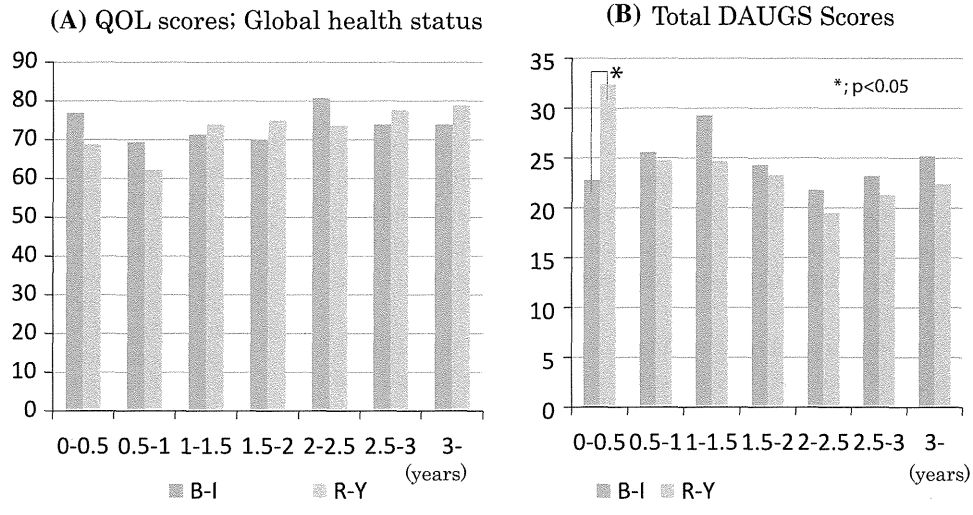

Fig. 5 There were significant differences in total DAUGS 20 scores during the first 6 months (B-I 22.8 ± 13.7, R-Y 32.4 ± 8.9, $p = 0.04$). There was no significant difference in global health status and total DAUGS 20 scores at other periods between the B-I group and the R-Y group

optimal operative approach. The Japanese version of the EORTC QLQ-C30 has been developed and validated. Kobayashi et al. [20] used this version to prospectively compare postoperative health-related QOL among gastrectomy patients and found clear difference among the operative procedures.

The DAUGS 20 scale was designed to objectively assess gastrointestinal dysfunction after surgery for upper gastrointestinal cancer. The scale has already been validated in the field of upper intestinal cancer [17, 18]. We found no significant difference between R-Y and B-I procedures in terms of overall postoperative dysfunction. However, there were significant differences in total DAUGS 20 scores during the first 6 months (B-I 22.8 ± 13.7, R-Y 32.4 ± 8.9, $p = 0.04$). Especially each score of food passage dysfunction and nausea and vomiting tended to be worse in the R-Y group (not significant). This may be weakened to gastrointestinal motility and delayed gastric emptying with R-Y. In this series, the frequency of nausea, vomiting, and discontinuation of food intake were significantly lower in the B-I group than in the R-Y group (3.7 vs. 12.4%, $p = 0.0027$; 3.1 vs. 8.9%, $p = 0.022$; 4.3 vs. 12.4%, $p = 0.0064$, respectively). Frequency of delayed gastric emptying in the B-I group was lower than in the R-Y group (4.3 vs. 9.5%, $p = 0.057$). In general, Roux en Y stasis occurred within the postoperative 1st month. Minor symptoms could not be detected during the hospital stay, and small amounts of nausea or vomiting might have occurred at home. Our questionnaire survey might detect this small difference between B-I and R-Y related gastrointestinal motility during the first 6 months.

The EORTC QLQ-C30 showed significant differences only in dyspnea. This symptom seemed to be physiologically unrelated to postoperative complications. Patients who received B-I gastrectomy sometimes complained of heartburn. This score seemed to be affected by esophagitis caused by bile and gastric juice reflux. In the analysis of partial items of the DAUGS, reflux symptoms also obviously appeared in the B-I group. If this limitation can be overcome, we can feel confident in continuing to perform B-I reconstructions. While Shibata reported that semifundoplication following B-I reconstruction prevented this difficulty, further surgical intervention following gastrectomy is less than ideal [21].

The questionnaire survey in the current study was performed only once for each patient and at varying time points after surgery, since co-investigators in this multiinstitutional study did not agree to perform the survey several times and at regular intervals. Such a design would have delivered more convincing data, but would have been too much of a burden for the co-investigators. An alternative design would have been to perform all the surveillances at a fixed time point, such as at 1 year postoperatively. However, it was not possible to decide on the optimal time point for performing the surveillance at the time this study was designed. It has now become clear how the scores vary at different time points, and further study to confirm the differences between B-I and R-Y can now be designed and proposed.

In general, from the point of view of the surgeon, B-I reconstruction is considered to be simple and relatively easy. For the patient, nutritional and hormonal advantages might exist in this physiological route. It is easier to treat common bile duct stones using a gastrointestinal fiberscope after B-I reconstruction. In contrast, the advantage of R-Y reconstruction is thought to be less anastomotic leakage and infrequent reflux esophagitis and gastritis. However, disadvantages include a more complicated surgical procedure as well as delayed gastric emptying, so-called Roux-en-Y stasis. All surgeons recognize these issues, and their decisions on which approach to use are based on individual experience. Clinical randomized trials are very important

in providing surgeons with information to facilitate their decision-making. Ishikawa et al. [12] conducted a randomized trial and showed that B-I reconstruction was superior to R-Y in terms of shorter postoperative hospital stay. At the time it was carried out, this study was the first and most important trial comparing B-I and R-Y reconstructions, and many surgeons have referred to its results as clinical evidence. Our study should also be useful in assisting surgeons with deciding between the two procedures.

In summary, this questionnaire survey using the EORTC QLQ-C30 and DAUGS 20 scales revealed that the B-I and R-Y reconstruction approaches were nearly equal in terms of postoperative QOL and dysfunction. It is noteworthy, however, that B-1 was significantly better regarding the total DAUGS 20 score during the first 6 months after surgery. The current study revealed differences in QOL and postoperative dysfunction scores between the two modes of reconstruction at various time points. More refined prospective trials with improved designs based on these results have to be proposed.

Acknowledgments The authors thank Dr. Tomoyuki Sugimoto at Osaka University for help with the statistical analysis. Participating institutions and chief participants: Sakai Municipal Hospital (H. Furukawa, H. Imamura), Osaka University (Y. Doki, S. Takiguchi), Osaka National Hospital (T. Tsujinaka, K. Fujitani), Toyonaka Municipal Hospital (J. Fujita, Kawanishi), Osaka Medical Center for Cancer and Cardiovascular Diseases (M. Yano, I. Miyashiro), Kinki Central Hospital of the Mutual Aid Association of Public School Teachers (K. Kobayashi), NTT West Osaka Hospital (Y. Kimura), Yao Municipal Hospital (Y. Fukushima, H. Matsuyama), Hyogo Prefectural Nishinomiya Hospital (H. Yano, H. Taniguchi), Kansai Rosai Hospital (S. Tamura, H. Miki), Ikeda Municipal Hospital (K. Shibata, T. Hirao), Belland General Hospital (K. Demura), SEMPOS Seamen's Insurance hospital (Y. Tsukahara), Saiseikai Senri Hospital (H. Fukunaga), Nishinomiya Municipal Hospital (H. Oka), Suita Municipal Hospital (C. Ebisui, K. Okada), Itami Municipal Hospital (M. Hiratsuka), and Mino Municipal Hospital (S. Iijima, Y. Makari).

Conflict of interest None of the authors have financial or personal conflicts of interest to disclose.

References

1. Yoshino K. History of gastric cancer surgery. J Jpn Surg Soc. 2000;101:855–60.
2. Weil PH, Buchberger R. From Billroth to PCV: a century of gastric surgery. World J Surg. 1999;23:736–42.
3. Osugi H, Fukuhara K, Tagada N, et al. Reconstructive procedure after distal gastrectomy to prevent remnant gastritis. Hepatogastroenterology. 2004;51:1215–8.
4. Kauer WK, Peters JH, DeMeester TR, et al. Composition and concentration of bile acids reflux into the esophagus of patients with gastroesophageal reflux disease. Surgery. 1997;122:874–81.
5. Svensson JO. Duodenogastric reflux after gastric surgery. Scand J Gastroenterol. 1983;18:729–34.
6. Fein M, Peters JH, Chandrasoma P, et al. Duodenoesophageal reflux induces esophageal adenocarcinoma without exogenous carcinogen. J Gastrointest Surg. 1998;2:260–8.
7. Taylor PR, Mason RC, Filipe MI, et al. Gastric carcinogenesis in the rat induced by duodenogastric reflux without carcinogens, morphology, mucin histochemistry, polyamine metabolism, and labeling index. Gut. 1991;32:1447–54.
8. Sato T, Miwa K, Sahara H. The sequential model of Barrett's esophagus and adenocarcinoma induced by duodeno-esophageal reflux without exogenous carcinogens. Anticancer Res. 2002;22:39–44.
9. Mackman S, Lemmer KE, Morrissey JF. Postoperative reflux alkali gastritis and esophagitis. Am J Surg. 1971;121:694–7.
10. Gillison EW, Kusakari K, Bombeck CT, et al. The importance of bile in reflux oesophagitis and the success in its prevention by surgical means. Br J Surg. 1972;59:794–8.
11. Mathias JR, Fernandez A, Sninsky CA, et al. Nausea, vomiting, and abdominal pain after Roux-en-Y anastomosis: motility of the jejuna limb. Gastroenterology. 1985;88:101–7.
12. Ishikawa M, Kitayama J, Kaizaki S, et al. Prospective randomized trial comparing Billroth-I and Roux-en-Y procedures after distal gastrectomy for gastric cancer. World J Surg. 2005;29:1415–20.
13. Japanese Gastric Cancer Association. Japanese classification of gastric carcinoma—2nd English edition. Gastric Cancer. 1998;1:10–24.
14. Kobayashi K, Takeda F, Teramukai S, et al. A cross-validation of the European Organization for Research and Treatment of Cancer QLQ-C30 (EORTC QLQ-C30) for Japanese with lung cancer. Eur J Cancer. 1998;34:810–5.
15. Wu C, Chiou J, Ko F, et al. Quality of life after curative gastrectomy for gastric cancer in a randomized controlled trial. Br J Cancer. 2008;98:54–9.
16. Aaronson NK, Ahmedzai S, Bergman B, et al. The European Organization for Research and Treatment of Cancer QLQC30: a quality of life instrument for use in international clinical trials in oncology. J Natl Cancer Inst. 1993;85:365–76.
17. Nakamura M, Kido Y, Yano M, Hosoya Y. Reliability and validity of a new scale to assess postoperative dysfunction after resection of upper gastrointestinal carcinoma. Surg Today. 2005;35:535–42.
18. Nakamura M, Hosoya Y, Yano M, Doki Y, Miyashiro I, Kurashina K, Morooka Y, Kishi K, Lefor AT. Extent of gastric resection impacts patient quality of life: the Dysfunction after Upper Gastrointestinal Surgery for Cancer (DAUGS32) Scoring System. Ann Surg Oncol. 2011;18:314–20.
19. Kim YW, Baik YH, Yun YH, et al. Improved quality of life outcomes after laparoscopy-assisted distal gastrectomy for early gastric cancer: results of a prospective randomized clinical trial. Ann Surg. 2008;248:721–7.
20. Kobayashi D, Kodera Y, Fujiwara M, Koike M, Nakayama G, Nakao A. Assessment of quality of life after gastrectomy using EORTC QLQ-C30 and STO22. World J Surg. 2011;35:357–64.
21. Shibata Y. Effect of semifundoplication with subtotal gastrectomy for prevention of postoperative gastroesophageal reflux. J Am Coll Surg. 2004;198:212–7.

Ann Surg Oncol (2012) 19:1560–1567
DOI 10.1245/s10434-011-2157-6

Annals of
SURGICAL ONCOLOGY
OFFICIAL JOURNAL OF THE SOCIETY OF SURGICAL ONCOLOGY

ORIGINAL ARTICLE – GASTROINTESTINAL ONCOLOGY

DOK2 as a Marker of Poor Prognosis of Patients with Gastric Adenocarcinoma After Curative Resection

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ABSTRACT

Background. DOK2 is known as the substrate of chmeric p210bcr/abl oncoprotein characterizing chronic myelogenous leukemia with Philadelphia chromosome. Reduced DOK2 expression was recently reported in lung adenocarcinoma, suggesting that this protein acts as a tumor suppressor in solid tumors. The purpose of this study was to determine the significance of DOK2 in gastric cancer.

Methods. The study subjects were 118 patients who underwent curative surgery for gastric cancer, as well as 7 gastric cancer cell lines. The tissues and cell lines were analyzed for DOK2 gene and protein expressions by histopathology and immunohistochemistry, and also using a microsatellite marker for loss of heterozygosity. Correlation of survival with clinicopathological parameters was investigated by univariate and multivariate analyses.

Results. DOK2 expression was confirmed in the normal gastric mucosa. Considerable differences in the gene expression were noted among the gastric cell lines. Positive DOK2 expression was noted in the noncancerous regions of all pathological specimens, whereas 59 (50.0%) specimens of 118 patients were negatively stained in the tumor. Loss of heterozygosity was observed in 54.5% of DOK2(−) cases. DOK2(−) patients were more likely to develop recurrence than DOK2(+) and showed poorer 5-year overall survival (59.1%) than DOK2(+) (76.4%, $P = .0403$). Multivariate analysis identified pT (hazard ratio [HR] = 2.748, 95% confidence interval [95% CI] = 1.061–8.927, $P = .0361$), pN (HR = 2.486, 95% CI = 1.264–4.932, $P = .0086$), and DOK2(−) (HR = 2.343, 95% CI = 1.211–4.727, $P = .0112$) as significant and independent determinants of poor survival.

Conclusions. Our data suggest the potential usefulness of DOK2 as a marker of poor prognosis in patients with gastric cancer after curative resection.

Gastric cancer is a global health problem with estimated new stomach cancer cases of 989,600 and cancer-related deaths of 738,000 in 2008 alone, with the highest incidence in Eastern Asia, Eastern Europe, and South America.[1] Advances in the diagnosis and treatment have offered excellent long-term survival for patients with early diagnosis of gastric cancer; however, the prognosis of those with advanced cancer remains poor and heterogeneous. Assessment of prognosis through clinicopathological features remains inadequate, especially with advanced cancer even when using the staging system of tumor-node-metastasis (TNM) classification, because of the considerable variability and heterogeneity within the same stage.[2,3] Therefore, it is necessary to identify novel biological markers that allow a more accurate identification of high-risk population for recurrent disease and help in the design of appropriate treatment strategies for individual population.

DOK1–3 are adaptor proteins that function in feedback loops to modulate tyrosine kinase signaling including epidermal growth factor receptor, platelet-derived growth factor receptor, c-Kit, Tie2, and Her2/*Neu*.[4–10] The interaction between DOK1–3 and these tyrosine kinases in hematopoietic cells is well described. DOK1 and DOK2 are crucial regulators in chronic myelogenous leukemia (CML) with Philadelphia chromosome.[8] Furthermore,

Electronic supplementary material The online version of this article (doi:10.1245/s10434-011-2157-6) contains supplementary material, which is available to authorized users.

First Received: 18 June 2011;
Published Online: 1 December 2011

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Dok-1/Dok-2 double-knockout mutants spontaneously developed transplantable CML-like myeloproliferative disease, and thus both are considered to be involved in leukemogenesis.[11,12]

Little is known about DOK1–3 expression in nonhematopoietic cells and solid tumors. Recently, Berger et al.[13] reported the presence of low expression of DOK2 in lung adenocarcinoma, suggesting that this protein acts as a tumor suppressor. The purpose of the present study was to determine the expression of DOK2 in gastric cancer and gastric cancer cell lines. The results indicated the clinical significance of DOK2 in the evaluation of prognosis of patients with gastric cancer.

MATERIALS AND METHODS

Patients

From October 2001 to March 2004, 118 consecutive patients diagnosed histopathologically with gastric adenocarcinoma underwent surgery at Osaka University Hospital. In all patients, the gastric adenocarcinoma was newly diagnosed, and none had received chemotherapy or radiotherapy before surgery; the tumor was resected curatively and pathologically diagnosed as pStage I–III. After surgery, the patients were surveyed every 3 months by clinical examination, and serum tumor markers [carcinoembryonic antigen (CEA), CA19-9], every 6 months by computed tomography (CT) scan and abdominal ultrasonography, and annually by endoscopy until tumor relapse was evident. Patients with tumor relapse received chemotherapy as long as their systemic condition permitted. Adjuvant chemotherapy was provided for 24 patients; 1 patient received TS-1 (tegafur, gimeracil, oteracil potassium) with paclitaxel, and the other 23 patients received oral fluoropyrimidines TS-1, 5-FU (fluorouracil), UFT (tegafur, uracil) or 5′DFUR (doxifluridine) for at least 6 months. The median follow-up period after surgery was 63.8 months (range, 5.5–132.4 months). Various clinicopathological parameters, such as age, gender, histological classification (according to the World Health Organization [WHO]), depth of invasion, nodal metastasis, pathological-tumor-metastasis (pTNM) stage (according to TNM Classification System of Malignant Tumors, 7th ed. [UICC]), and lymphovascular invasion status were evaluated by reviewing the medical and pathologic reports, and hematoxylin and eosin (H&E)-stained tumor tissue sections.[2] There were 6 frozen samples (N1–N6) chosen at random from 118 patients for reverse transcription polymerase chain reaction (RT-PCR) analysis described below. This study adhered to the guidelines established by the Declaration of Helsinki, and all patients provided informed written consent.

Gastric Cancer Cell Line

The gastric cancer cell lines MKN74, MKN45, MKN45P, MKN7, NUGC3, RERFGC1B, and AGS were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air in RPMI 1640 (Life Technologies, Grand Island, NY) with 10% (v/v) FBS (GIBCO BRL, Grand Island, NY) and penicillin/streptomycin (1000 units/mL; GIBCO BRL).

Laser Microdissection

Six frozen samples (N1–N6) were sectioned with a cryostat (Leica Microsystems, Wetzlar, Germany) at 6 μm thickness. The sliced samples were immediately fixed with a mixture of 100% ethanol and acetic anhydride (18:1). After staining with H&E, only normal gastric mucosal cells were differentially dissected from stromal cells and lymphocyte using a laser microdissection (LMD) system (Leica LMD System, Leica Microsystems).

Reverse Transcription Polymerase Chain Reaction Analysis

Total RNA from frozen normal gastric mucosa dissected by LMD was extracted using an RNeasy mini kit (Qiagen, Hilden, Germany), and total RNA from gastric cancer cell line was extracted using Trizol reagent (Gibco BRL, Grand Island, NY) following the instructions provided by the manufacturer. Total RNA was reverse transcribed to cDNA in a 20 μl volume using Reverse Transcription System (A3500 Promega, Madison, WI). The reaction condition was based on the information provided by the manufacturer.

RT-PCR was carried out in a reaction mixture containing 2 μl of cDNA, 12.5 μl ampliTaq GOLD (Applied Biosystems, Foster City, CA), and 10.5 μl water. The cycling conditions were 95°C for 5 min followed by 35 cycles of 95°C for 30 s, 68°C (58°C for B2M) for 30 s, 72°C for 2 min, and a final extension at 72°C for 7 min. Equal amounts of PCR products were electrophoresed on 1.5% agarose gels and visualized by ethidium bromide staining. Peripheral mononuclear blood cells (PMBC) were prepared as a positive control with standard Ficoll-Hypaque density gradient separation techniques. The details of DOK2 gene specific primers were reported by Favre et al.[14] To verify the quality and integrity of synthesized cDNA, the *beta-2 microglobulin* (*B2M*) gene was used as an internal control: 5′-TGTCTTTCAGCAAGGACTGG-3′ (sense) and 5′-CCTCCATGATGCTGCTTACA-3′ (antisense) (NCBI-Acc. AF072097, nucl. pos. 1021–1168).

Immunohistochemical Staining

DOK2 protein contents were examined by immunohistochemical staining of formalin-fixed and paraffin-embedded gastric cancer tissue and normal gastric mucosa sections (3.5 μm). One representative slide with the deepest tumor invasion was selected from each patient and subjected to immunohistochemistry. Briefly, after deparaffinization in xylene and dehydration in graded ethanol solutions, tissue sections were heated at 121°C for 20 min in ethylenediaminetetraacetic acid (EDTA)-tris buffer, pH 9.0, for antigen retrieval. Then, endogenous peroxidase activity was blocked by incubation with 30 ml/l hydrogen peroxide for 20 min. After overnight incubation with mouse monoclonal primary antibody DOK2 (sc-17830 Santa Cruz Biotechnology, Santa Cruz, CA, dilution 1:200) at 4°C, staining was performed by the labeled streptavidin-biotin (LSAB) method. Negative controls of immunohistochemical reactions included omission of the primary antibody. Lymphocytes were used as positive control. DOK2 staining in each gastric cancer sample was judged positive when the cancer cells in the section were immunoreactive to DOK2. All slides were assessed independently by 2 pathologists and then by consensus in case of disagreement. Both pathologists were blinded to the clinicopathological data.

Microsatellite Analysis

A total of 30 frozen paired normal and tumor tissue samples were randomly selected from 118 patients described previously. The DNA was extracted using QIAmp DNA Mini Kit (Qiagen). The microsatellite marker D8S560, which is located at 164 kbp downstream of DOK2 gene, was selected for loss of heterozygosity (LOH) analysis. The specific PCR primer was designed as follows: 5′-GGCATTTCAGAGGACC-3′ (sense) and 5′-TGCAAA GATGGGCTCAG-3′ (anti-sense). The cycling conditions were 95°C for 5 min followed by 40 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s, and a final extension at 72°C for 7 min. Equal amounts of PCR products were electrophoresed on 15% SDS-polyacrylamide gel and visualized by cyber green staining. In each case, the results were analyzed by visual inspection. In addition, cases presenting equivocal results and cases delimiting critical regions of loss were analyzed by comparison of allele intensities in matched normal/tumor DNA using scanning densitometry with a computerized ChemiDoc XRS Plus (Bio-Rad Laboratories, Hercules, CA). Quantification was performed using the Quantity One program. The relative ratio of both tumor and normal alleles was determined, normalized, and then compared. LOH was assigned when the intensity ratio of the two tumor sample alleles differed by at least 30% from that observed on normal DNA.[15]

Quantitative Real-Time PCR

Total RNA was extracted from the same 30 frozen paired normal and tumor samples used for microsatellite marker analysis, by the Trizol reagent (GIBCO). Real-time monitoring of PCRs was performed using the LightCycler FastStart DNA Master SYBR-Green I kit (Roche Diagnostics, Tokyo, Japan) for cDNA amplification of DOK2 and B2M. The amplification protocol consisted of 35 cycles of denaturation at 95°C for 15 s, annealing at 66°C for 18 s, and elongation at 72°C for 30 s. The products were then subjected to a temperature gradient from 55 to 95°C at 0.1°C/s, with continuous fluorescence monitoring to produce product melting curves. The expression ratio of mRNA copies in tumor and normal tissues was calculated and normalized against B2M mRNA expression.

Statistical Analysis

Correlations between DOK2 expression and various clinicopathological parameters were evaluated by the χ^2 test and Fisher exact probability test. Prognostic variables were assessed by log-rank test, and overall survival (OS) and relapse-free survival (RFS) were analyzed by the Kaplan and Meier method. Cox proportional hazards regression model was used to evaluate the independent prognostic factors. These analyses were carried out using JMP version 8.0.1 (SAS Institute, Cary, NC) for Windows. A P value of less than .05 denoted the presence of statistical significance.

RESULTS

DOK2 Gene Expression in Normal Gastric Mucosa and Gastric Cancer Cell Lines by RT-PCR

DOK1 mRNA expression was observed in all normal gastric mucosa and gastric cancer cell lines, while DOK3 mRNA expression was hardly observed in both. DOK2 mRNA expression was observed in all normal gastric mucosa samples (N1–N6) (Supplementary Fig. 1a). In gastric cancer cell lines, the expression was strong in MKN7 and AGS, and weak in MKN74. On the other hand, MKN45, MKN45P, NUGC3, and RERFGC1B were negative for DOK2 (Supplementary Fig. 1b). There were considerable differences in DOK2 expression level between gastric cell lines. Thus, we focused on DOK2 and assessed the correlation between DOK2 expression and clinicopathological outcome.

DOK2 Protein Expression in Gastric Cancer by Immunohistochemistry

Samples from the entire group of 118 patients (Table 1) that contained both cancerous and noncancerous tissues were evaluated for DOK2 protein expression by immunohistochemistry. Abundant DOK2 protein expression was detected in the mucosa of all normal gastric tissues (100%; Fig. 1a, b). The cytoplasm of the normal gastric parietal cells stained strongly for DOK2, while that of chief cells showed moderate reactivity.

In cancer cells, 59 of 118 patients (50.0%) showed positive DOK2 expression (Fig. 1c, d), mainly in the cytoplasm of tumor cells while the remaining 59 (50.0%) were negative (Fig. 1a, b). The pattern of DOK2 expression in cancer cells was almost similar to that in normal gastric chief cells. Positive staining was almost homogeneous at single cancer nest and among different areas (surface, central, and deepest areas) of the cancer lesion. There was a close agreement between the two pathologists on the pathological assessment with interobserver variation of less than 5%.

TABLE 1 Correlation between DOK2 expression and various clinicopathological parameters

Parameters	DOK2 expression		*P* value
	Positive	Negative	
All cases	59	59	
Age (<66/≥66)	24/35	34/25	.0971
Gender (female/male)	21/38	15/44	.3175
Differentiation (poorly/well & mod)	40/19	21/38	.0008
pT (T3–4a/T1–2)	39/20	39/20	1.0000
pN (N2–3/N0–1)	20/39	17/42	.6918
pStage (I/II/III)	18/17/24	16/20/23	.8259
Lymphatic infiltration (positive/negative)	49/10	46/13	.6428
Venous invasion (positive/negative)	22/37	26/33	.5742
Relapse cases ($n = 35$)	12	23	.0430
Lymph node ($n = 10$)	3	7	.3220
Peritoneum ($n = 10$)	5	5	1.0000
Hematogenous ($n = 18$)	4	14	.0105
Liver ($n = 14$)	2	12	.0083

Hematogenous included bone, lung, brain, and liver metastases as the first site of metastasis

Well & Mod well and moderately differentiated carcinomas (including papillary adenocarcinomas), *Poorly* poorly differentiated carcinomas (including signet ring cell carcinomas and mucinous adenocarcinoma), *pT pN pStage* (pathological classification) according TNM Classification of Malignant Tumors (UICC) 7th ed

DOK2 mRNA Expression Evaluated by Quantitative Real-Time PCR

To assess the relevance of DOK2 protein level to DOK2 mRNA expression, we conducted quantitative real-time PCR in 30 frozen paired normal and tumor samples from the 118 cases. As shown in Fig. 2, DOK2 mRNA expression was significantly lower in DOK2(−) cases than DOK2(+) (fold change 2.41, $P < .001$), which was consistent with protein expression evaluated by immunohistochemistry.

Relationship Between Loss of Heterozygosity and Low DOK2 Expression

To assess the relevance of the reduced DOK2 expression to genomic status, microsatellite analysis was performed in 30 patients with gastric cancer. Allelic loss of DOK2 was observed in 6 of 30 cases, while the results in 7 cases were noninformative. DOK2 protein expression level correlated significantly with allelic loss of DOK2. The loss was not detected in any of the patients with positive DOK2 expression, but was detected in 6 of 11 cases (54.5%) negative for DOK2 expression (Table 2). Correlation of the results of microsatellite analysis and histopathological grade showed that the six cases with allelic loss comprised five cases with differentiated tumors and one with poorly differentiated tumor (Table 2). The 17 heterozygous cases consisted of 6 (35.3%) with differentiated tumors and 11 (64.7%) with poorly differentiated tumors. In DOK(−) cases with heterozygosity, three of five cases (60.0%) had poorly differentiated tumors, which was similar to DOK(+) cases ($P = 1.000$).

Correlation Between DOK2 Expression and Clinicopathological Parameters

Table 1 lists the correlations between DOK2 expression and various clinicopathological parameters. Tumors of the differentiated type (including papillary and well and moderately differentiated adenocarcinomas), based on histopathological grade, were significantly more likely to be negative for DOK2 expression than those positive for DOK2 [DOK2(−): 66.7%, DOK2(+): 33.3%, $P = .0008$]. There were no significant correlations with other parameters, including age, gender, pT, pN, pStage, lymphatic infiltration, and venous invasion (Table 1).

Correlation Between DOK2 Expression and Clinical Outcome

Disease relapse was diagnosed after surgery in 35 of 118 patients (29.7%), and the median time to relapse was 14.8 months. The relapse rate in DOK2(−) patients was

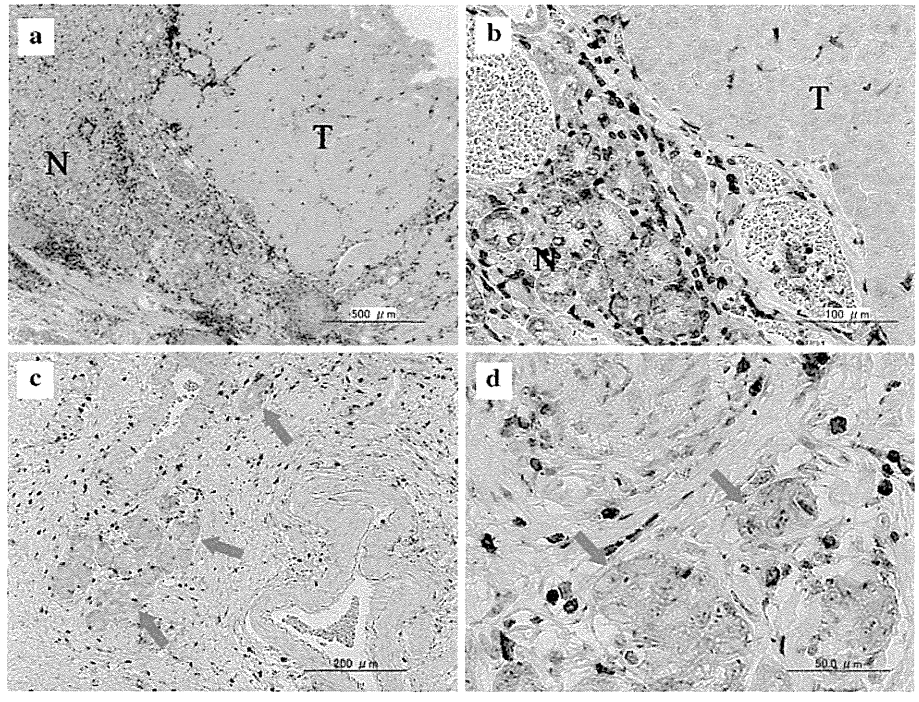

FIG. 1 DOK2 expression by immunohistochemistry. **a** Low magnification of representative DOK2-negative gastric adenocarcinoma (T) diagnosed as moderately differentiated adenocarcinoma, and adjacent normal gastric mucosa (N) positive for DOK2 (original magnification ×40). **b** High-magnification view of **a** (original magnification ×200×). **c** Representative DOK2-positive gastric adenocarcinoma (*arrows*) diagnosed as poorly differentiated adenocarcinoma (original magnification ×100) **d** High-magnification view of **c** showing cytoplasmic staining of tumor cells (*arrows*) (original magnification ×400)

significantly higher compared with those with DOK2(+) tumors ($P = .0430$). Significantly poorer OS and RFS were noted in patients with DOK2(−) tumors than those with DOK2(+) [5-year OS: DOK2(−) 59.1%, DOK2(+) 76.4%, $P = .0403$, 5-year RFS: DOK2(−) 58.1%, DOK2(+) 73.0%, $P = .0334$] (Fig. 3a, b).

Stratification analysis by histopathological type showed similar tendency in all types, including differentiated type, and poorly differentiated type (including poorly differentiated adenocarcinomas, signet ring cell carcinomas, and mucinous adenocarcinomas) [differentiated type; 5-year OS: DOK2(−) 57.6%, DOK2(+) 76.8%, $P = .2857$, poorly differentiated type; 5-year OS: 61.9%: 76.2%, respectively, $P = .0897$, data not shown].

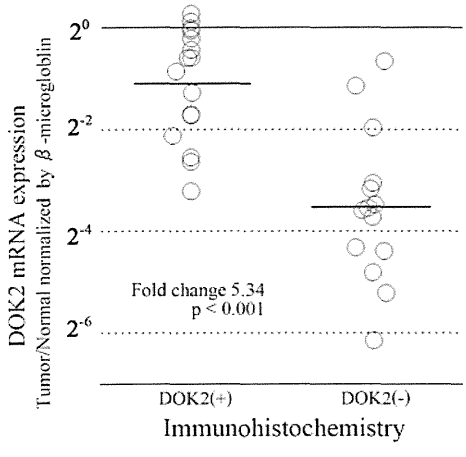

FIG. 2 DOK2 mRNA expression in clinical tissue specimens. Quantitative real-time reverse transcriptase-polymerase chain reaction on 30 paired clinical samples (normalized by *beta-2 microglobulin*). The mean DOK2 mRNA expression in DOK2(−) cases evaluated by immunohistochemistry was significantly lower compared with that of DOK(+) cases ($P < .001$; *t* test)

Univariate analysis showed significant relationship between OS and pT (hazard ratio [HR] = 3.354, 95% confidence interval [95% CI] = 1.509–8.900, $P = .0020$), pN (HR = 2.720, 95% CI = 1.437–5.131, $P = .0024$), lymphatic infiltration (HR = 3.167, 95% CI = 1.143–13.133, $P = .0239$), and negative DOK2 expression (HR = 1.960, 95% CI = 1.032–3.873, $P = .0395$), but not with age, gender, histology, venous invasion, or adjuvant chemotherapy (Table 3).

Multivariate analysis using the aforementioned 4 significant parameters identified pN (HR = 2.486, 95% CI = 1.264–4.932, $P = .0086$) as the poorest prognostic factor, followed by negative DOK2 expression (HR = 2.343, 95% CI = 1.211–4.727, $P = .0112$) and pT (HR = 2.748, 95% CI = 1.061–8.927, $P = .0361$) (Table 3).

DISCUSSION

The present study showed that while DOK2 is always expressed in the normal epithelium, its expression in gastric cancer cell varies considerably among patients, and the expression was downregulated in approximately half of the patients. This result suggested that loss of DOK2 expression might possibly play a role in the development of gastric cancer. LOH analysis also showed the involvement of reduced DOK2 expression in allelic loss of 8p21.3. High

TABLE 2 Results of microsatellite marker analysis (D8S560)

	LOH	Heterozygosity	Noninformative	*P* value
DOK2 expression				.005
Positive ($n = 16$)	0	12	4	
Negative ($n = 14$)	6	5	3	
Histological grade				.069
Well & Mod ($n = 15$)	5	6	4	
Poorly ($n = 15$)	1	11	3	

P value was calculated by Fisher exact test excluding noninformative cases

LOH loss of heterozygosity, *Well & Mod* well and moderately differentiated carcinomas (including papillary adenocarcinomas), *Poorly* poorly differentiated carcinomas (including signet ring cell carcinomas and mucinous adenocarcinoma)

frequency of the allelic loses has been reported in gastric cancer and many other cancers.[13,15–25] Various tumor suppressor genes are considered to reside on this locus, and the simultaneous loss of these tumor suppressors may trigger tumorigenesis, lending support to the tumor suppressive role of this gene.[13,26–29] The results of this study point to the importance of loss of DOK2 expression in histological grade of gastric cancer. Interestingly, most of the DOK2(−) cancers with allelic loss were of the differentiated type, while the DOK2(−) cancers without allelic loss did not correlate with histological grade. This result indicates that the pathologic differentiation of gastric cancer involves other genes in this locus but not DOK2 expression. How allelic loss determine the histopathological grade is not well understood at present, and further studies are needed to clarify the relationship.

Furthermore, clinicopathological analysis in this study revealed that patients with DOK2(−) tumors were at a significantly high risk for relapse and showed poorer overall survival than patients with DOK2(+) tumors. The prediction of recurrence and metastasis after curative resection could allow us to determine the need for intensive follow-up and adjuvant therapy. In gastric cancer therapy, it is important to prevent metachronous metastasis after curative surgical resection. In this study, 35 of 118 patients had a relapse, similar to the number of relapses reported in a previous study.[30] Recent studies described the beneficial effects of certain adjuvant chemotherapies for the treatment of recurrence in certain stages.[31,32] Staging according to TNM classification has been successful to some extent but is still insufficient. Thus, the use of predictive markers of tumor recurrence and metastasis that are independent of traditional TNM classification is clinically important. The present study indicated that DOK2 is not only an independent prognostic factor but also a candidate predictor of metachronous metastasis. The results showed no difference in OS between patients with and without DOK2 expression in the first 2 years after surgery [2-year OS: DOK2(−) 82.6%, DOK2(+) 87.85%, $P = .4717$], although differences in RFS were present between the 2 groups [2-year RFS: DOK2(−) 65.5%, DOK2(+) 84.4%, $P = .0152$]. In the present study, the liver was the first site for metastasis, accounting for 52% in DOK2(−) patients, which was much higher than 16.7% in DOK2(+) patients (Table 1). Prognosis after liver metastasis might occur in general after a longer period than after metastases at other locations, most likely because of the ease of detection and availability of effective therapeutic options such as surgical resection or radiofrequency ablation.[33–39] The present study also showed that prognosis after relapse was better for patients with liver metastasis than peritoneal metastasis (15–16 vs.

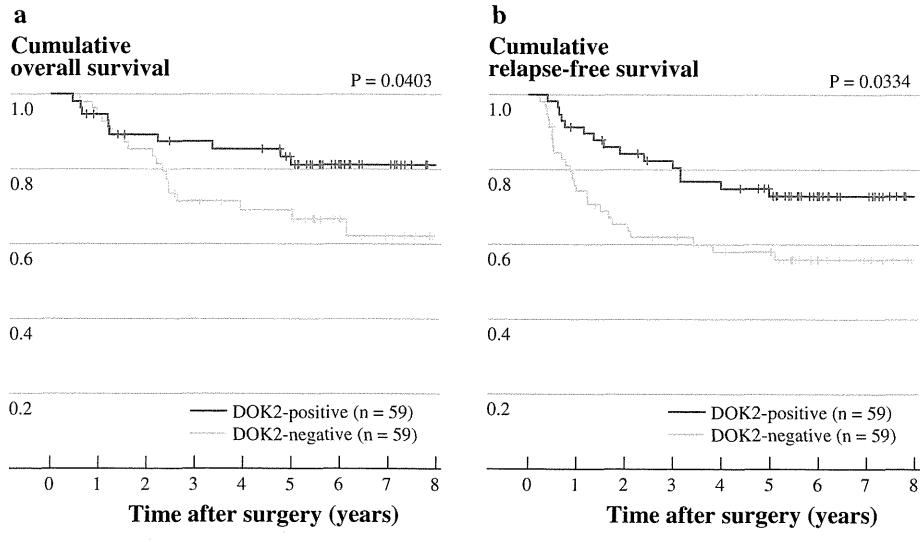

FIG. 3 Kaplan–Meier curves for overall and relapse-free survival according to DOK2 expression. Overall survival curve (**a**) and relapse-free survival curve (**b**) according to DOK2 expression for all patients. Differences between the two groups were evaluated by log-rank test. *Ordinate* survival rate, *abscissa* time after surgery (years)

TABLE 3 Results of univariate and multivariate survival analyses of overall survival by Cox proportional hazards model

Parameter	*n*	Univariate analysis			Multivariate analysis		
		HR	95% CI	*P* value	HR	95% CI	*P* value
DOK2 expression (negative/positive)	59/59	1.960	1.032–3.873	.0395	2.343	1.211–4.727	.0112
Age (<66/≥66)	58/60	1.584	0.842–3.031	.1535			
Gender (female/male)	36/82	0.511	0.219–1.059	.0721			
Differentiation (poorly/well & mod)	57/61	0.857	0.454–1.612	.6306			
pT (T3–4a/T1–2)	78/40	3.354	1.509–8.900	.0020	2.748	1.061–8.927	.0361
pN (N2–3/N0–1)	37/81	2.720	1.437–5.131	.0024	2.486	1.264–4.932	.0086
Lymphatic infiltration (positive/negative)	96/22	3.167	1.143–13.133	.0239	1.063	0.266–5.346	.9343
Venous invasion (positive/negative)	48/70	1.643	0.873–3.101	.1226			
Adjuvant chemotherapy (yes/no)	24/94	0.882	0.357–1.884	.7613			

6 months). These short-term outcomes might result from the association between DOK2 expression and predisposition to relapse pattern.

In conclusion, the present study demonstrated the expression of DOK2 in normal gastric mucosa and 50% of gastric cancer samples. Our data pointed to the potential usefulness of DOK2 as a marker for prediction of prognosis of patients with gastric cancer after curative resection. Further prospective studies are necessary to clarify the clinical significance of such prediction. Moreover, the potential mechanism of poor prognosis in patients with low DOK2 expression should be evaluated. The present findings could open the door for exploration of efficacious treatment strategies and the development of new therapeutic modalities for gastric cancer.

DISCLOSURES Hiromichi Miyagaki and all authors of the manuscript: "Evaluation of DOK2 protein expression in gastric adenocarcinoma" declare no conflict of interest and no financial ties to disclose.

REFERENCES

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. *CA Cancer J Clin.* 2011;61:69–90.
2. Sobin LH, Gospodarowicz MK, Wittekind C, International Union against Cancer. TNM classification of malignant tumours. Chichester: Wiley-Blackwell; 2010.
3. Japanese Research Society for Gastric Cancer. Japanese classification of gastric carcinoma (in Japanese). 14th ed. Kyoto: Japanese Research Society for Gastric Cancer; 2010.
4. Zhang Y, Yan Z, Farooq A, Liu X, Lu C, Zhou MM, et al. Molecular basis of distinct interactions between Dok1 PTB domain and tyrosine-phosphorylated EGF receptor. *J Mol Biol.* 2004;343:1147–55.
5. Jones N, Dumont DJ. Recruitment of Dok-R to the EGF receptor through its PTB domain is required for attenuation of Erk/MAP kinase activation. *Curr Biol.* 1999;9:1057–60.
6. Van Slyke P, Coll ML, Master Z, Kim H, Filmus J, Dumont DJ. Dok-R mediates attenuation of epidermal growth factor-dependent mitogen-activated protein kinase and Akt activation through processive recruitment of c-Src and Csk. *Mol Cell Biol.* 2005;25:3831–41.
7. Zhao M, Janas JA, Niki M, Pandolfi PP, Van Aelst L. Dok-1 independently attenuates Ras/mitogen-activated protein kinase and Src/c-Myc pathways to inhibit platelet-derived growth factor-induced mitogenesis. *Mol Cell Biol.* 2006;26:2479–89.
8. Carpino N, Wisniewski D, Strife A, Marshak D, Kobayashi R, Stillman B, et al. P62(Dok): a constitutively tyrosine-phosphorylated, GAP-associated protein in chronic myelogenous leukemia progenitor cells. *Cell.* 1997;88:197–204.
9. Jones N, Dumont DJ. The Tek/Tie2 receptor signals through a novel Dok-related docking protein, Dok-R. *Oncogene.* 1998;17:1097–108.
10. Dankort D, Jeyabalan N, Jones N, Dumont DJ, Muller WJ. Multiple ErbB-2/Neu phosphorylation sites mediate transformation through distinct effector proteins. *J Biol Chem.* 2001;276:38921–8.
11. Niki M, Di Cristofano A, Zhao M, Honda H, Hirai H, Van Aelst L, et al. Role of Dok-1 and Dok-2 in leukemia suppression. *J Exp Med.* 2004;200:1689–95.
12. Yasuda T, Shirakata M, Iwama A, Ishii A, Ebihara Y, Osawa M, et al. Role of Dok-1 and Dok-2 in myeloid homeostasis and suppression of leukemia. *J Exp Med.* 2004;200:1681–7.
13. Berger AH, Niki M, Morotti A, Taylor BS, Socci ND, Viale A, et al. Identification of Dok genes as lung tumor suppressors. *Nat Genet.* 2010;42:216–23.
14. Favre C, Gerard A, Clauzier E, Pontarotti P, Olive D, Nunes JA. DOK4 and DOK5: new Dok-related genes expressed in human T cells. *Genes Immun.* 2003;4:40–5.
15. Baffa R, Santoro R, Bullrich F, Mandes B, Ishii H, Croce CM. Definition and refinement of chromosome 8p regions of loss of heterozygosity in gastric cancer. *Clin Cancer Res.* 2000;6:1372–7.
16. Peng DF, Sugihara H, Mukaisho K, Tsubosa Y, Hattori T. Alterations of chromosomal copy number during progression of diffuse-type gastric carcinomas: metaphase- and array-based comparative genomic hybridization analyses of multiple samples from individual tumours. *J Pathol.* 2003;201:439–50.
17. Kong G, Oga A, Park CK, Kawauchi S, Furuya T, Sasaki K. DNA sequence copy number aberrations associated with histological subtypes and DNA ploidy in gastric carcinoma. *Jpn J Cancer Res.* 2001;92:740–7.
18. Sheffer M, Bacolod MD, Zuk O, Giardina SF, Pincas H, Barany F, et al. Association of survival and disease progression with chromosomal instability: a genomic exploration of colorectal cancer. *Proc Natl Acad Sci USA.* 2009;106:7131–6.

19. Brown MR, Chuaqui R, Vocke CD, Berchuck A, Middleton LP, Emmert-Buck MR, et al. Allelic loss on chromosome arm 8p: analysis of sporadic epithelial ovarian tumors. *Gynecol Oncol.* 1999;74:98–102.
20. Macoska JA, Trybus TM, Benson PD, Sakr WA, Grignon DJ, Wojno KD, et al. Evidence for three tumor suppressor gene loci on chromosome 8p in human prostate cancer. *Cancer Res.* 1995;55:5390–5.
21. El Gammal AT, Bruchmann M, Zustin J, Isbarn H, Hellwinkel OJ, Kollermann J, et al. Chromosome 8p deletions and 8q gains are associated with tumor progression and poor prognosis in prostate cancer. *Clin Cancer Res.* 2010;16:56–64.
22. Matsuyama H, Oba K, Matsuda K, Yoshihiro S, Tsukamoto M, Kinjo M, et al. Haploinsufficiency of 8p22 may influence cancer-specific survival in prostate cancer. *Cancer Genet Cytogenet.* 2007;174:24–34.
23. Stoehr R, Wissmann C, Suzuki H, Knuechel R, Krieg RC, Klopocki E, et al. Deletions of chromosome 8p and loss of sFRP1 expression are progression markers of papillary bladder cancer. *Lab Invest.* 2004;84:465–78.
24. Armes JE, Hammet F, de Silva M, Ciciulla J, Ramus SJ, Soo WK, et al. Candidate tumor-suppressor genes on chromosome arm 8p in early-onset and high-grade breast cancers. *Oncogene.* 2004;23:5697–702.
25. Chan KL, Lee JM, Guan XY, Fan ST, Ng IO. High-density allelotyping of chromosome 8p in hepatocellular carcinoma and clinicopathologic correlation. *Cancer.* 2002;94:3179–85.
26. Takada H, Imoto I, Tsuda H, Sonoda I, Ichikura T, Mochizuki H, et al. Screening of DNA copy-number aberrations in gastric cancer cell lines by array-based comparative genomic hybridization. *Cancer Sci.* 2005;96:100–10.
27. Chitale D, Gong Y, Taylor BS, Broderick S, Brennan C, Somwar R, et al. An integrated genomic analysis of lung cancer reveals loss of Dusp4 in EGFR-mutant tumors. *Oncogene.* 2009;28: 2773–83.
28. Rubio-Moscardo F, Blesa D, Mestre C, Siebert R, Balasas T, Benito A, et al. Characterization of 8p21.3 chromosomal deletions in B-cell lymphoma: TRAIL-R1 and TRAIL-R2 as candidate dosage-dependent tumor suppressor genes. *Blood.* 2005;106:3214–222.
29. Seibold S, Rudroff C, Weber M, Galle J, Wanner C, Marx M. Identification of a new tumor suppressor gene located at chromosome 8p21.3-22. *FASEB J.* 2003;17:1180–2.
30. Bonenkamp JJ, Hermans J, Sasako M, van de Velde CJ, Welvaart K, Songun I, et al. Extended lymph-node dissection for gastric cancer. *N Engl J Med* 1999;340:908–14.
31. Sakuramoto S, Sasako M, Yamaguchi T, Kinoshita T, Fujii M, Nashimoto A, et al. Adjuvant chemotherapy for gastric cancer with S-1, an oral fluoropyrimidine. *N Engl J Med.* 2007;357: 1810–20.
32. Paoletti X, Oba K, Burzykowski T, Michiels S, Ohashi Y, Pignon JP, et al. Benefit of adjuvant chemotherapy for resectable gastric cancer: a meta-analysis. *JAMA.* 2010;303:1729–37.
33. Jin SS, Beak J, Kim H, Park J, Kim G, Kim D, et al. Prognostic factors in patients with recurrent gastric cancer after curative gastrectomy. *J Clin Oncol.* 2008;26:suppl;abstr 15612.
34. Hwang SE, Yang DH, Kim CY. Prognostic factors for survival in patients with hepatic recurrence after curative resection of gastric cancer. *World J Surg.* 2009;33:1468–72.
35. Lee CC, Lo SS, Wu CW, Shen KH, Li AF, Hsieh MC, et al. Peritoneal recurrence of gastric adenocarcinoma after curative resection. *Hepatogastroenterology.* 2003;50:1720–2.
36. Kerkar SP, Kemp CD, Avital I. Liver resections in metastatic gastric cancer. *HPB (Oxford).* 2010;12:589–96.
37. Sakamoto Y, Sano T, Shimada K, Esaki M, Saka M, Fukagawa T, et al. Favorable indications for hepatectomy in patients with liver metastasis from gastric cancer. *J Surg Oncol.* 2007;95:534–9.
38. Kim HR, Cheon SH, Lee KH, Ahn JR, Jeung HC, Lee SS, et al. Efficacy and feasibility of radiofrequency ablation for liver metastases from gastric adenocarcinoma. *Int J Hyperth.* 2010;26: 305–15.
39. An JY, Kim JY, Choi MG, Noh JH, Choi D, Sohn TS, et al. Radiofrequency ablation for hepatic metastasis from gastric adenocarcinoma. *Yonsei Med J.* 2008;49:1046–51.

Journal of Surgical Oncology 2012;105:780–785

Oncofetal Protein, IMP-3, a Potential Marker for Prediction of Postoperative Peritoneal Dissemination in Gastric Adenocarcinoma

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Background: The aim of this study was to determine the expression of insulin-like growth factor-II messenger RNA (mRNA)-binding protein-3 (IMP-3) and its clinical significance in gastric cancers, as well the prognostic value of its expression in the peritoneal lavage fluid after surgery.

Methods: IMP-3 expression was examined by immunohistochemistry in 96 primary gastric tumors. IMP-3 mRNA expression in peritoneal lavage fluid obtained at laparotomy was determine by real-time quantitative reverse transcriptase-polymerase chain reaction (RT-PCR).

Results: Positive staining for IMP-3 was observed in 74% (71/96) of the tumors. IMP-3 expression in gastric tumors correlated significantly with worst overall survival (OS) and recurrence-free survival. Multivariate analyses identified pathological N stage and IMP-3 expression as significant independent prognostic factors for disease-free survival. Eight (28%) of 36 peritoneal lavage samples were cytologically negative but positive for IMP-3 mRNA expression by RT-PCR. The OS of patients with IMP-3-positive peritoneal lavage was significantly worse than of those with negative expression.

Conclusions: IMP-3 expression in primary gastric tumors was an independent poor prognostic factor. IMP-3 mRNA expression in peritoneal lavage fluid was a predictor of recurrence after surgery in gastric cancer and a marker of poor prognosis.

J. Surg. Oncol. 2012;105:780–785. © 2011 Wiley Periodicals, Inc.

KEY WORDS: gastric cancer; IMP-3; peritoneal dissemination; RT-PCR

INTRODUCTION

The human insulin-like growth factor (IGF) II mRNA-binding protein (IMP-3), also known as K homology domain-containing protein overexpressed in cancer (KOC), is a member of the IMP family (which also includes IMP-I and IMP-2). IMP-3 is a newly identified oncofetal mRNA-binding protein [1]. The IMP-3 gene, located on chromosome 7q11.5, encodes a 4,350-bp mRNA and a 580 amino acid protein [2]. IMP family members play important roles in RNA trafficking and stabilization, cell growth, and cell migration during the early stages of embryogenesis [1–3]. IMP-3 is expressed in developing epithelia, myocytes, and placenta during the early stage of human and mouse embryogenesis, but it is expressed at low or undetectable levels in adult tissues [2,3]. Overexpression of IMP-3 has been recently described in many malignant tumors including pancreas, lung, colon, endometrium, ovary, kidney, and soft tissue sarcomas [4–9]. Furthermore, IMP-3 was reported to promote cancer cell motility, invasion, and migration through the endothelial layer and induces the highly malignant phenotype in hepatocellular carcinoma [10]. Based on these results, IMP-3 may be a useful prognostic factor in many malignant solid tumors. In gastric cancer, Jeng et al. [11] reported that overexpression of IMP-3 correlated with older age, larger tumor size, deeper tumor invasion, and lymph node metastasis and was an independent prognostic factor in gastric cancer.

Although the incidence of gastric cancer has decreased especially in Western countries, it still ranks as the fourth most common cancer with the second most common cancer-related deaths [12,13]. The prognosis of patients with advanced gastric cancer remains poor even after curative operation, and in these cases, peritoneal dissemination caused mainly by seeding of free cancer cells from the primary gastric cancer is the most common mode of metastasis [14,15]. The peritoneum is the most frequent site of recurrence in patients with advanced gastric cancer who undergo curative resection [16]. Therefore, identification of suitable biomarkers for early prediction of peritoneal recurrence and prognosis is important in the overall management of patients with advanced gastric cancer.

Cytological examination of peritoneal lavage fluid obtained at laparotomy is performed to predict peritoneal spread [17–19]. The majority of cases with positive cytology on peritoneal lavage develop peritoneal metastasis, although peritoneal recurrence also occurs in patients with negative cytological results [17–19]. These results indicate that cytological examination lacks sensitivity for detection of residual cancer cells and prediction of peritoneal spread. Recent studies described the use of reverse transcriptase-polymerase chain reaction (RT-PCR) analysis for detection of cancer micrometastasis [20–22]. RT-PCR is more sensitive than conventional cytological examination [23–25]. Based on several reports, the results of RT-PCR of peritoneal lavage correlate strongly with peritoneal recurrence and prognosis after curative surgery in patient with advanced gastric cancer [23,25–30]. We and others reported the utility of a carcinoembryonic antigen (CEA) mRNA and cytokeratin-20 (CK-20) as molecular markers for early detection of peritoneal recurrence after

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Received 31 May 2011; Accepted 10 September 2011

DOI 10.1002/jso.22108

Published online 19 October 2011 in Wiley Online Library (wileyonlinelibrary.com).

gastric cancer surgery. However, CEA and CK-20 are not cancer-specific markers and some parts of the gastric tumor tissue do not express of CEA or CK-20. Therefore, other marker(s) are needed to improve the sensitivity and specificity of prediction of peritoneal recurrence in gastric cancer.

In this study, we examined the expression of IMP-3 protein in gastric cancer specimens and assessed the correlations between IMP-3 overexpression and clinicopathological characteristics. In addition, we investigated the expression of IMP-3 mRNA in peritoneal lavage specimens obtained during gastric cancer surgery by real-time quantitative RT-PCR and assessed its clinical utility for the prediction of peritoneal recurrence after curative resection of gastric cancer.

MATERIALS AND METHODS

Patients and Specimens

Gastric cancer tissues were obtained from 96 patients who underwent gastrectomy at Department of Gastroenterological Surgery, Osaka University Hospital during the period from 2001 to 2006. All tumors were confirmed to be gastric adenocarcinoma by histopathological examination. The patients comprised 66 men and 30 women, with a mean age of 63 years (range; 33–91) (Table I). None had received preoperative treatment including chemotherapy and/or radiotherapy before surgery. Peritoneal lavage specimens were obtained from 46 patients with serosa-invaded gastric tumor who underwent gastrectomy during the period from 2002 to 2009. Cytological results with peritoneal lavage at laparotomy showed positive in 10 patients and showed negative in 36 patients, who were subjected to molecular diagnosis with RT-PCR.

Clinicopathological Characteristics

The specimens were classified pathologically based on the 13th edition of the Japanese Classification of Gastric Cancer [31]. Histopathologically, 45 were undifferentiated tumors, including poorly differentiated and signet ring cell carcinoma, while 51 were differentiated tumors. The tumor-node-metastasis (TNM) stage was classified as I in 38 cases, II in 26 cases, III in 29 cases, and IV in 3 cases according to the Japanese Classification of Gastric Cancer (Table I).

TABLE I. Associations Between IMP-3 Expression and Various Clinicopathologic Factors in 96 Patients With Gastric Cancer

Variable	Entire group (n = 96)	IMP-3 expression: Negative (n = 25)	IMP-3 expression: Positive (n = 71)	*P*-value
Age (years)				0.162
<65	48	16	32	
≥65	48	9	39	
Gender				0.8041
Male	66	18	48	
Female	30	7	23	
Depth of tumor invasion				0.624
pT1–2	64	18	46	
pT3–4	32	7	25	
Lymph node metastasis				0.102
Negative	43	15	28	
Positive	53	10	43	
Vessel invasion				0.642
No	57	16	41	
Yes	39	9	30	
Histopathology				1.000
Differentiated	51	13	38	
Undifferentiated	45	12	33	
Stage				0.812
1–2	63	17	46	
3–4	33	8	25	

Immunohistochemical Analysis

The expression of IMP-3 was evaluated by immunohistochemical (IHC) analyses in 4-μm thick sections of the 10% formalin-fixed and paraffin-embedded blocks. For IHC staining, tissue slides were deparaffinized in xylene and then rehydrated through graded ethanol. For antigen retrieval, these slides were incubated by autoclave in 10 mM Tris and 1 mM ethylenediaminetetraacetic acid (EDTA) buffer (pH 9.0) for 20 min. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in methanol for 20 min. Nonspecific binding was blocked with 10% normal serum for 20 min. Subsequently, the tissue slides were incubated overnight with IMP-3 antibody (anti-human L523S, dilution 1:200, Dako Cytomation, Carpinteria, CA) at 4°C in a moist chamber. Sites of antibody binding were visualized with the ABC peroxidase detection system (Vector Laboratories, Burlingame, CA). Finally, the sections were incubated in 3,3′-diaminobenzidine tetrahydrochloride with 0.05% H_2O_2 for 3 min and counterstained with 0.1% hematoxylin. The percentage of cancer cells stained with the antibody was evaluated. We classified the results of immunohistochemistry for IMP-3 into three grades as follows: 0; positive cells less than 10%, +1; positive cells more than 10% and less than 50%, and +2; positive cells more than 50%. Expression of IMP-3 protein was judged as positive when more than 10% of cancer cells stained positive for IMP-3.

RNA Extraction

Crushed surgical specimens were dissolved in TRIZOL Reagent (Invitrogen, Carlsbad, CA). Total RNA was extracted using the method supplied by the manufacturer. Total cellular RNA was extracted from cell pellets of peritoneal lavage fluid samples and cancer cell lines using TRIZOL reagent according to the protocol provided by the manufacturer. In brief, the mixture was minced with disposable homogenizers (IEDA^{TM}, Tokyo, Japan), mixed with 0.2 ml chloroform and centrifuged at 12,000*g* for 15 min. The supernatant was transferred to a fresh tube and mixed with 0.5 ml 100% isopropyl alcohol. After incubation for 10 min at room temperature, RNA was precipitated by centrifugation, washed with 75% ethanol, and diluted with DEPC (diethyl pyrocarbonate)-treated water.

Real-Time Quantitative RT-PCR Analysis

The primer sequences for PCR amplification were as follows, IMP-3: 5′-AAGACTTAGGAAGACTGGTGGA-3′ (forward) and 5′-TCCCACTGTAAATGAGGCGGGATA-3′ (reverse), CEA: 5′-TCTGGAACTTCTCCTGGTCTCTCTCAGCTGG-3′ (forward) and 5′-TGTAGCTGTTGCAAATGCTTTAAGGAAGAAGC-3′ (reverse), CK20: 5′-GGTCGCGACTACAGTGCATATTACA-3′ (forward) and 5′-CCTCAGCAGCCAGTTTAGCATTATC-3′ (reverse). The housekeeping gene, porphobilinogen deaminase (PBGD) was used as an internal control: PBGD: 5′-TGTCTGGTAACGGCAATGCGGCTGCAAC-3′ (forward) and 5′-TCAATGTTGCCACCACACTGTCCGTCT-3′ (reverse). The integrity of all RNA samples was verified by quantitative RT-PCR for PBGD in each sample. The emission intensity of SYBR Green was detected in real-time with *LightCycler 3.5* instrument (Roche Diagnostics, Mannheim, Germany). The external standards were prepared by serial dilution (1:1–1:10,000) of cDNA from the MKN45 cell line. IMP-3 expression was reported relative to the expression of PBGD in each sample. The detection sensitivity was

calculated as follows: PBGD mRNA was detected in at least 10^2 cells of MKN45. IMP-3 and CEA mRNAs were detected in at least 10^1 cells of MKN45. CK20 mRNA was detected in more than 10^3 cells of MKN45.

Statistical Analysis and Ethical Considerations

Statistical analysis was performed with JMP® software (JMP version 8.0.2, SAS Institute, Cary, NC). The relationship between IMP3 expression and various clinicopathological parameters was assessed by the χ^2 test. Disease-free survival (DFS) and overall survival (OS) were assessed with the Kaplan–Meier method and compared by the log-rank test. All parameters that were found to be significant on univariate analysis using the Cox proportional hazard model were entered into multivariate survival analysis. *P*-values of <0.05 were considered significant.

The study protocol was approved by the Human Ethics Review Committee of Osaka University and a signed consent form was obtained from each subject with regard to biopsy and peritoneal lavage sampling.

RESULTS

Correlations Between Protein and mRNA Expressions of IMP-3 in Gastric Cancer Tissues

Ten gastric tumor specimens were subjected to RT-PCR and IHC analyses for IMP-3. Eight tumors expressed IMP-3 mRNA and out of them six tumors showed positive for IHC for IMP-3 protein. Two tumors with no expression for IMP-3 mRNA also showed negative for IMP-3 IHC analysis (Fig. 1).

IMP-3 Protein Expression in Gastric Cancer Tissues

The normal gastric mucosa showed nonspecific and weak staining for IMP-3 (Fig. 2A). Among the 96 specimens of gastric cancer, 71 (74%) were diagnosed as positive and the staining was mainly in the cytoplasm of tumor cells (Fig. 2B,C). The remaining 25 (26%) were negatively stained for IMP-3 (Fig. 2D). The IMP-3-positive cells were detected in various areas of the tumor such as the surface, central, and deep areas of gastric walls.

Correlations Between IMP-3 Expression in Gastric Cancer and Clinicopathological Parameters

Table I shows the correlations between IMP-3 expression in gastric tumors and various clinicopathological parameters. The frequency of IMP-3-positive cases did not correlate with any clinicopathological parameter. However, IMP-3 expression correlated

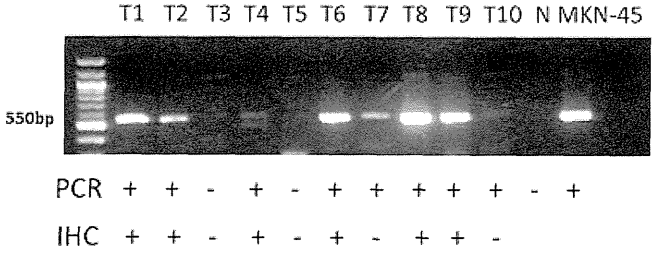

Fig. 1. Correlations between protein and mRNA expressions of IMP-3 in gastric cancer tissues. T, gastric tumor tissue; N, normal gastric epithelium; MKN-45, gastric cancer cell line; PCR, polymerase chain reaction for IMP-3; IHC, immunohistochemistry; and bp, base pairs.

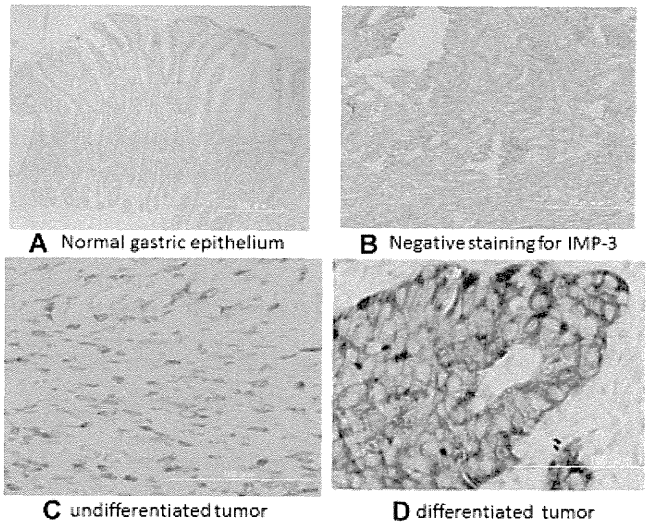

Fig. 2. Representative immunohistochemical staining for IMP-3 antibody.

significantly with poor OS and recurrence-free survival times (Fig. 3A,B).

Prognostic Significance of IMP-3 Expression in Gastric Cancer and Overall/Recurrence-Free Survival

The relation between various clinicopathological parameters and OS was examined by Cox's proportional hazard model (Table II). Univariate analysis showed that pathological N stage and IMP-3 expression correlated with OS (HR = 5.13 and 3.17, respectively). However, multivariate analysis identified pathological N stage as the only significant independent prognostic predictor of OS (HR = 4.70).

The relation between various clinicopathological parameters and DFS was examined by Cox's proportional hazard model (Table III). Univariate analysis showed that gender, pathological N stage, and IMP-3 expression correlated with DFS (HR = 2.62, 4.92, and 3.54, respectively). Multivariate analysis identified gender, pathological N stage, and IMP-3 expression as significant independent prognostic predictors (HR = 2.59, 4.27, and 3.02, respectively).

IMP-3 mRNA in Peritoneal Lavage Specimens

Finally, we examined the expression of IMP-3, CEA, and CK-20 mRNAs in peritoneal lavage specimens obtained from 36 patients with gastric cancer by real-time quantitative RT-PCR. All peritoneal lavage specimens showed negative cytology. Among the specimens, 10 (28%) were positive for CEA expression, 6 (17%) were positive for CK-20 expression, and 8 (28%) were positive for IMP-3 expression. Furthermore, 4 (11%) specimens were positive for both CEA and IMP-3 expression, and 14 (22) were positive for either of the two (Table IV). Figure 4 shows the comparative OS rates for all patients according to the results of RT-PCR of peritoneal lavage fluid for CEA, IMP-3, and CK-20. The OS of patients positive for IMP-3 was significantly worse than that of patients those negative for IMP-3. Furthermore, the OS of patients positive for both CEA and IMP-3 was significantly worse than that of others. Out of eight patients whose peritoneal lavage showed positive with IMP-3 mRNA, five suffered recurrence after surgery including four peritoneal recurrence. Out of 28 patients whose peritoneal lavage showed negative with IMP-3 mRNA, 10 suffered recurrence including 6 peritoneal recurrence and 4 liver metastases.

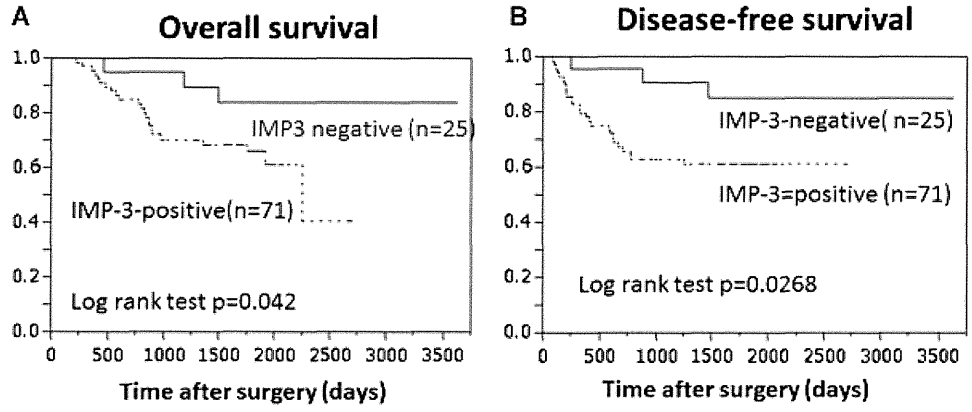

Fig. 3. Kaplan–Meier analyses of overall survival and disease-free survival according to the results of IMP-3 expression. Median follow-up was 5.5 years. Median overall survival was 3.5 years in patients with positive IMP-3 and 4.3 years in patients with negative IMP-3. Median disease-free survival was 3.1 years in patients with positive IMP-3 and 4.2 years in patients with negative IMP-3.

DISCUSSION

Our study demonstrated the expression of IMP-3 in three fourths of gastric adenocarcinoma and that such expression correlates significantly with shorter DFS and tends to correlate with shorter OS. These results confirm the findings reported by Jeng et al. [10] and suggest that IMP-3 expression in gastric adenocarcinoma could be involved in tumor progression and metastasis. Liao et al. [32] reported that IMP-3 acts as a translational activator of IGF II leader-3 mRNA and that IMP-3 plays a critical role in the regulation of cell proliferation via an IGF II-dependent pathway. Other studies reported that IMP-3 is highly expressed, at both mRNA and protein levels, in most cancer cell lines and cancer tissues, but barely detectable in most normal adult tissues except for testis and placenta [5,33]. IMP-3 is an oncofetal protein expressed in various malignancies including gastric cancer, which could be a potential target for immunotherapy. Suda et al. [34] identified highly immunogenic human IMP-3-derived peptides, which induced cytotoxic T lymphocytes. Kono et al. [35] performed a phase I study with peptide vaccine therapy using three types of peptides including IMP-3 for patients with esophageal squamous cell carcinoma who failed to respond to standard therapy. They reported that the cancer vaccine therapy provided a satisfactory, safe, and good immunogenicity as well as promising efficacy.

In this study, we used the cancer-specific oncofetal molecule, IMP-3, as a molecular marker for prediction of peritoneal recurrence after curative surgery for gastric cancer. In this regard, the CEA

TABLE II. Results of Univariate and Multivariate Analyses of Overall Survival by Cox' Proportional Hazard Model

		Univariate analysis			Multivariate analysis		
Variable	n	HR	CI (95%)	*P*-value	HR	CI (95%)	*P*-value
Age (<65/≥65) (years)	48/48	1.57	0.68–3.30	0.307			
Gender (F/M)	30/66	2.17	0.88–6.53	0.096			
pT (1–2/3–4)	64/32	2.04	0.93–4.42	0.076			
pN (0/1–3)	43/53	5.13	1.95–17.6	<0.001	4.70	1.78–16.2	0.001
p Stage (1–2/3–4)	63/33	2.48	1.14–5.43	0.023			
Histopathology (differentiated/undifferentiated)	51/45	0.98	0.45–2.13	0.956			
IMP-3 (−/+)	25/71	3.17	1.09–13.5	0.032	2.66	0.90–11.4	0.079

TABLE III. Results of Univariate and Multivariate Analyses of Disease-Free Survival Analyzed by Cox' Proportional Hazard Model

		Univariate analysis			Multivariate analysis		
Variables	n	HR	CI (95%)	*P*-value	HR	CI (95%)	*P*-value
Age (<65/≥65) (years)	48/48	1.57	0.75–3.34	0.227			
Gender (F/M)	30/66	2.62	1.08–7.79	0.031	2.59	1.07–7.71	0.033
pT (1–2/3–4)	64/32	2.04	0.98–4.26	0.058			
pN (0/1–3)	43/53	4.92	2.03–14.6	<0.001	4.27	1.76–12.7	<0.001
p Stage (1–2/3–4)	63/33	2.55	1.22–5.35	0.013			
Histopathology (differentiated/undifferentiated)	51/45	0.83	0.39–1.72	0.613			
IMP-3 (−/+)	25/71	3.54	1.25–14.9	0.015	3.02	1.05–12.7	0.038

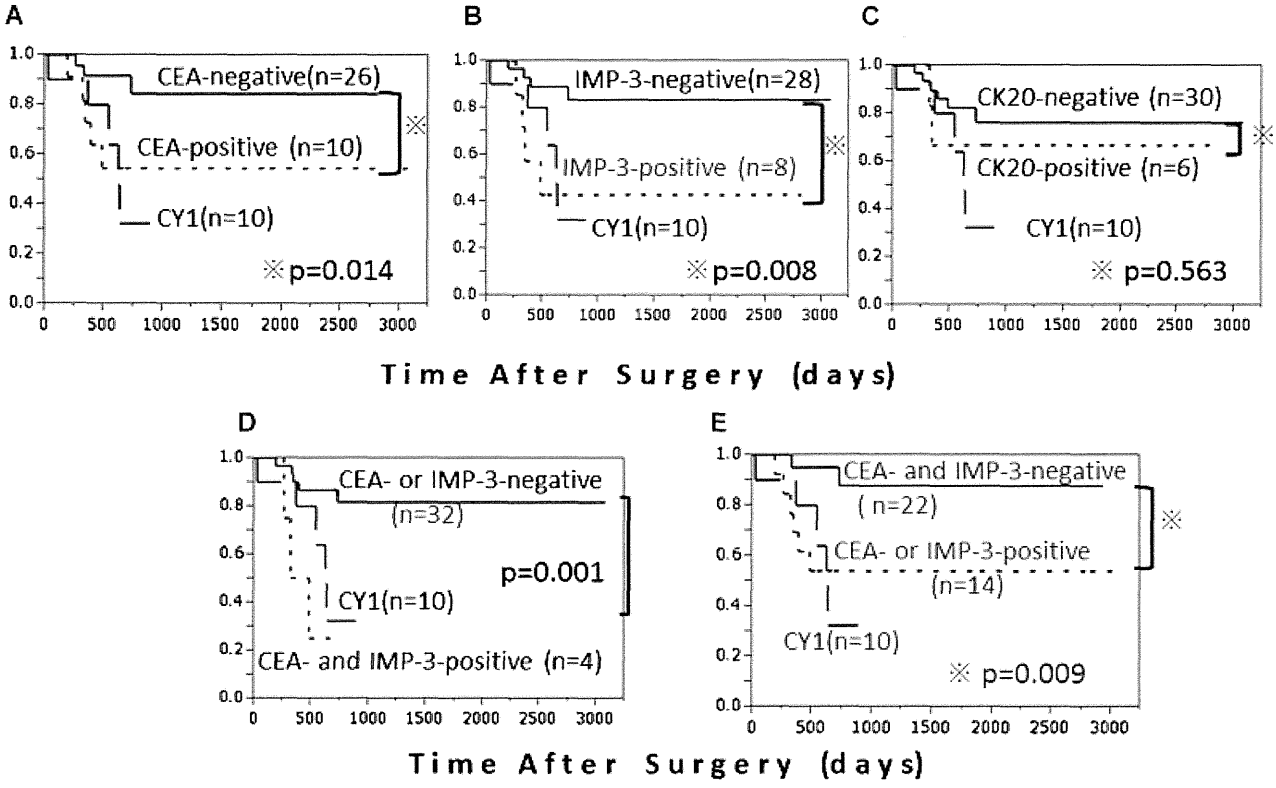

Fig. 4. Overall survival according to the results of RT-PCR using three molecular markers.

mRNA has been commonly used for RT-PCR-based molecular detection of such recurrence. However, some gastric cancer tumors do not express CEA mRNA and other markers such as CK-20 have also been used in some reports [24,36]. In the present study, we tested the expression of IMP-3 mRNA as well as CEA and CK-20. The proportions of tumors positive for CEA, IMP-3, and CK-20 were 28%, 22%, and 17%, respectively. Patients positive for IMP-3 mRNA expression showed significantly worse OS, similar to those positive for CEA mRNA, although positivity for CK-20 did not correlate significantly with OS. RT-PCR for IMP-3 mRNA identified four more patients with high risk for peritoneal recurrence, who were otherwise negative for CEA mRNA. Furthermore, patients positive for both CEA and IMP-3 had the worst prognosis, which almost similar to that of patients with positive cytology and those with peritoneal carcinomatosis. Thus, the use of this assay may allow early surgery followed by aggressive treatment.

In conclusion, the present study identified the expression of IMP-3 as a potential marker of gastric adenocarcinoma. Furthermore, IMP-3 might be a cancer-specific genetic marker for detection of minimal spread of cancer micrometastasis and further study will be urgently needed to clarify its clinical utility.

TABLE IV. Expression of CEA, CK20, and IMP3 mRNAs in Peritoneal Lavage Fluid

Variable	n = 36	%
CEA	10/36	28
CK20	6/36	17
IMP-3	8/36	22
CEA and IMP3	4/36	11
CEA or IMP3	14/36	22

REFERENCES

1. Nielsen J, Christiansen J, Lykke-Andersen J, et al.: A Family of insulin-like growth factor II mRNA-binding proteins represses translation in late development. Mol Cell Biol 1999;19:1262–1270.
2. Mueller-Pillasch F, Pohl B, Wilda M, et al.: Expression of the highly conserved RNA binding protein KOC in embryogenesis. Mech Dev 1999;88:95–99.
3. Nielsen FC, Nielsen J, Christiansen J: A family of IGF-II mRNA binding proteins(IMP) involved in RNA trafficking. Scand J Clin Lab Invest 2001;61:93–99.
4. Schaeffer D, Owen D, Lim H, et al.: Insulin-like growth factor 2 mRNA binding protein 3 (IGF2BP3) overexpression in pancreatic ductal adenocarcinoma correlates with poor survival. BMC Cancer 2010;10:59.
5. Wang T, Fan L, Watanabe Y, et al.: L523S, an RNA-binding protein as a potential therapeutic target for lung cancer. Br J Cancer 2003;88:887–894.
6. Dawei L, Dongwang Y, Huaamei T, et al.: IMP3 is a novel prognostic marker that correlates with colon cancer progression and pathogenesis. Ann Surg Oncol 2009;16:3499–3506.
7. Zheng W, Yi X, Fadare O, et al.: The oncofetal protein IMP3: A novel biomarker for endometrial serous carcinoma. Am J Surg Pathol 2008;32:304–315.
8. Köbel M, Xu H, Bourne PA, et al.: IGF2BP3 (IMP3) expression is a marker of unfavorable prognosis in ovarian carcinoma of clear cell subtype. Mod Pathol 2009;22:469–475.
9. Jiang Z, Chu PG, Woda BA, et al.: Analysis of RNA-binding protein IMP3 to predict metastasis and prognosis of renal-cell

carcinoma: A retrospective study. Lancet Oncol 2006;7:556–564.
10. Jeng YM, Chang CC, Hu FC, et al.: RNA-binding protein insulin-like growth factor II mRNA-binding protein3 expression promotes tumor invasion and predicts early recurrence and poor prognosis in hepatocellular carcinoma. Hepatology 2008;48: 1118–1127.
11. Jeng YM, Wang TH, Lu SH, et al.: Prognostic significance of insulin-like growth factor II mRNA-binding protein 3 expression in gastric adenocarcinoma. Br J Surg 2009;96:66–73.
12. Ferlay J, Shin HR, Bray F, et al.: Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer 2010;127:2893–2917.
13. Parkin DM, Bray F, Ferlay J, et al.: Global cancer statistics, 2002. CA Cancer J Clin 2005;55:74–108.
14. Dupont JB, Jr., Lee JR, Burton GR, et al.: Adenocarcinoma of the stomach: Review of 1,497 cases. Cancer 1978;41:941–947.
15. Yamada E, Miyaishi S, Nakazato H, et al.: The surgical treatment of cancer of the stomach. Int Surg 1980;65:387–399.
16. Yoo CH, Noh SH, Shin DW, et al.: Recurrence following curative resection for gastric carcinoma. Br J Surg 2000;87: 236–242.
17. Bando E, Yonemura Y, Takeshita Y, et al.: Intraoperative lavage for cytological examination in 1,297 patients with gastric carcinoma. Am J Surg 1999;178:256–262.
18. Benevolo M: Diagnostic and prognostic value of peritoneal immunocytology in gastric cancer. J Clin Oncol 1998;16:3406–3411.
19. Koga S, Kaibara N, Iitsuka Y, et al.: Prognostic significance of intra-peritoneal free cancer cells in gastric cancer patients. J Cancer Res Clin Oncol 1984;108:236–238.
20. Noura S, Yamamoto H, Ohnishi T, et al.: Comparative detection of lymph node micrometastases of stage II colorectal cancer by reverse transcriptase polymerase chain reaction and immunohistochemistry. J Clin Oncol 2002;20:4232–4241.
21. Yoshioka S, Fujiwara Y, Sugita Y, et al.: Real-time rapid reverse transcriptase-polymerase chain reaction for intraoperative diagnosis of lymph node micrometastasis: Clinical application for cervical lymph node dissection in esophageal cancers. Surgery 2002;132:34–40.
22. Okada Y, Fujiwara Y, Yamamoto H, et al.: Genetic detection of lymph node micrometastases in patients with gastric carcinoma by multiple-marker reverse transcriptase-polymerase chain reaction assay. Cancer 2001;92:2056–2064.
23. Sugita Y, Fujiwara Y, Taniguchi H, et al.: Quantitative molecular diagnosis of peritoneal lavage fluid for prediction of peritoneal recurrence in gastric cancer. Int J Oncol 2003;23: 1419–1423.
24. Majima T, Ichikura T, Mochizuki H: Prognostic significance of the cytologic features of free cancer cells in the peritoneal cavity of patients with gastric cancer. Surg Today 2002;32: 35–39.
25. Nakanishi H, Kodera Y, Torii A, et al.: Detection of carcinoembryonic antigen-expressing free tumor cells in peritoneal washes from patients with gastric carcinoma by polymerase chain reaction. Jpn J Cancer Res 1997;88:687–692.
26. Nakanishi H, Kodera Y, Yamamura Y, et al.: Rapid quantitative detection of carcinoembryonic antigen-expressing free tumor cells in the peritoneal cavity of gastric-cancer patients with real-time RT-PCR on the LightCycler. Int J Cancer 2000;89:411–417.
27. Yonemura Y, Fujimura T, Ninomiya I, et al.: Prediction of peritoneal micrometastasis by peritoneal lavaged cytology and reverse transcriptase-polymerase chain reaction for matrix metalloproteinase-7 mRNA. Clin Cancer Res 2001;7:1647–1653.
28. Schmidt P, Thiele M, Rudroff C, et al.: Detection of tumor cells in peritoneal lavages from patients with gastrointestinal cancer by multiplex reverse transcriptase PCR. Hepatogastroenterology 2001;48:1675–1679.
29. Kodera Y, Nakanishi H, Yamamura Y, et al.: Prognostic value and clinical implications of disseminated cancer cells in the peritoneal cavity detected by reverse transcriptase-polymerase chain reaction and cytology. Int J Cancer 1998;79:429–433.
30. Kodera Y, Nakanishi H, Yamamura Y, et al.: Quantitative detection of disseminated free cancer in peritoneal washes with real-time reverse transcriptase polymerase chain reaction. Ann Surg 2002;235:499–506.
31. Japanese Gastric Cancer A. Japanese classification of gastric carcinoma—2nd English edition. Gastric Cancer 1998;1:10–24.
32. Liao B, Hu Y, Herrick DJ, et al.: The RNA-binding protein IMP-3 is a translational activator of insulin-like growth factor II leader-3 mRNA during proliferation of human K562 leukemia cells. J Biol Chem 2005;280:18517–18524.
33. Mueller-Pillasch F, Lacher U, Wallrapp C, et al.: Cloning of a gene highly overexpressed in cancer coding for a novel KH-domain containing protein. Oncogene 1997;32:379–388.
34. Suda T, Tsunoda T, Daigo Y, et al.: Identification of human leukocyte antigen-A24-restricted epitope peptides derived from gene products upregulated in lung and esophageal cancers as novel targets for immunotherapy. Cancer Sci 2007;98:1803–1808.
35. Kono K, Mizukami Y, Daigo Y, et al.: Vaccination with multiple peptides derived from novel cancer-testis antigens can induce specific T-cell responses and clinical responses in advanced esophageal cancer. Cancer Sci 2009;100:1502–1509.
36. Kodera Y, Nakanishi H, Ito S, et al.: Prognostic significance of intraperitoneal cancer cells in gastric carcinoma: Detection of cytokeratin 20 mRNA in peritoneal washes, in addition to detection of carcinoembryonic antigen. Gastric Cancer 2005;8:142–148.

Japanese Journal of Clinical Oncology Advance Access published March 1, 2012

JJCO Japanese Journal of Clinical Oncology

Jpn J Clin Oncol 2012
doi:10.1093/jjco/hys025

Clinical Trial Note

A Phase III Trial to Evaluate the Effect of Perioperative Nutrition Enriched with Eicosapentaenoic Acid on Body Weight Loss after Total Gastrectomy for T2–T4a Gastric Cancer

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Received November 15, 2011; accepted February 9, 2012

This randomized Phase III trial will evaluate whether perioperative nutrition enriched with eicosapentaenoic acid can prevent body weight loss after total gastrectomy for gastric cancer. The patients who enroll in this study will be randomly assigned to Group A: no supplementation with oral nutrients (standard diet) or Group B: standard diet with eicosapentaenoic acid-enriched supplementation for 7 days before surgery and for 21 days after surgery. For both groups, patients will undergo total gastrectomy with Roux-en Y reconstruction. The extent of dissection will principally follow the third edition of the Gastric Cancer Treatment Guideline published by the Japanese Gastric Cancer Association. When patients are diagnosed with pathological Stage II or III disease, adjuvant chemotherapy with S-1 will be initiated within 6 weeks after surgery and administered for 1 year. The primary endpoint will be the body weight loss at 1 and 3 months after surgery (double primary endpoints). The secondary endpoints will be the relative performance of the supplement, loss of lean body mass at 1 and 3 months after surgery, the lowest serum albumin level, quality of life, the incidence of surgical morbidity and mortality, and the incidence of surgical site infection.

Key words: gastric cancer – EPA – gastrectomy – weight loss

INTRODUCTION

Gastric cancer is the second leading cause of cancer death in the world and is the most common malignancy in Japan, South America, and Eastern Europe (1). Complete resection is essential for the cure of gastric cancer (2). Total gastrectomy with lymph node dissection is necessary when tumors are located in the upper to middle third of the stomach. After total gastrectomy, the body weight loss is commonly observed, and patients often experience a loss of >10% of their preoperative body weight (3). The body weight loss correlates with a decline in postoperative quality of life and is the most reliable indicator of malnutrition (4). Various mechanisms possibly underlying this weight loss after gastrectomy have been considered, such as inflammatory reactions due to surgical stress, reduced food intake due to loss of reservoir function and reduction in the blood ghrelin level (4). A reduction in the lean body mass is considered to be responsible for the body weight loss (5).

Ghrelin administration may be effective for decreasing weight loss (6), but is currently available only for experimental studies. Moreover, continuous intravenous administration is necessary because ghrelin is unstable in humans. Gastric substitution using a jejunal pouch has been tried and examined (4); however, its impact on weight loss is still controversial. Enteral nutrition may be useful for patients with reduced food intake after surgery; however, physicians do not consider a nasal tube or jejunostomy for enteral nutrition because most patients are discharged 8–10 days after surgery (7). A simple and convenient method is therefore required to prevent body weight loss after surgery.

Eicosapentanoic acid (EPA), a long-chain polyunsaturated fatty (PUFA) acid of the omega-3 (n-3) family, has both anabolic and immunomodulatory properties, making it attractive for use during the postoperative period (8). When EPA is consumed in quantities above the normal dietary levels, it replaces arachidonic acid, an *n*-6 PUFA, in cell membrane phospholipids, and is a substrate for the synthesis of the 3-series prostaglandins and the 5-series leukotrienes (9). These products are less immunoinflammatory than the respective 2- and 4-analogs normally synthesized from arachidonic acid (9). Anticatabolic properties of EPA have been reported in patients with advanced cancer due to the inhibition of the production of inflammatory cytokines (10). Moreover, Ryan et al. (11) reported that enteral nutrition including EPA preserved the lean body mass after esophageal cancer surgery in a randomized study, suggesting that EPA has benefits even for patients who undergo major surgery.

Based on these studies, we planned a randomized Phase III trial to evaluate whether perioperative administration of an EPA-enriched supplement can prevent body weight loss after total gastrectomy for gastric cancer.

PROTOCOL DIGEST OF THE STUDY

Purpose

The purpose of the study is to evaluate whether perioperative administration of EPA-enriched supplement can preserve the patient body weight after total gastrectomy for gastric cancer.

Study Setting and Protocol Review

The study is an open-label, randomized Phase III clinical trial. The protocol has been approved by the Protocol Review Committee of Kanagawa Standard Anti-cancer Therapy Support System (non-profit organization KSATTS).

Resources

Research grants are from the KSATTS.

Endpoints

The primary endpoint is the body weight loss at 1 and 3 months after surgery (double primary endpoints). The secondary endpoints are the relative performance of the supplement, loss of lean body mass at 1 and 3 months after surgery, the lowest serum albumin level, the patient quality of life, the incidence of surgical morbidity and mortality, and the incidence of surgical site infections. The patient quality of life is evaluated by EORTC-QLQ C30 and STO22.

Eligibility Criteria

Tumors will be staged according to the 14th edition of the Japanese Gastric Cancer Classification (12). The inclusion criteria are as follows:

(i) Histologically proven adenocarcinoma of the stomach.
(ii) Clinical T2–T4a disease with no distant metastasis.
(iii) R0 resection is possible by open total gastrectomy.
(iv) Sufficient oral intake.
(v) Age ranging between 20 and 80 years.
(vi) ECOG performance status of 0–1.
(vii) No cancer of the remnant stomach.
(viii) Sufficient organ function: AST $\leq$100 IU/l, ALT $\leq$100 IU/l, total bilirubin $\leq$2.0 mg/dl, serum creatinine $\leq$1.5 mg/dl.
(ix) Written informed consent provided.

The exclusion criteria are as follows:

(i) Treatment with drugs including EPA (ethyl icosapentate).
(ii) Synchronous or metachronous cancer (synchronous multiple cancers in the stomach included).
(iii) Active inflammation requiring systemic treatment.
(iv) Hemorrhagic tendency.
(v) Lactose intolerance.
(vi) Allergic reaction to milk and soy beans.
(vii) Uncontrolled diabetes mellitus.
(viii) Systemic treatment with a corticosteroid.
(ix) Unstable angina or cardiac infarction within 6 months.
(x) Pulmonary disorder requiring oxygen.
(xi) Females with an on-going pregnancy or breast-feeding, or who are contemplating pregnancy.
(xii) Mental disorders which may affect the ability or willingness to provide informed consent or abide by the study protocol.

Registration

Participating investigators are instructed to send an eligibility criteria report to the Data Center at the non-profit organization KSATTS. Eligible patients will be registered and then randomized to one of the two groups described in the next section by a centralized dynamic method using the following factors: cT (T2/T3/T4a), C-reactive protein ($<$0.5 mg/dl/$\geq$),

albumin (<3.5 g/dl/≥), and institution as balancing variables. The accrual was started in October 2011 and is to continue for 3 years.

Treatment Methods

The patients enrolled in this study will receive treatment within 28 days. Group A will be given no nutritional supplementation perioperatively (standard diet). Group B will be given an EPA-enriched supplement (ProSure, Abbott Japan, Japan) in addition to their standard diet. This supplement includes 600 kcal with 2.2 g/day of EPA. The hospital diet will not be restricted. The amount of the supplements consumed will be counted. The supplement will be given from 7 days to 1 day before surgery. When patients undergo gastrectomy, regardless of whether it is a curative resection, the supplement will be given for 21 days when oral intake is initiated after surgery.

For both groups, patients will undergo total gastrectomy with Roux-en Y reconstruction. The extent of dissection will principally follow the third edition of the Gastric Cancer Treatment Guideline published by the Japanese Gastric Cancer Association (12). Spleen-preserving D2 total gastrectomy is permitted in this study. When patients are diagnosed with pathological Stage II or III disease, adjuvant chemotherapy using S-1 will be initiated within 6 weeks after surgery and will be administered for 1 year.

STUDY DESIGN AND STATISTICAL METHODS

The present study is a randomized Phase III trial to evaluate the preventive effect of an EPA-enriched supplement on body weight loss after total gastrectomy for gastric cancer. The primary endpoint is the % change in the body weight from baseline at 1 and 3 months after surgery (double primary endpoints). We judge that this treatment is effective when at least one of primary endpoints is confirmed. The power was computed with a Bonferroni correction (two sided $\alpha = 0.05/2 = 0.025$). Test treatment takes almost 20 000 Yen, but few adverse events are expected. We decided that 30% reduction in % body weight loss is necessary for this test treatment, considering the balance between the risk and benefit. From the retrospective data in our institution, we estimated % body weight loss as 8.5% at 1 month and 11.0% at 3 months in the control arm. Considering 30% risk reduction, % body weight loss was estimated as 6.0% at 1 month and 7.7% at 3 months in the test arm. Thus, we assumed that the expected difference in the % body weight loss between both arms would be 2.5% (SD 4.0%) and 3.3% (SD 5.5%) at 1 and 3 months, respectively. In this situation, the sample size required to ensure an at least 80% probability for both hypotheses was 110 patients, with 55 patients per arm. Considering the likelihood of enrolling ineligible patients, the number of patients to be accrued was set at 120 in total.

For the primary endpoints, arithmetic means and their 95% confidence intervals will be estimated and compared with Student's *t*-test. The relative performance of the supplement, loss of lean body at 1 and 3 months after surgery, and the lowest serum albumin level will also be analyzed in the same manner. With regard to the quality of life, a logistic regression analysis will be performed with a 10-point deterioration of the Global Health Status scale considered as an event. The incidence of surgical morbidity and mortality, and the incidence of surgical site infection will be calculated as proportions with exact confidence intervals, and compared with Fisher's exact test.

INTERIM ANALYSIS AND MONITORING

The Data and Safety Monitoring Committee (DSMC) will independently review the reports of trial monitoring regarding the efficacy and safety data from the present study. Based on the monitoring, the DSMC can consider early termination of a treatment regimen if the TRD exceeds 5% (3 patients) in each group during the enrollment. The protocol compliance, safety, and on-schedule study progress will also be monitored by the DSMC.

Funding

This work is supported, in part, by KSATTS.

Conflict of interest statement

None declared.

References

1. Pisani P, Parkin DM, Bray F, Ferlay J. Estimates of the worldwide mortality from 25 cancers in 1990. *Int J Cancer* 1999;83:18–29.
2. Sasako M. Principles of surgical treatment for curable gastric cancer. *J Clin Oncol* 2003;21:274s–5.
3. Noguchi YTA, Makino T, Fukuzawa K, Nomura K, Yoshikawa T, Xu HM, et al. Metabolic alterations in totally gastrectomised patients—caloric intake and energy consumption. *Asian J Surg* 1992;15:97–102.
4. Liedman B. Symptoms after total gastrectomy on food intake, body composition, bone metabolism, and quality of life in gastric cancer patients—is reconstruction with a reservoir worthwhile? *Nutrition* 1999;15:677–82.
5. Liedman B, Henningsson A, Mellstrom D, Lundell L. Changes in bone metabolism and body composition after total gastrectomy: results of a longitudinal study. *Dig Dis Sci* 2000;45:819–24.
6. Adachi S, Takiguchi S, Okada K, Yamamoto K, Yamasaki M, Miyata H, et al. Effects of ghrelin administration after total gastrectomy: a prospective, randomized, placebo-controlled phase II study. *Gastroenterology* 2010;138:1312–20.
7. Yamada T, Hayashi T, Cho H, Yoshikawa T, Taniguchi H, Fukushima R, et al. Usefulness of enhanced recovery after surgery protocol as compared with conventional perioperative care in gastric surgery. *Gastric Cancer* 2012;15:34–41.
8. Myrianthefs PM, Batistaki C. Cancer cachexia and immunomodulation. *J BUON* 2005;10:181–8.
9. Calder PC. N-3 polyunsaturated fatty acids and inflammation: from molecular biology to the clinic. *Lipids* 2003;38:343–52.

10. Mantovani G. Randomised phase III clinical trial of 5 different arms of treatment on 332 patients with cancer cachexia. *Eur Rev Med Pharmacol Sci* 2010;14:292–301.
11. Ryan AM, Reynolds JV, Healy L, Byrne M, Moore J, Brannelly N, et al. Enteral nutrition enriched with eicosapentaenoic acid (EPA) preserves lean body mass following esophageal cancer surgery: results of a double-blinded randomized controlled trial. *Ann Surg* 2009;249:355–63.
12. Japanese Gastric Cancer Association. Japanese gastric cancer treatment guidelines 2010 (ver. 3). *Gastric Cancer* 2011;14:113–23.