma protransglutaminase) in the physiological coagulation cascade, and it is also important for the proliferation and immigration

of fibroblasts in wound healing. Since factor XIII is required for normal wound healing, we investigated the temporal changes in plasma factor XIII following esophagectomy. The plasma factor XIII activity on POD 14 was significantly decreased in the patients with postoperative leakage. Notably, the activity of factor XIII on POD 7 in the patients with postoperative pneumonia was significantly lower than that in those without pneumonia. Taking into consideration that three of six patients with postoperative pneumonia suffered subsequent anastomotic insufficiency, depression of the activity of factor XIII associated with pneumonia might be related to poor wound healing of the anastomotic site. Factor XIII has been used in the treatment of ulceration caused by pressure, large burns, sepsis, and acute liver disorders.<sup>25</sup> Further clinical investigations on the effectiveness of factor XIII for patients with anastomotic leakage are warranted. Other factors that have been reported to be useful markers for predicting postoperative complications include serum acute phase reactants and several cytokines such as interleukin-6 and gene polymorphism.<sup>26</sup> However, the measurement of these factors, other than acute phase reactants, is complex and expensive. Thus, we measured the general peripheral blood values in patients with esophageal cancer, and the results are considered meaningful from the viewpoint of cost-effectiveness.

In conclusion, the perioperative peripheral blood values in patients undergoing surgery for esophageal cancer revealed decreased WBC and lymphocyte counts in patients who received preoperative treatment, lower levels of serum transferrin in patients who suffered postoperative pneumonia, and a positive association of decreased postoperative plasma factor XIII with anastomotic insufficiency. Further studies on the roles of these and other markers in a larger group of patients with esophageal cancer are required to fully understand the role of peripheral blood measurements in the perioperative management.

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## A Survey of the Effects of Sivelestat Sodium Administration on Patients with Postoperative Respiratory Dysfunction

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### Abstract

**Purpose.** To clarify the clinical significance of sivelestat sodium (SIV) administration, we surveyed the status of 40 patients treated with SIV for respiratory dysfunction following surgery.

**Methods.** The subjects were patients who received SIV administration due to systemic inflammatory response syndrome (SIRS) and respiratory dysfunction ( $\text{PaO}_2/\text{FiO}_2$  ratio  $\leq 300$  mmHg) after surgery at the Department of Surgery and Science, Kyushu University, and related facilities between April and December 2008.

**Results.** The most frequent underlying condition was perforation of the digestive tract, followed by cancer of the upper digestive organs. The main causes of SIRS were surgical stress and infection. The mean P/F ratio at the initiation of SIV administration was  $185.5 \pm 72.0$  mmHg. The ratio increased, and the number of SIRS-related factors decreased with time after SIV administration. Sivelestat sodium was administered within 24 h after the onset of respiratory dysfunction in 87.5% of the patients, and the survival rate at 28 days after the initiation of SIV administration was 90.0%.

**Conclusion.** Our findings suggest that multidisciplinary postoperative management, including the administration of SIV, during the early phase after the onset of respiratory dysfunction leads to improvements in respiratory function and survival.

**Key words** Surgical stress · Systemic inflammatory response syndrome ·  $\text{PaO}_2/\text{FiO}_2$  ratio · Postoperative management · Sivelestat sodium

### Introduction

Systemic inflammatory response syndrome (SIRS) persists and progresses in some cases due to surgical stress and complications of trauma and infection. Protraction of the inflammatory responses can diminish patient recovery from surgery, and may lead to multiorgan failure (MOF), resulting in death.<sup>1,2</sup> These inflammatory reactions involve activation of various inflammatory cells and cytokine/chemokine production by these cells.<sup>3,4</sup> Inflammatory reactions play an important role as defensive reactions, but organ impairment may be induced when the responses become excessive.<sup>5</sup>

The involvement of neutrophils in postoperative acute lung injury (ALI)/acute respiratory distress syndrome (ARDS) has been attracting increasing attention.<sup>6</sup> Activated neutrophils release neutrophil elastase, which can injure vascular endothelial cells and increase permeability, inducing further neutrophil infiltration.<sup>7</sup> By binding specifically to neutrophil elastase, sivelestat sodium (SIV) inhibits the neutrophil elastase-induced pulmonary vascular endothelial cell injury, stops the increase in pulmonary vascular permeability, and decreases the alveolar epithelial cell injury, resulting in a therapeutic effect that can be useful for the treatment of ALI/ARDS.

In this study, we surveyed the postoperative status of patients who were administered SIV for postoperative respiratory dysfunction following surgery in our department and related facilities. The aim of the study was to clarify the clinical significance of SIV administration for patients with respiratory dysfunction following surgery.

### Patients and Methods

The subjects of this study were 51 patients who underwent surgery at the Department of Surgery and Science,

Graduate School of Medical Science, Kyushu University, and 19 related facilities between April and December 2008, and were treated with SIV after meeting the following two conditions after surgery: (A) two or more of the four items of the SIRS diagnostic criteria — (1) body temperature  $>38^{\circ}\text{C}$  or  $<36^{\circ}\text{C}$ , (2) heart rate  $>90$  beats/min, (3) respiratory rate  $>20$  times/min or  $\text{PaCO}_2$   $<32$  mmHg, (4) white blood cell count  $>12000/\mu\text{l}$  or  $<4000/\mu\text{l}$  or band cells  $>10\%$ ; and (B) a decreased  $\text{PaO}_2/\text{F}_2\text{O}_2$  (P/F ratio) (P/F ratio  $\leq 300$  mmHg). Sivelestat sodium was administered intravenously at a rate of 0.2 mg/kg per hour. Out of the 51 patients, 11 had experienced respiratory dysfunction prior to surgery due to their comorbidities. In order to focus on postoperative respiratory dysfunction, these 11 patients were excluded from the analysis.

Physicians filled in a survey form consisting of the following items: patient background (sex, age, underlying disease, surgical division [emergency/scheduled], cause of SIRS [infection, surgical stress, pancreatitis, other], inflammatory markers, number of organs with failure), respiratory function/management, treatment, respiratory function (P/F ratio), chest X-ray score (the number of infiltrated lung quadrants<sup>8</sup>), time-course number of SIRS-positive items, duration of SIRS (days), duration of artificial respiratory management, and outcome after 28 days. Organ failure was judged based on the method reported by Tamakuma et al.<sup>9</sup> (Table 1).

Data are presented as mean  $\pm$  standard deviation. For statistical analysis, Fisher's exact test, the unpaired *t*-test, the Wilcoxon signed-rank test, and the Cochran-Armitage trend test were employed, and  $P < 0.05$  was regarded as significant.

**Table 1.** Diagnostic criteria for organ failure

Impaired organ	Diagnostic criteria
Heart	Reduced blood pressure ( $<100$ mmHg) The circulating blood volume is normal, and no response to the normal dose of inotropic agents is noted
Liver	Serum bilirubin $>5$ mg/dl or ALT $>200$ U
Kidney	BUN $>50$ mg/dl or serum creatinine $>3$ mg/dl
Intestine	Intestinal hemorrhage requiring transfusion
Central nervous system	2 digits or higher on the Japan Coma Scale (3-3-9-step system)
Blood coagulation system	DIC based on the criteria established by the Ministry of Health, Labor and Welfare

ALT, alanine aminotransferase; BUN, blood urea nitrogen; DIC, disseminated intravascular coagulation

## Results

### Background of Patients

The background of the patients is shown in Table 2. Male patients accounted for 70.0% of the subjects, and the mean age of all patients was 67.1 years. Perforation of the digestive tract was the most frequent underlying disease, and was present in 10 patients (25.0%), followed by cancer of the upper digestive organs in 9 patients (22.5%). Half of the cases underwent emergency surgery. Surgical stress was the most frequent cause of SIRS, occurring in 26 patients (65.0%), followed by infection in 18 patients (45.0%). The number of organs with failure (other than the lungs) was 0 or 1 in 67.5% of the cases.

The respiratory function/management at the initiation of SIV administration is shown in Table 3. The P/F ratio was 200–300 mmHg in 18 cases (45.0%), 100–200 mmHg in 14 (35.0%), and 100 mmHg or lower in 8 (20.0%). On chest X-ray examination, infiltration was noted in 30 cases (75.0%). Regarding the respiratory management method, invasive positive pressure ventilation was employed in 36 patients (90.0%) and noninvasive positive pressure ventilation was used in the other 4 patients (10.0%).

### Treatment

The postoperative treatments are shown in Table 4. Drugs administered concomitantly were antimicrobial agents in 39 patients (97.5%), protease inhibitors in 18 (45.0%), diuretics in 14 (35.0%), and steroids in 13 (32.5%). Hemocatharsis by endotoxin adsorption was performed in 3 patients (7.5%). Sivelestat sodium was administered within 24 h after the onset of SIRS in 29 cases (72.5%), within 24 h after the initiation of respiratory management in 29 (72.5%), and within 24 h after the occurrence of respiratory dysfunction in 35 (87.5%).

### Changes in the P/F Ratio, Chest X-Ray Score, and Number of SIRS-Positive Items

The changes in the P/F ratio, chest X-ray score, and number of positive items on the SIRS list are shown in Fig. 1. The P/F ratio significantly improved with time after the initiation of SIV administration. The number of positive items on the SIRS checklist also significantly decreased after the initiation of SIV administration.

### Duration of SIRS, Duration of Artificial Respiratory Management, and Outcome After 28 Days

The duration of SIRS was  $6.7 \pm 6.9$  (range 1–35) days, and that of artificial respiratory management was  $9.1 \pm$

**Table 2.** Patient background

Background factors	n = 40
Sex: M/F	28 (70.0%)/12 (30.0%)
Age (years old)	67.1 ± 12.5
Under 40	1 (2.5%)
40s	3 (7.5%)
50s	7 (17.5%)
60s	7 (17.5%)
70s	17 (42.5%)
80 or older	5 (12.5%)
Underlying disease (including overlaps)	
Cancer of upper digestive organs	9 (22.5%)
Cancer of lower digestive organs	4 (10.0%)
Perforation of the digestive tract	10 (25.0%)
Acute pancreatitis	1 (2.5%)
Liver disease (liver failure, hepatic cirrhosis, other)	5 (12.5%)
Cardiovascular disease (abdominal aortic aneurysm, other)	5 (12.5%)
Other	6 (15.0%)
Surgical division (emergency/scheduled)	20 (50.0%)/20 (50.0%)
Cause of SIRS (including overlaps)	
Infection	18 (45.0%)
Surgical stress	26 (65.0%)
Pancreatitis	2 (5.0%)
Other	7 (17.5%)
C-reactive protein (mg/dl)	13.8 ± 8.7
Number of organs with failure other than the lungs	
0	17 (42.5%)
1	10 (25.0%)
2	9 (22.5%)
3	3 (7.5%)
Not investigable	1 (2.5%)

SIRS, systemic inflammatory response syndrome

**Table 3.** Respiratory function and management at the initiation of sivelestat sodium administration

	n = 40
PaO <sub>2</sub> /F <sub>i</sub> O <sub>2</sub>	
≤100mmHg	8 (20.0%)
100–200mmHg	14 (35.0%)
200–300mmHg	18 (45.0%)
Chest X-ray score	
0 point	10 (25.0%)
1 point	6 (15.0%)
2 points	14 (35.0%)
3 points	6 (15.0%)
4 points	4 (10.0%)
Respiratory management method	
Invasive positive pressure ventilation	36 (90.0%)
Non-invasive positive pressure ventilation	4 (10.0%)

8.3 (range 1–35) days. The survival rate after 28 days was 90.0% (36/40).

#### *Analysis of the Background of the Surviving and Fatal Cases*

The backgrounds at the initiation of SIV administration of patients who had survived versus those who had died by

**Table 4.** Treatments

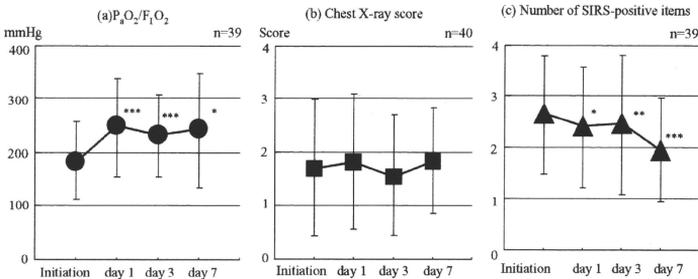
	n = 40
Combination therapy (including overlaps)	
Steroids	13 (32.5%)
Antimicrobial agents	39 (97.5%)
Protease inhibitors	18 (45.0%)
Diuretics	14 (35.0%)
Hemocatharsis (endotoxin adsorption)	3 (7.5%)
Sivelestat sodium administration	
After SIRS onset	0.8 ± 1.8 days
Within 24h	29 (72.5%)
1–3 days	7 (17.5%)
4 days or later	4 (10.0%)
After the initiation of respiratory management	0.8 ± 2.0 days
Within 24h	29 (72.5%)
1–3 days	7 (17.5%)
4 days or later	4 (10.0%)
After the onset of respiratory dysfunction	0.6 ± 1.8 days
Within 24h	35 (87.5%)
1–3 days	1 (2.5%)
4 days or later	4 (10.0%)

**Table 5.** Patient background by outcome

Background factors	Survival ( <i>n</i> = 36)	Death ( <i>n</i> = 4)	<i>P</i> value
Sex: Male/female	25/11	3/1	1.0000 <sup>a</sup>
Age (years)	66.4 ± 12.6	73.5 ± 11.2	0.2851 <sup>b</sup>
Number of positive items for SIRS	2.6 ± 1.2	3.0 ± 1.2	0.5262 <sup>b</sup>
PaO <sub>2</sub> /F <sub>i</sub> O <sub>2</sub> (mmHg)	184.6 ± 70.0	193.5 ± 100.7	0.8171 <sup>b</sup>
Chest X-ray score	1.6 ± 1.3	3.0 ± 0.8	0.0310 <sup>b</sup>
Number of impaired organs other than the lungs	0.8 ± 0.9	2.3 ± 0.5	0.0044 <sup>b</sup>
C-reactive protein level (mg/dl)	13.0 ± 8.3	20.2 ± 10.4	0.1220 <sup>b</sup>
Surgery (emergency/scheduled)	17/19	3/1	0.6050 <sup>a</sup>
Steroid (treated/untreated)	11/25	2/2	0.5839 <sup>a</sup>

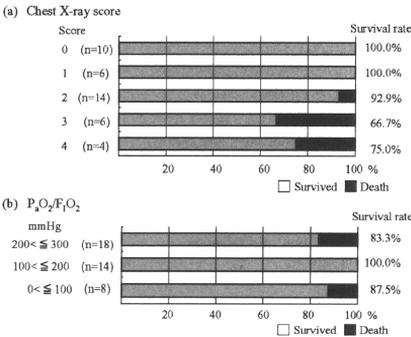
<sup>a</sup>Fisher exact test

<sup>b</sup>Unpaired *t*-test



**Fig. 1.** Changes in the **a** P/F ratio, **b** chest X-ray score, and **c** number of positive items on the systemic inflammatory response syndrome (SIRS) criteria during the postoperative

course. Wilcoxon signed-rank test; \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001 vs initiation



**Fig. 2.** Patient background by outcome in relation to **a** chest X-ray score and **b** P/F ratio. **a** Cochran–Armitage trend test, *P* = 0.0308; **b** Cochran–Armitage trend test, *P* = 0.4917

28 days after SIV administration are shown in Table 5 and Fig. 2. Of the patient background factors, the chest X-ray score was significantly higher in fatal cases in comparison to the patients who survived (*P* = 0.0310). The mean number of impaired organs other than the lungs was 0.8 in the surviving cases, but 2.3 in fatal cases, which was significantly higher (*P* = 0.0044). The increases in the chest X-ray score and mortality rate significantly correlated (*P* = 0.0308). The survival rate was 100% in cases in which the chest X-ray score was 0–1 (no infiltration or infiltration in one quadrant of one lung, if any), and 83.3% in cases in which the score was 2 or higher (apparent shadows were present). No significant correlation was noted between the increase in the P/F ratio and the mortality rate. The chest X-ray scores in surviving and fatal cases with a P/F ratio of 200–300 mmHg at the initiation of treatment were 1.1 ± 1.2 and 3.3 ± 0.6, respectively.

**Discussion**

Various factors, such as infection, may cause complications after surgery. The earliest acute complication that

can affect the prognosis is ALI/ARDS. Impairment of the lungs may lead to dysfunction of important organs, such as the liver and kidneys, due to a lack of oxygen supply, resulting in MOF. The mortality rate from ARDS has been reported to be 30%–65%,<sup>10–13</sup> indicating that treatment before the occurrence of ARDS or during the early stage of ARDS is important. Accordingly, various perioperative management methods, including respiratory management, have been investigated, and countermeasures have also been taken.

In Japan SIV, a selective neutrophil elastase inhibitor, became commercially available in 2002. Sivelestat sodium is administered to treat ALI/ARDS accompanying SIRS induced by various underlying diseases and factors, such as pneumonia<sup>14</sup> and sepsis,<sup>14,15</sup> and its usefulness has been confirmed in numerous clinical trials. Regarding postoperative SIV treatment, administration has also been reported after surgery for esophageal cancer<sup>16–18</sup> and cardiopulmonary bypass.<sup>19</sup> However, the clinical significance of SIV administration for patients with respiratory dysfunction following surgery is unclear.

We surveyed the effects of using SIV for postoperative respiratory dysfunction in patients who underwent surgery at the multiple facilities that make up our hospital to investigate the efficacy of SIV. Of the 40 cases included in our study, the P/F ratio was 200mmHg or lower in 22 patients, and the chest X-ray score was 2 or higher in 24 patients, showing that this was a relatively ill patient population. This survey was performed in selected cases treated with SIV at multiple facilities during a 9-month period, and was not a comparative study with a control group. Therefore, it was not possible to completely clarify the usefulness of SIV in this study, as it is not known how untreated patients would have fared. However, the survival rate after 28 days (90.0%) was favorable, suggesting that postoperative multidisciplinary management, including SIV treatment, was superior to conventional postsurgical survival in patients with ARDS and SIRS. Since SIV was administered early after the occurrence of respiratory dysfunction, SIV may have contributed to the improvement of the prognosis.

When we stratified the patients according to outcome, the chest X-ray score and the number of impaired organs at the initiation of SIV administration correlated with the outcome, showing that these factors are related to the patient prognosis. In contrast, no correlation was noted between the P/F ratio and outcome.

In the USA–Europe joint AECG conference in 1994, the definition and diagnostic criteria for ALI were proposed.<sup>20</sup> Acute lung injury was defined as conditions with: (1) acute development, (2) a P/F ratio  $\leq 300$ mmHg, (3) bilateral infiltrations in the frontal view on chest radiography, and (4) pulmonary arterial wedge pressure

$\leq 18$ mmHg or the absence of clinical left arterial pressure elevation. However, microinfiltration that is only detectable by computed tomography scan may not be found by X-ray examination. Sixteen cases with a P/F ratio  $\leq 300$ mmHg and chest X-ray score of 1 or lower were included in this study. The early administration of SIV may have improved the prognosis in these cases, thus suggesting that intensive care, in accordance with that for ALI/ARDS patients, is already necessary for cases of SIRS with a P/F ratio of  $\leq 300$ mmHg.

We have herein demonstrated that the initiation of SIV administration in the early phase after the onset of respiratory dysfunction following surgery improves the prognosis for patients. Further investigations, including controlled clinical studies, will be necessary to fully determine the efficacy of using SIV for ALI/ARDS with SIRS following surgery.

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## Original Article

# Curative Surgery Improves the Survival of Patients with Perforating Colorectal Cancer

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### Abstract

**Purpose.** Colorectal cancers that manifest as a perforation are generally regarded as carrying a poor prognosis. We conducted this study to assess the outcome of colorectal cancer complicated by perforation.

**Methods.** Between 1996 and 2004, 848 patients underwent surgery for colorectal cancers in our department. We reviewed 22 (2.6%) consecutive patients who presented with perforation at one institution.

**Results.** Fifteen (69%) patients underwent potentially curative resection. The overall operative morbidity and mortality rates were 50% and 9%. The overall 5-year survival rate was 17.4%, although by excluding patients who either had stage IV disease at diagnosis or who died during or soon after surgery ( $n = 7$ ), the 5-year survival rate increased to 32% ( $n = 15$ ). Furthermore, the 5-year survival rate of patients who underwent a potentially curative resection (36.9%) was significantly better than that of those who underwent a noncurative resection (0%,  $P = 0.0093$ ).

**Conclusions.** Perforating colorectal cancers are associated with high postoperative mortality and poor long-term survival. However, the intensive management of radical lymph node dissection and surgical resection are recommended to improve the long-term prognosis.

**Key words** Curative surgery · Perforated colorectal cancer

### Introduction

Colorectal cancer remains the third most common type of cancer in Japan. Each year, approximately 94 000 new

cases are diagnosed and almost 39 000 people die of the disease.<sup>1</sup> The overall survival rate is poor: many patients have locally advanced or metastatic disease at the time of presentation, and only half of those who undergo a potentially curative resection survive for 5 years.

Perforation, obstruction, bleeding, and invasion of the adjoining organs are the major complications associated with locally advanced colorectal cancer. Perforation has been reported to occur in 3%–9% of colorectal cancers, and is associated with a high postoperative mortality and a poor long-term outcome.<sup>2–7</sup> This presentation adds the morbidity and mortality of sepsis and peritonitis to that already associated with emergency surgery for colorectal cancer, and appears to reduce the 5-year survival by 20%.<sup>2</sup>

The aim of the present study was to elucidate the postoperative morbidity and long-term outcome of patients presenting with perforation in the setting of colorectal cancer, and to search for the indicators of prognosis and possible causes of death. To evaluate the risk of this complication, we compared the data of patients with perforated colorectal cancers with those of patients with uncomplicated colorectal cancers.

### Patients and Methods

Between 1996 and 2004, 22 patients underwent emergency surgery for perforated colorectal cancer, among a total of 848 patients with colorectal cancer treated for colorectal cancer during the same period at one institution. Their clinical data, prospectively stored in a database, were retrieved and analyzed for this study. The tumors were classified according to site as follows: lesions of the cecum, ascending colon, and hepatic flexure were classified as right-sided lesions, whereas lesions of the transverse colon, splenic flexure, and descending colon were classified as left-sided colon lesions. Tumor staging was assessed by the TNM and Dukes' classification based on histological examinations

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of resected specimens and the findings of radiological examinations, such as chest X-ray and computed tomography.

Patients were deemed to have undergone a curative resection if the surgeon considered that there was no macroscopic residual tumor once the resection was completed. Patients with distant metastases who underwent resection and patients in whom inadequate local clearance was achieved were deemed to have undergone a palliative resection. The curability and type of lymph node dissection were described according to the General Rules for Clinical and Pathological Studies on Cancer of the Colon, Rectum, and Anus.<sup>8</sup>

Follow-up examination after discharge was conducted either at our hospital or at a private physician's office every 3 months for 2 years, every 6 months for the next 3 years, and annually thereafter. Concurrent follow-up was also performed in our tumor registry. Proportions were compared using the chi-squared test for large samples and Fisher's exact probability test for small samples. Continuous data were compared using a non-parametric Wilcoxon test. Differences were considered significant at a *P* value of less than 0.05. Survival curves were analyzed by the Kaplan-Meier method and tests of significance were done using the log-rank test.

## Results

Of the 848 patients with colorectal cancer, retrospectively analyzed, 22 (2.6%) presented with pathologically proved perforated lesions. The pathological findings confirming perforation included ulceration and splitting of all layers of the bowel wall, necrosis or chronic inflammatory reaction, and abscess formation around the perforation site. The incidence of the various clinicopathological factors are shown in Table 1. The primary tumor was most often located on the left side of the colon (*n* = 21, 96%), especially the sigmoid colon and rectum (*n* = 16, 73%). The perforation was located at the tumor site in 20 (91%) patients, and proximal to the obstructed primary lesion in the other 2 (9%) patients. We were able to diagnose the colorectal cancers preoperatively by computed tomography in only 12 (54.5%) patients.

All 22 patients underwent surgery; however, the resectability rate was 86%, as 3 patients underwent only a palliative colostomy. Fourteen patients (64%) underwent a potentially curative resection and 6 (27%) had metastatic disease at the time of surgery. Distant metastases were found in the liver in 3 patients, the peritoneum in 3, and the lung in 1. One of these six patients underwent a relatively curative resection combined with an adenectomy for an ovarian metastasis. Seven of the 13 patients who underwent potentially curative surgery received oral adjuvant chemotherapy. The other

**Table 1.** Patients and tumor characteristics

Characteristic	No. of patients (%)
Total	22 (100)
Sex	
Male	10 (45)
Female	12 (55)
Median (range) age (years)	71.8 (34-92)
Cancer site	
Right	1 (4)
Left	5 (23)
Sigma	9 (41)
Rectum	7 (32)
Median (range) tumor diameter (cm)	6.6 (2-18)
TNM stage	
I	0 (0)
II	11 (50)
III	5 (23)
IV	6 (27)
Dukes' stage	
A	1 (5)
B	13 (59)
C	2 (9)
D	6 (27)
Lymph node dissection	
D0	3 (14)
D1	7 (32)
D2	10 (4)
D3	2 (9)
Curability	
A	14 (64)
B	1 (5)
C	7 (31)
Surgical procedure	
Hartmann's procedure	11 (50)
Primary resection	
With anastomosis	6 (27)
With a colostomy	2 (9)
Without a colostomy	4 (18)
Without anastomosis	2 (9)
Colostomy alone	3 (14)

patients did not receive adjuvant chemotherapy because of either complications or their age. The operative morbidity and mortality rates are shown in Table 2. Septic shock accounted for two early deaths. The patients with perforated colorectal cancer had significantly higher postoperative morbidity and mortality rates than the patients with uncomplicated cancers.

The mean follow-up was 18.9 months (range, 1-133 months). At the time of writing, four patients were alive without recurrence, three were alive with liver metastasis, nine had died of relapse of colorectal cancer (as liver metastasis in four, peritoneal dissemination in three, brain metastasis in one, and bone metastasis in one), and four had died of unrelated causes. The survival data of our study (Fig. 1) revealed an overall 5-year survival rate of 17.4% for the 22 cases; however, the exclusion of seven patients with either stage IV disease at the time of perforation, or who died during or soon after their