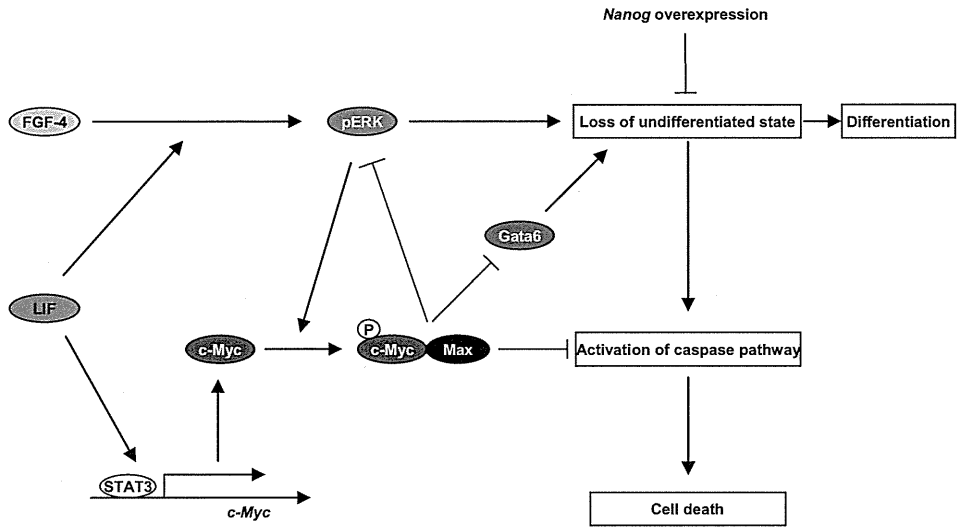

Figure 7. Proposed Model of the c-Myc/Max Complex Functions that Maintain the Pluripotent State of ESCs

c-Myc mRNA levels are transcriptionally boosted by LIF signaling through the JAK-STAT3 pathway, while the c-Myc protein is stabilized by pERK-mediated phosphorylation. The c-Myc/Max complex supports the self-renewal of pluripotent ESCs by inhibiting MAPK signaling that is activated by LIF and FGF and negatively affects the pluripotent state of ESCs. The complex also directly suppresses *Gata6* gene expression to prevent differentiation, demonstrated by Smith et al. (2010). Another role of c-Myc/Max is to support the viability of cells that have lost the undifferentiated state.

whether the requirement of c-Myc/Max is absolute for preserving the remarkable properties of ESCs in any culture condition.

Here, we report the establishment of *Max*-null ESCs and demonstrate that *Max* gene expression ablation in an empirical culture condition induces a loss of the undifferentiated state and caspase-dependent cell death in that order. This hierarchy possibly also occurs in cancer stem cells, as indicated by the fact that the *Max*-null condition in ESCs leads to impairment of the Myc module and ESC-like gene expression programs that are commonly activated in ESCs and cancer stem cells in a Myc-dependent manner (Kim et al., 2010; Wong et al., 2008). Therefore, we could speculate that our findings may lead to a new cancer therapy that eradicates cancer stem cells by impairing the Myc-dependent undifferentiated state. It will be important to determine whether this rule is restrictively enforced in *Max*-null ESCs or if this is the general death mechanism in ESCs. Although *Max*-null ESCs undergo extensive cell death, *c-Myc/N-Myc* dKO ESCs are viable, but these cells fail to preserve a pluripotent state. One explanation is that L-Myc might be responsible for the differences in phenotype and cell viability due to functional L-Myc being in dKO ESCs but not in *Max*-null ESCs. However, unlike c-MycRX (a mutant used in Figure 1I), the corresponding L-Myc mutant, L-MycRX, could not exhibit any noticeable effect on either pluripotency or cell viability of *Max*-null ESCs with MaxRX (data not shown). The pheochromocytoma cell line PC12 bears no functional Max protein because of a homozygous gene mutation. Notwithstanding, forced expression of c-Myc induces extensive cell death (Wert et al., 2001). Therefore, the same mechanism possibly operates in *Max*-null ESCs.

One of the important findings of our study is that the c-Myc/Max complex in ESCs suppresses ERK signaling as well as *Gata6* expression (Smith et al., 2010) to prevent ESC differentiation. MAPK phosphorylates c-Myc at Ser62, leading to the stabilization of the c-Myc protein (Lee et al., 2008; Sears et al., 2000). In agreement with the previous observation (Ying et al., 2008), our analyses revealed that PD0325901 treatment profoundly reduced c-Myc protein levels in ESCs (Figure 3G), indicating that MAPK-dependent stabilization of the c-Myc protein also occurs in ESCs. Based on our findings and previous reports from other groups (Cartwright et al., 2005; Smith et al., 2010; Ying et al., 2008), we propose a hierarchical model depicted in Figure 7. In this model, the *c-Myc* gene is induced by LIF via STAT3 signaling and the resulting c-Myc protein is stabilized via MAPK signaling activated by FGF and/or LIF. The stabilized c-Myc protein then suppresses MAPK signaling to prevent ESC differentiation, although the molecular basis of this suppression is not currently known.

Finally, our data demonstrate that the c-Myc/Max complex is dispensable for preserving the ESC status when exposed to the 2i condition. This finding is somewhat surprising because the c-Myc/Max complex, like Oct3/4, is widely regarded as a crucial regulator for sustaining the ESC status (Cartwright et al., 2005; Hu et al., 2009; Singh and Dalton, 2009; Smith et al., 2010; Varlakhanova et al., 2010). We found that, like the 2i condition, forced *Nanog* expression also conferred unlimited ESC self-renewal in the absence of Myc/Max complexes. However, there is a significant difference between these two rescues, with *Nanog* expression boosting Myc module activity and Core module activity to the normal levels, while the 2i condition rescued *Max*-null ESCs without significantly affecting Myc module activity.

Empirical culture medium containing LIF and serum to sustain the ESC status is only effective with a few specific inbred mouse strains such as the 129 line, but not for rat and most other mouse ESC lines (Buehr et al., 2008; Hanna et al., 2009; Li et al., 2008). However, the application of 2i or related conditions appears to be much broader than conventional culture condition with LIF

and serum. Indeed, it has been reported that self-renewal and pluripotency can be permanently maintained in cells from rats and nonobese diabetic mice (Buehr et al., 2008; Hanna et al., 2009; Li et al., 2008). Therefore, our data raises an interesting possibility that a wider application of 2i or related conditions compared with empirical culture conditions may be attributable to the independence of Myc/Max transcriptional complexes to preserve the ESC status.

EXPERIMENTAL PROCEDURES

Plasmid Constructions

For tet-off expression of the *Max* gene, *Max* cDNA was subcloned into the XhoI/NotI sites of the exchange vector pZhCSfi (Masui et al., 2005). For pCAG-IH-HA-cMycWT, pCAG-IH-HA-c-MycEG, pCAG-IH-HA-c-MycRX, pCAG-IP-FLAG-MaxWT, pCAG-IP-FLAG-MaxEG, and pCAG-IP-FLAG-MaxRX, the ORFs for the wild-type, EG mutant, and RX mutant of c-Myc and Max were PCR amplified from the corresponding cDNA-containing plasmids provided by Dr. Bruno Amati. For pCAG-IP-NanogWT and pCAG-IP-KLF4, PCR-amplified ORFs for Nanog and KLF4 by means of total RNA from mouse ESCs were sequenced and subcloned into pCAG-IP (Niwa et al., 2002) or pCAG-IH, which conferred puromycin or hygromycin resistance, respectively. Mouse Nanog^{D67G} mutagenesis was performed with Pfu and DpnI.

A targeting vector for the *Max* gene was designed to replace exons 3, 4, and 5 that encode from the 21st amino acid onward and contains the entire basic DNA-binding region and HLH-Z domain, with a β-galactosidase/neomycin-resistance gene (β-geo). To construct a targeting vector for the *Max* gene, the 5′ and 3′ homologous arms were PCR amplified with BAC DNA carrying the entire *Max* gene (RP23-246K11) as a template. These homologous arms were introduced into a Bluescript-based vector containing a negative selection marker, diphtheria toxin A subunit (DTA), together with the β-geo reporter gene.

ESC Culture and Transfection

EBRTcH3 ESCs (Masui et al., 2005) and their derivatives were cultured via standard medium containing fetal bovine serum (FBS) and LIF unless indicated otherwise. Expression plasmids were transfected into ESCs with a Nucleofector (Lonza). The expression plasmids (5 μg) were introduced with the A-23 Nucleofector program.

Cell Cycle Analysis

BrdU labeling was performed with anti-BrdU according to the manufacturer's instructions. The labeled cells were stained with propidium iodide (PI) and analyzed on a FACS Calibur flow cytometer with doublet discrimination. The data were analyzed with CellQuest software from Becton Dickinson.

Detection of Intracellular ROS

ROS levels were determined by incubating cells with 10 μM DCF-DA for 30 min at 37°C in the presence of 20 μM verapamil. The cells were washed twice in PBS and then trypsinized. Fluorescence was measured with the flow cytometer.

Blastocyst Injection

2i/Nam-rescued *Max*-null ESCs bearing Dox-regulatable *Max* cDNA and those lacking the cDNA were labeled with fluorescent Kusabira-Orange protein. These labeled cells were injected into blastocysts and transferred into surrogate C57BL/6 mice. Embryos at ~E6.5 and E9.5 were recovered and inspected by microscopy.

Microarray Analysis

Biotin-labeled cRNA synthesized according to the Affymetrix guidelines were hybridized to Affymetrix GeneChip Mouse Genome 430 2.0 arrays. For scanning and intensity data analyses, see Supplemental Experimental Procedures.

ACCESSION NUMBERS

DNA microarray data are deposited in NCBI's Gene Expression Omnibus under accession number GSE27881.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and four tables and can be found with this article online at doi:10.1016/j.stem.2011.04.020.

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A Randomized Phase II Trial to Test the Efficacy of Intra-peritoneal Paclitaxel for Gastric Cancer with High Risk for the Peritoneal Metastasis (INPACT Trial)

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Owing to its peculiar pharmacological characteristics, paclitaxel attains substantial intra-peritoneal concentration for a prolonged period when delivered intra-peritoneally, and is active against peritoneal metastasis of ovarian cancer. It is also considered promising against disseminated gastric cancer. However, the fact that the intra-peritoneal paclitaxel has not been approved in Japan has rendered its evaluation by a formal clinical trial impossible. The authors designed a randomized phase II trial using the Kodo Iryo Hyoka system, a new system to legally test an yet unapproved mode of treatment. It is hoped that this trial will result in a breakthrough in the treatment of peritoneal carcinomatosis from gastric cancer.

Key words: paclitaxel – clinical trial – gastric cancer

TRIAL BACKGROUNDS AND RATIONALE

Curatively resected gastric cancer patients often suffer from recurrence as peritoneal carcinomatosis. This could be caused by cancer cells that had already been shed from the serosal surface at the time of surgery, sometimes detectable by examining the peritoneal washes, or those that were disseminated during surgical procedures. In addition to extensive irrigation of the peritoneal cavity (1), intra-peritoneal (IP) instillation of effective anticancer drugs could eliminate these cells to the extent that the recurrences could be prevented. Repeated IP administration of paclitaxel (PTX) has been shown to be safe and effective for disseminated ovarian cancer, another cancer type where peritoneal disease often turns out to be a major cause for disease failure (2). Since its efficacy when administered intravenously (DIV) against gastric cancer has been proved (3) and its potential advantage when given intra-peritoneally has been robustly shown pharmacologically (4,5), IP PTX has been considered promising also to eliminate peritoneal metastasis from gastric cancer.

Formal clinical trials to prove the efficacy of this approach have been hindered by the fact that the IP administration of PTX has not been approved by the Ministry of Health, Labour and Welfare in Japan. When using such drugs outside of the medical insurance system, all other expenses such as the cost of medical services at the outpatient clinic, including drugs such as steroids, H2 blockers and anti-emetics will have to be covered also by the individual researcher or the patient. The authors attempted to overcome this problem by making an official request to conduct a multi-institutional trial by using a system known as the 'Kodo Iryo Hyoka' system. Using this system, unapproved or experimental medical practice whose cost is covered by the individuals can be delivered simultaneously with general medical procedures that are covered by the insurance. To use this system, the study protocol will have to be scrutinized and approved by a committee appointed by the Ministry. Furthermore, a trial thus performed is expected to be designed so as to generate an evidence for future approval of

the treatment by the Ministry. A one-arm single-institutional phase II trial to confirm the efficacy of a regimen that includes IP PTX (6) has already been approved and is ongoing using the 'Kodo Iryo Hyoka' system. To add further evidence in support of the IP treatment and to ultimately establish a basis for the future approval by the Ministry, a head-to-head comparison of IP and DIV of the same drug under the same schedule was considered mandatory. Since the patients so allocated will then have to be treated by IP PTX alone for a fixed period of time, patients who are deemed eligible for the trial had to have a significant risk to develop peritoneal carcinomatosis, while harbouring no gross lesions that immediately call for systemic administration of the anticancer drugs.

The authors held a few meetings to finally compile a protocol for a clinical trial to evaluate IP PTX, as described in the following section. The study is called INPACT, in which INPACT is an abbreviation for 'IP administration of chemotherapeutic agent'.

PROTOCOL DIGEST OF THE STUDY

Purpose

The purpose of this study is to show a prognostic impact of repeated IP of PTX over the DIV on the identical treatment schedule, among patients who are considered to have a high risk of developing peritoneal carcinomatosis. In the event of detecting a survival advantage, this study should be one of valuable evidence based on which to request the Ministry of Health, Labour and Welfare for approval of the IP administration. The establishment of various combinations incorporating IP PTX to combat all types of metastatic gastric cancer and a subsequent randomized trial to prove their survival benefits would then be expected.

Resources

Data centre services and statistical supervision are funded by a non-profit organization, the Epidemiological and Clinical Research Information Network (ECRIN), Kyoto, Japan. All treatments with the exception of PTX-administered IP have been approved as a general practice within the scope of general medical insurance. IP administration of PTX has been approved by the Ministry of Health, Labour and Welfare as of July 2010, exclusively for the participants of this trial, using the Kodo Iryo Hyoka system. Bristol- Myers Squibb has kindly agreed to supply PTX to be given intra-peritoneally.

Endpoints

The primary endpoint is the 2-year overall survival (OS) rate. The secondary endpoints are the incidence of adverse events, progression-free survival time, and OS time.

Eligibility for Participating in the Trial

Approval of the protocol by the institutional review board is a prerequisite to participate in the trial. In addition, each participating institution is requested to fill in and send an application form to the Ministry of Health, Labour and Welfare via Nagoya University to obtain final approval by the government to join the Kodo Iryo Hyoka system.

Eligibility Criteria for the Enrollment

Inclusion criteria for primary registration:

(i) Histologically confirmed adenocarcinoma of the stomach.
(ii) Either macroscopically defined as Type 3 with a diameter >8 cm or Type 4 (linitis plastica), or defined as the other macroscopic type, but is considered highly suspicious for serosal invasion or peritoneal seeding.
(iii) Patients without the following findings on computerized tomography: cervical or mediastinal lymphadenopathy, bulky metastasis to suprapancreatic or retroperitoneal lymph nodes, distant organ metastasis, thoracic effusion, ascites spreading beyond the pelvic cavity.
(iv) No previous history of chemotherapy or radiation.
(v) Eastern Cooperative Oncology Group performance status of 0 or 1.
(vi) Age ≥ 20.
(vii) Adequate organ function is defined as follows: a white blood cell count of 3000–12 000/m^3, neutrophil count of $>1500/m^3$, platelet count of $>100\,000/m^3$, AST and ALT ≤ 100 IU/l, total bilirubin ≤ 1.5, serum creatinin level ≤ 1.5 mg/dl, serum albumin level ≥ 3.0 g/dl.
(viii) Surgery planned within 1 month of registration.
(ix) Written informed consent.

Exclusion criteria for primary registration:

(i) Serious comorbidities include the following:
  (a) Ischemic heart disease and arrhythmia needing treatment.
  (b) Myocardial infarction within 6 months of onset.
  (c) Liver cirrhosis.
  (d) Interstitial pneumonitis.
  (e) Gastrointestinal bleeding in need of repeated blood transfusion.
  (f) Uncontrolled diabetes mellitus.
(ii) Bowel obstruction rendering treatment with oral drugs impractical.
(iii) Active synchronous cancer or disease-free metachronous cancer within 5 years of onset.
(iv) Signs of acute infection or inflammatory disease
(v) Systemic treatment with corticosteroids
(vi) Hypersensitivity to Cremophor EL.

(vii) Women who are pregnant, contemplating pregnancy or amid breast-feeding.
(viii) Mental disorders which may affect ability or willingness to provide informed consent.
(ix) History of severe hypersensitivity to any drugs.
(x) History of alcoholic anaphylaxis.
(xi) Peripheral neuropathy.
(xii) Patients otherwise considered inappropriate for inclusion in the study.

Inclusion criteria for secondary registration:

(i) Considered resectable either at laparotomy or laparoscopy.
(ii) If the macroscopic type was not Type 3 with a diameter >8 cm or Type 4 (linitis plastica), peritoneal seeding or positive cytology of the peritoneal washes need to be confirmed during surgery.
(iii) Placement of the IP reservoir is possible.

Registration

Participating investigators are instructed to send an eligibility criteria report to the data centre at the non-profit organization ECRIN for the primary registration within 1 month of the scheduled surgery. Investigators are then requested to proceed to the secondary registration by telephone upon laparotomy or laparoscopy, when the eligibility criteria such as resectability, peritoneal metastasis and peritoneal washing cytology findings were confirmed. Patients are randomized during surgery to one of the two treatment groups by a centralized dynamic method using the following factors as balancing variables: macroscopical Type (Types 3 and 4/others), curability of surgery (R0 and R1/R2), age (<75 years/≥75 years) and institution. Follow-up data including compliance to the treatment, adverse reactions and survival are to be reported to the data centre through clinical report forms.

The first 10 cases are to receive the IP PTX exclusively as a feasibility test, which will be evaluated only for toxicity and will be not included in the survival analysis. If more than four successful IP deliveries are conducted in less than 5 of the 10 patients, the study will either be terminated or modified appropriately.

The study has been registered in the University hospital Medical Information Network (UMIN) as No. 000002957.

Treatment Methods

Patients enrolled in this study are randomized to receive one of the following regimens of chemotherapy after gastrectomy.

Group A: IP administration group:

PTX: 60 mg/m2 IP on the day of surgery (day 1) and on days 15, 22, 29, 43, 50 and 57. The dose of IP PTX is based on a phase I trial performed in the USA for ovarian cancer patients, and its safety when given weekly has been confirmed by a phase II trial (2).

Group B: Intravenous administration group:

PTX: 80 mg/m^2 DIV on the day of surgery (day 1) and on days 15, 22, 29, 43, 50, and 57.

These regimens of treatment are to be followed after 2–3 weeks by a standard systemic chemotherapy for advanced gastric cancer which, at the time the trial started, would be either S-1 monotherapy or a combination of S-1 and cisplatin (CDDP) (7). S-1 is generally recommended after R0/R1 resection and S-1/CDDP after R2 resection, but the selection is left to the discretion of the physician in charge. When patients randomized into Group A failed to receive IP chemotherapy for reasons other than allergic reaction to PTX, they are expected to continue with intravenous PTX according to the predetermined schedule, so that the subsequent systemic chemotherapy will be started at the same time as in other patients.

Study Design and Statistical Methods

The current study is a randomized phase II trial applying selection design as proposed by Simon et al. with selection probability of around 80% (8). The primary analysis in this study is aimed to select an appropriate treatment arm for further evaluation, and the sample size was calculated on the hypothesis that the 2-year OS rate of the DIV arm, estimated to be 30–40%, could be improved by 10% in the IP arm. The selection probability is estimated to be 82–83% when a total sample size is 80 and 84–85% when a sample size is 100. Since the first 10 cases will be treated by IP therapy as a feasibility phase and will be excluded from the survival analysis, the total sample size will be 90–110 and 50–60 patients will receive IP therapy.

Interim Analysis and Monitoring

The Data and Safety Monitoring Committee (DSMC) independently review the report of trial monitoring regarding efficacy and safety data. The first interim analysis will be performed at 1 year after registration of the last patient and DSMC will decide whether or not to publish the results based on futility analysis and safety data.

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Conflict of interest statement

Dr Mitsuru Sasako received lecture fee and donation for promotion of education and research from Taiho Pharmaceutical Co., Ltd.

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Clinical Significance of Circulating Tumor Cells, Including Cancer Stem-Like Cells, in Peripheral Blood for Recurrence and Prognosis in Patients With Dukes' Stage B and C Colorectal Cancer

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See accompanying editorial on page 1508 and article on page 1556

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Terms in blue are defined in the glossary, found at the end of this article and online at www.jco.org.

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ABSTRACT

Purpose
Using multiple genetic markers, including cancer stem-like cells, we evaluated the clinical significance of circulating tumor cells (CTCs) as a prognostic factor for overall survival (OS) and disease-free survival (DFS) in the peripheral blood (PB) of patients with colorectal cancer (CRC) who had undergone curative surgery.

Patients and Methods
In a multi-institutional study, 735 patients with CRC were assigned to a retrospective training set (n = 420) or prospective validation set (n = 315). CTCs that expressed carcinoembryonic antigen (CEA), cytokeratin (CK) 19, CK20, and/or CD133 (CEA/CK/CD133) mRNA in PB were detected using real-time reverse transcription polymerase chain reaction assay.

Results
In the training sets, OS and DFS of patients who were positive for CEA/CK/CD133 were significantly worse than those of patients who were negative for these markers ($P < .001$). At each staging analysis, OS and DFS of patients with Dukes' stage B or C cancer who were positive for CEA/CK/CD133 were significantly worse than those of patients who were negative for these markers ($P < .003$ and $P < .001$ in Dukes' stage B; $P < .001$ in Dukes' stage C). In contrast, in patients with Dukes' stage A, no significant differences were seen between patients who were positive for these markers and those who were negative. Cox multivariate analysis demonstrated that CEA/CK/CD133 was a significant prognostic factor for OS (hazard ratio [HR], 3.84; 95% CI, 2.41 to 6.22; $P < .001$) and DFS (HR, 3.02; 95% CI, 1.83 to 5.00; $P < .001$). In particular, in patients with Dukes' stage B and C cancer, CEA/CK/CD133 demonstrated significant prognostic value. In validation sets, similar results were confirmed in patients with Dukes' stage B and C cancer.

Conclusion
In patients with Dukes' stage B and C CRC who require adjuvant chemotherapy, detection of CEA/CK/CD133 mRNA in PB is a useful tool for determining which patients are at high risk for recurrence and poor prognosis.

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INTRODUCTION

Despite advances in therapeutic approaches for patients with colorectal cancer (CRC), approximately 20% to 45% of those who undergo curative resection subsequently develop local tumor recurrence or metastasis at distant sites.[1] It is known that approximately 30% of the metastasis in CRC is liver metastasis.[2] To improve the prognosis, adjuvant chemotherapy such as fluorouracil (FU) plus leucovorin (LV; FU/LV) and uracil/tegafur plus LV have been established as generalized regimens, and FU/LV plus irinotecan and FU/LV plus oxaliplatin combined with molecular-targeted compounds such as bevacizumab and cetuximab have been approved for the treatment of patients with advanced-stage CRC.[3] Although these combination therapies involving chemotherapy and molecular-targeted agents have demonstrated survival benefits, the substantial financial cost of these treatments remains a serious problem.[4] Furthermore, chemotherapy without certain selection tends to lead to

overtreatment of patients with toxic agents that exert severe adverse effects. To facilitate individually tailored treatment for CRC, useful biomarkers for the determination of patients who are at high risk for recurrence and/or poor prognosis are required. In particular, markers for the determination of patients with Dukes' stage B CRC who are at high risk are desirable because the role of adjuvant chemotherapy in these patients remains controversial.[5]

Many investigators[6-9] have shown the utility of circulating tumor cells (CTCs) in predicting the clinical outcome of patients with various solid cancers. The usefulness of CTCs as a predictive tool for patients with metastatic breast cancer and those with CRC has been reported using the CellSearch system (Veridex, Raritan, NJ).[10,11] However, the prognostic relevance of CTCs in patients with early-stage disease without metastasis remains unclear.

Recently, a new concept relating to cancer stem cells has arisen and is expected to lead to the development of novel diagnosis and therapeutic procedures.[12] Stem cell markers are frequently overexpressed in the CTCs of patients with metastatic breast cancer, which suggests that metastasis may be evoked by the subpopulation of CTCs that have the cancer stem cells' marker.[13] Two interesting studies have demonstrated that CD133-positive cells in CRC have high tumorigenic ability in nude mice, and it is thought that CD133 is one of the key markers of cancer stem cells in CRC.[14,15] Furthermore, it was reported that cancer stem cells are often characterized by downregulation of epithelial markers, including the epithelial cell adhesion molecule (EpCAM) and cytokeratin.[16] In this study, we hypothesized that multimarkers, including the phenotypes of cancer stem-like cells (CD133), may be useful for detecting aggressive CTCs. However, large-scale multicenter studies that use these markers are still required.

Our study was a multicenter validation study that focused on clarifying the prognostic value of CTCs in the PB of patients with CRC who were treated with curative surgery, on the basis of real-time reverse transcription polymerase chain reaction (RT-PCR) using multimarkers that included cancer stem-like cells.

PATIENTS AND METHODS

Study Design

This study was undertaken at four clinical institutions in Japan (The National Cancer Center, Tokyo; Kyushu University, Oita; Kumamoto University, Kumamoto; and Teikyo University, Tokyo). The study protocol conformed to the guidelines of the ethics committee of each institution and was approved by each institution's review board; all patients provided written informed consent. A total of 735 patients with CRC who were undergoing curative surgery at these hospitals between January 2000 and December 2008 were enrolled. The diagnosis of CRC was confirmed in all patients by endoscopic biopsy, and the primary tumor stage was confirmed by histologic examination of the resected primary tumor. Overall survival (OS) was defined as the time between the date of diagnosis and the date of death. Disease-free survival (DFS) was defined as the length of time after surgical treatment for the cancer during which the patient survived with no sign of the cancer. The study consisted of a sequential retrospective training set and a prospective validation set. The first 420 patients were enrolled as a retrospective training set between January 2000 and December 2004. The cutoff values of carcinoembryonic antigen (CEA), cytokeratin 19 (CK19), cytokeratin 20 (CK20), and CD133 mRNA were determined from the training set by analyzing receiver-operating characteristic curves on the basis of mRNA levels of these markers and clinical follow-up data. The ability of these genetic markers to predict recurrence and prognosis on the basis of the predetermined cutoff values was then examined in the prospective validation set. Between January 2005 and December 2008, 315 patients were enrolled in this set. Disease status was assessed without knowledge of the CTC levels. Patients with Dukes' stage A and B cancer were not treated with chemotherapy. Patients with Dukes' stage C cancer were treated with a standard Roswell Park Memorial Institute 1640 regimen that involved administration of FU/LV for 6 months. As a follow-up, development of local recurrence, distant metastases, and death were re-evaluated in all patients at 3-month intervals during the first year and at 6-month intervals thereafter.

Blood Sampling and Quantitative Real-Time RT-PCR

PB samples were obtained at the presurgery stage before chemotherapy was begun. Ten-milliliter blood samples were collected and stored at −80°C. Extraction of total RNA and cDNA synthesis was performed according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). The expression of CEA, CK19, CK20, and CD133 mRNA of blood samples was examined; target genes of CTCs and glyceraldehydes-3-phosphate-dehydrogenase (GAPDH) mRNA were used as internal control genes. The expression levels of these mRNA were measured by real-time quantitative RT-PCR using the LightCycler instrument (Roche Diagnostics, Mannheim, Germany), as described previously.[9,17] All samples were measured in duplicate. The levels of CEA, CK19, CK20, and CD133 mRNA were normalized by GAPDH mRNA.

Statistical Analysis

The sample size was calculated by SAS statistical software (V 9; SAS Institute, Cary, NC) on the basis of the results of a small-scale test (n = 50) under which the one-sided P value of .025 would have 80% power to detect a difference between the DFS and OS curves of patients with and without CTC.

The cutoff values of markers were determined by receiver-operating characteristic curves that were constructed by plotting all possible sensitivity/1 − specificity pairs in the training sets. Sensitivities of genetic markers were calculated as a ratio of the number of patients with PCR and hematogenous metastasis relative to the number of patients who developed hematogenous metastasis. Specificities were calculated as the ratio of the number of patients without PCR and hematogenous metastasis relative to the number of all patients without hematogenous metastasis. The relationships between OS and DFS and genetic makers were analyzed by Kaplan-Meier survival curves and the log-rank test. Cox proportional hazards regression was used to determine univariate and multivariate hazard ratios for OS and DFS. The comparison of clinicopathologic factors in the training and validation sets were analyzed using the t test, χ^2 tests, and analysis of variance. All P values were two sided, and P values of less than .05 were considered statistically significant. Data were analyzed using JMP software (V 7; SAS Institute).

RESULTS

Clinicopathologic Characteristics and Genetic Markers

In the comparison of clinicopathologic characteristics of the patients in the training and validation sets, there were no significant differences found in tumor size, depth of invasion, lymphatic invasion, venous invasion, lymph node metastasis, histologic type, and tumor stage (Table 1). In the training set, cutoff values for each marker were predetermined (5.2×10^{-6} as CEA/GAPDH; 8.7×10^{-5} as CK19/GAPDH; 1.9×10^{-5} as CK20/GAPDH; and 3.4×10^{-4} as CD133/GAPDH), and the positive rates, sensitivities, and specificities of various combinations of genetic markers were examined (data not shown). In the single-marker groups, CEA showed the highest positive rates and highest sensitivity for hematogenous metastasis. In contrast, CD133 showed the highest specificity for hematogenous metastasis. In the multimarker groups, CEA^+, $CK19^+$, $CK20^+$, and/or $CD133^+$ (CEA/CK/CD133) showed the highest positivity, sensitivity, and specificity. Therefore, the CEA/CK/CD133 group was selected as representative of PCR positivity and used for the prognostic analysis. Between the training and validation sets, there were no significant differences in positive rates, sensitivity, and specificity of CEA/CK/CD133 (Table 1).

Table 1. Patient Characteristics in Training and Validation Sets

Characteristic	No. of Patients	Training Set		Validation Set		*P*
		No.	%	No.	%	
Total No. of patients	735	420		315		
Age (mean ± SD)		66.0 ± 12.4		67.5 ± 11.8		.524
Sex						
Women	336	196	46.67	140	44.44	.550
Men	399	224	53.33	175	55.56	
Tumor size						
< 5	440	252	60.00	188	59.68	.940
≥ 5	295	168	40.00	127	40.32	
Depth of invasion						
≤ pT2	188	107	25.48	81	25.71	.942
≥ pT3	547	313	74.52	234	74.29	
Lymphatic invasion						
Negative	511	295	70.24	216	68.57	.628
Positive	224	125	29.76	99	31.43	
Venous invasion						
Negative	387	217	51.67	170	53.97	.536
Positive	348	203	48.33	145	46.03	
Lymph node metastasis						
Negative	488	270	64.29	218	69.21	.162
Positive	247	150	35.71	97	30.79	
Histologic type						
Well	590	328	78.10	262	83.17	.086
Nonwell	145	92	21.90	53	16.83	
Dukes' stage						
A	169	94	22.01	75	24.35	.101
B	319	176	41.22	143	46.43	
C	247	150	35.13	97	31.49	
CEA/CK/CD133						
Positive rates*		106 of 427	25.24	75 of 308	23.81	.143
Sensitivity		55 of 67	82.09	34 of 46	73.91	.352
Specificity		302 of 353	85.55	228 of 269	84.76	.147

Abbreviations: SD, standard deviation; CEA, carcinoembryonic antigen; CK, cytokeratin.
*Ratio of number of CEA/CK19/CK20/CD133–positive patients (according to polymerase chain reaction) to number of all patients.

Kaplan-Meier OS and DFS Curve Analysis

In the training set, the average follow-up time for OS was 36.9 ± 19.3 months (95% CI, 35.0 to 38.8 months) and that of DFS was 32.7 ± 20.2 months (95% CI, 30.7 to 34.6 months). In the validation set, the average follow-up time was 37.1 ± 18.1 months (95% CI, 35.0 to 39.2 months) for OS and 33.4 ± 19.1 months (95% CI, 31.2 to 35.4 months) for DFS. In the training set, all patients with CEA/CK/CD133 positivity showed significantly worse OS and DFS than those with PCR negativity. The same results were found in the validation set (data not shown).

Figure 1 shows the OS and DFS at each tumor stage according to the PCR status in the training set. In patients with Dukes' stage A cancer, there were no significant differences in OS and DFS in the patients with CEA/CK/CD133 positivity as compared with those with negativity (data not shown). In all patients with Dukes' stage B cancer, the OS and DFS of those with CEA/CK/CD133 positivity were significantly worse than those of patients who were negative for these markers (Figs 1A and 1B). Furthermore, when patients with Dukes' stage B and CEA/CK/CD133 positivity who were considered to be at high risk were separated out on the basis of factors such as perforation or obstruction and/or lymphatic invasion, they demonstrated significantly worse OS and DFS than patients who were negative for these markers (Figs 1C and 1D). In patients with Dukes' stage C cancer, the OS and DFS of the group with CEA/CK/CD133 positivity were significantly worse than those of patients who were negative for these markers (Figs 1E and 1F). In the validation set, similar results were demonstrated for patients with Dukes' stage A (data not shown), B, and C (Figs 2A to 2F). These results suggest that the expression of CEA/CK/CD133 is associated with recurrence and poor prognosis in patients with Dukes' stage B and C cancer.

To clarify the clinical significance of CD133 addition in patients with Dukes' stage B and C cancer, we analyzed the OS and DFS in the general CTC markers (CEA, CK19, and/or CK20: CEA/CK), and in the CD133 single marker. In patients with Dukes' stage B cancer, no significant differences in OS and DFS were found between those who were positive for CEA/CK and those who were negative for CEA/CK, nor were significant differences seen between patients who were positive for CD133 as compared with those who were negative for CD133 (Data Supplement). In contrast, in patients with Dukes' stage C, significant differences were found in OS and DFS between those who were positive for CEA/CK and those who were negative for CEA/CK in both the training and the validation sets (Data Supplement). However, in the CD133 single-marker analysis, no significant differences in OS and DFS were found between the patients who were positive for

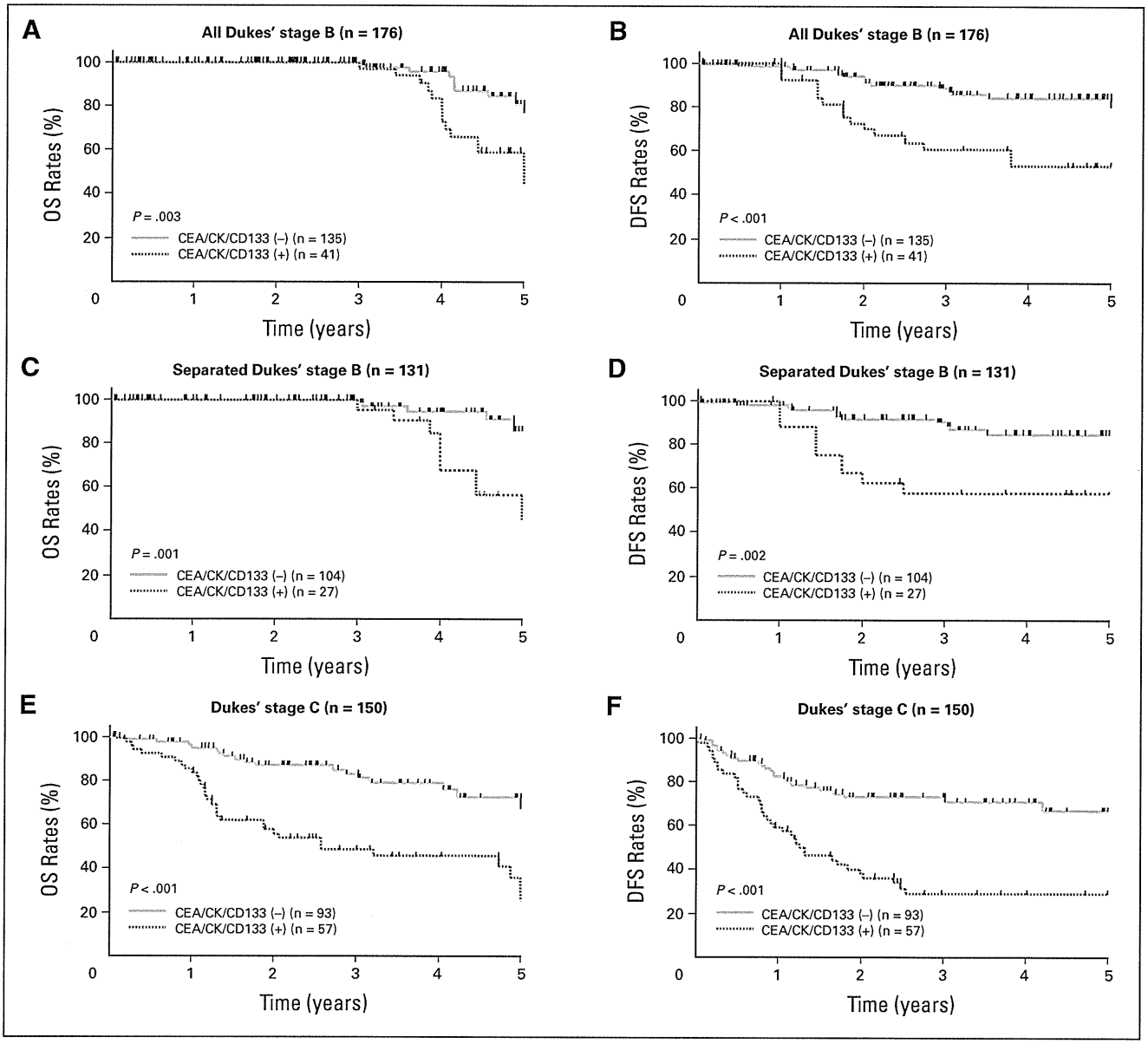

Fig 1. Kaplan-Meier survival curves for overall survival (OS) and disease-free survival (DFS) according to the CEA/CK/CD133 status in the training set. Patients with Dukes' stage B were separated into two groups as follows: all patients with Dukes' stage B and patients who were separated out on the basis of perforation or obstruction and/or lymphatic invasion (separated patients with Dukes' stage B). (A, B) OS and DFS of all patients with Dukes' stage B, (C, D) separated patients with Dukes' stage B, and (E, F) patients with Dukes' stage C were analyzed in the training set according to CEA/CK/CD133 mRNA levels.

CD133 and those who were negative for CD133 in either set. These results suggest that the addition of CD133 to general CTC markers is important for the determination of patients who are at high risk for recurrence and poor prognosis in Dukes' stage B cancer.

OS and DFS in Patients With Colon and Rectal Cancer

In patients with colon cancer, groups positive for CEA/CK/CD133 showed a significantly worse OS and DFS than those negative for CEA/CK/CD133 in the training and validation sets (Figs 3A, 3B, 3E, and 3F). In patients with rectal cancer, similar results were demonstrated in the training and validation sets (Figs 3C, 3D, 3G, and 3H). These results suggest that the prognostic value of CEA/CK/CD133 is the same in both colon and rectal cancer.

Univariate and Multivariate Cox Analysis

Table 2 shows the results of multivariate Cox analysis of various factors for OS and DFS in all of the patients. Multivariate analysis was performed for factors that showed significance in univariate analysis. In the training set, CEA/CK/CD133 and Dukes' stage showed significance for OS, and CEA/CK/CD133, Dukes' stage, and serum CEA protein showed significance for DFS. In the multivariate analysis of the validation set, CEA/CK/CD133 and Dukes' stage showed significance

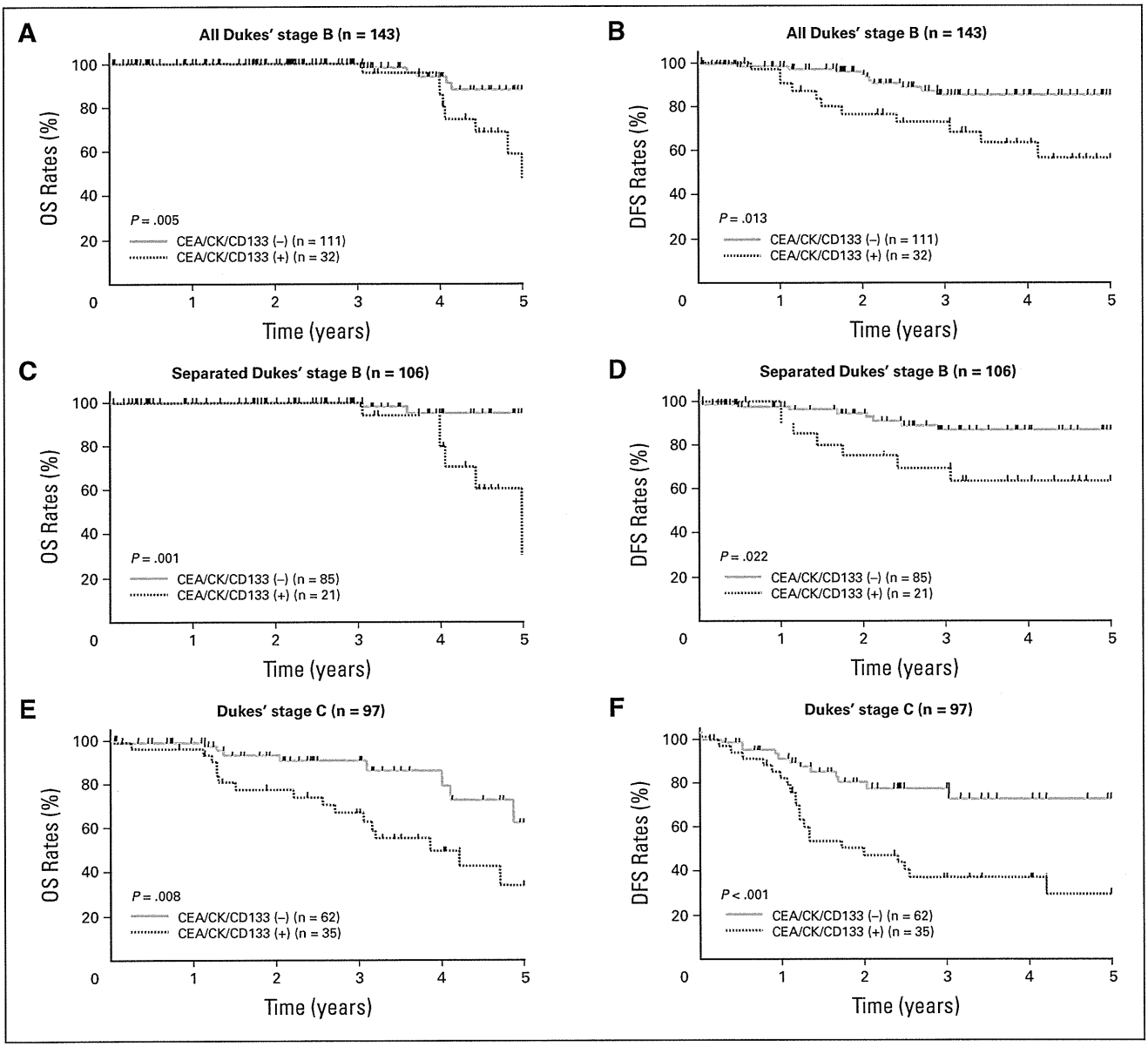

Fig 2. Kaplan-Meier survival curves of overall survival (OS) and disease-free survival (DFS) on the basis of the CEA/CK/CD133 status in the validation set. (A, B) OS and DFS of all patients with Dukes' stage B, (C, D) patients with Dukes' stage B who were separated out on the basis of factors such as perforation or obstruction and/or lymphatic invasion, and (E, F) patients with Dukes' stage C were analyzed in the validation set according to CEA/CK/CD133 mRNA levels.

for OS, and CEA/CK/CD133, Dukes' stage, and serum CEA showed significance for DFS.

Table 3 shows the results of multivariate Cox analysis of various factors for OS and DFS in the patients with Dukes' stage B and C cancer. In the analysis of all patients with Dukes' stage B and patients with Dukes' stage B who were separated out on the basis of perforation or obstruction and/or lymphatic invasion, the CEA/CK/CD133 showed significance for OS and DFS in the training and validation sets. In patients with Dukes' stage C cancer, CEA/CK/CD133 levels showed significance for OS, and CEA/CK/CD133 and serum CEA levels showed significance for DFS in the multivariate analysis of the training set. In the validation set, similar results were demonstrated. These results suggest that CEA/CK/CD133 is a significant predictor for poor prognosis and recurrence in patients with Dukes' stage B and C.

DISCUSSION

Our multi-institutional validation study demonstrates that the detection of CEA/CK/CD133 mRNA in PB samples has prognostic value in patients with Dukes' stage B and C colorectal cancer.

Although many assays have been developed to detect CTCs, the two main approaches that are used involve either cytology that is based on immunocytochemical staining or PCR-based analysis.[18-21] Using

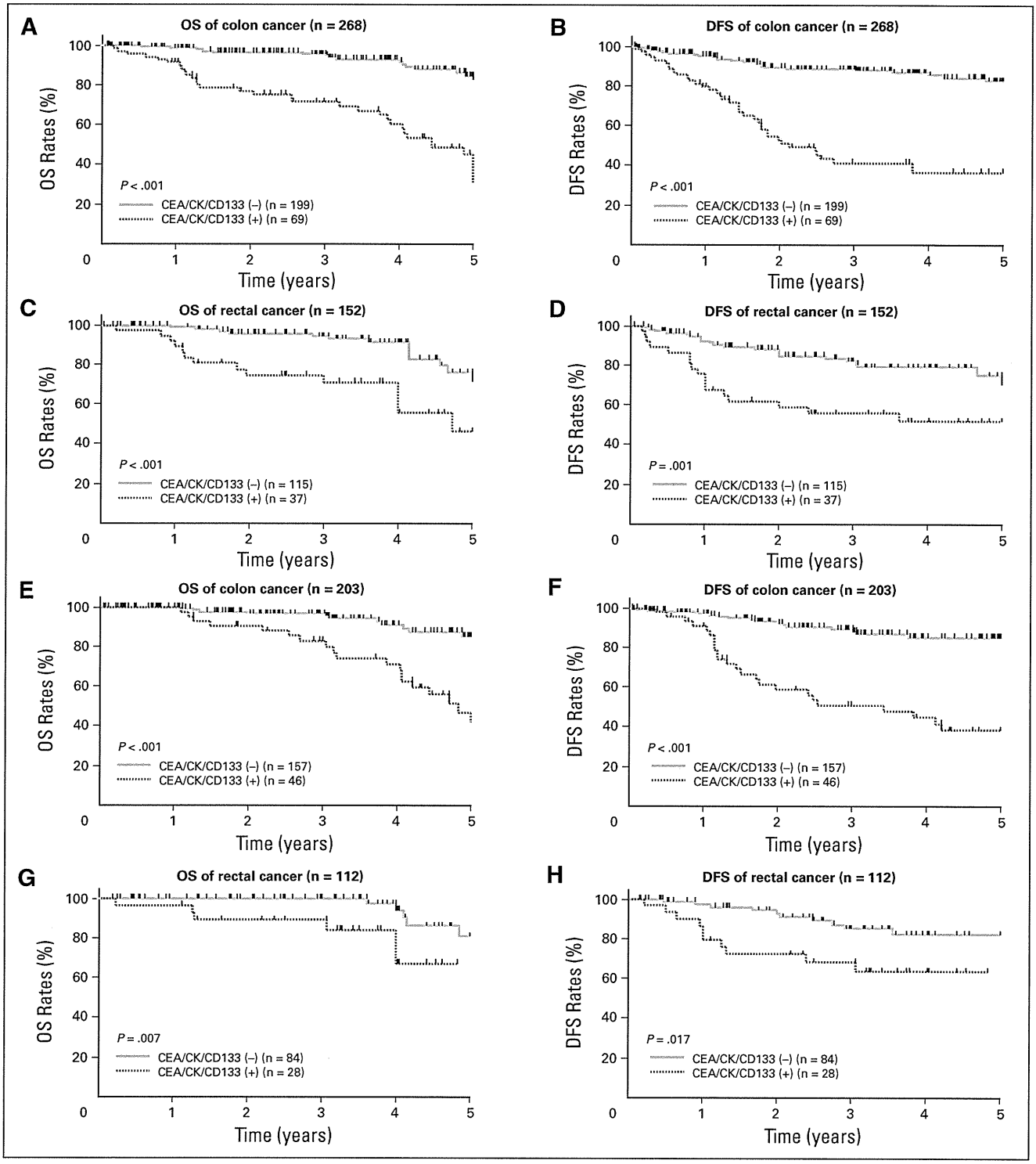

Fig 3. Kaplan-Meier survival curves of overall survival (OS) and disease-free survival (DFS) in patients with colon and rectal cancer. (A, B, E, F) OS and DFS of patients with colon and (C, D, G, H) rectal cancer in training and validation sets were analyzed according to CEA/CK/CD133 mRNA levels.

the US Food and Drug Administration–approved CellSearch system, the usefulness of CTCs as a predictive marker for survival has been reported in metastatic breast cancer and metastatic CRC.[9,10] This automatic cell analysis system is based on immunocytochemical staining, and the antibodies against EpCAM and cytokeratin are used to capture and detect the CTCs. However, the fact that epithelial-mesenchymal transition–transformed tumor cells and cancer stem cells are often characterized by downregulation of epithelial markers

Table 2. Multivariate Analysis for OS and DFS of All Patients in Training and Validation Sets

Characteristic	OS			DFS		
	HR	95% CI	*P*	HR	95% CI	*P*
Training set						
CEA/CK/CD133	3.84	2.41 to 6.22	< .001*	3.02	1.83 to 5.00	< .001*
Tumor size	1.06	0.66 to 1.71	.798	0.97	0.58 to 1.58	.899
Depth of invasion	1.92	0.88 to 4.82	.103	2.01	0.97 to 4.73	.061
Dukes' stage	3.23	2.00 to 5.34	< .001*	2.87	1.75 to 4.76	< .001*
Lymphatic invasion	1.35	0.82 to 2.22	.240	1.22	0.71 to 2.08	.475
Venous invasion	1.05	0.62 to 1.82	.857	0.79	0.46 to 1.38	.410
Histologic type	0.30	0.13 to 1.71	.071	0.49	0.30 to 1.83	.088
Serum CEA	1.71	0.71 to 4.23	.230	1.96	1.20 to 3.22	.007*
Validation set						
CEA/CK/CD133	3.20	1.67 to 6.31	< .001*	3.04	1.79 to 5.22	< .001*
Tumor size	1.08	0.56 to 2.08	.828	1.02	0.60 to 1.73	.933
Depth of invasion	0.99	0.41 to 2.67	.992	1.57	0.73 to 3.74	.259
Dukes' stage	3.39	1.64 to 7.27	< .001*	2.13	1.20 to 3.8	.010*
Lymphatic invasion	1.24	0.65 to 2.78	.536	1.09	0.62 to 1.92	.769
Venous invasion	1.34	0.62 to 2.78	.373	1.30	0.74 to 2.34	.362
Histologic type	0.41	0.21 to 1.81	.081	0.63	0.33 to 1.05	.105
Serum CEA	1.64	0.85 to 3.20	.142	1.85	1.09 to 3.19	.023*

Abbreviations: OS, overall survival; DFS, disease-free survival; HR, hazard ratio; CEA, carcinoembryonic antigen; CK, cytokeratin.
*$P < .05$.

that include EpCAM and cytokeratin suggests that general CTC markers are not able to detect the most aggressive tumor cells in circulation.[22-24] These results may be related to the difficulty of using the CellSearch system as a screening tool for detection of new primary cancer or early tumor cell dissemination. In this study, we used a real-time RT-PCR assay, which has the advantage of improved sensitivity compared with cell-based assays. The limitation of this technique lies in the fact that it does not allow for individual cell analysis. However, we think that it has the potential to detect primitive tumor-initiating cells that may be EpCAM negative because this assay does not depend on the expression of EpCAM.

Whether CTCs have stem cell features is of interest. The cancer stem cell concept hypothesizes that tumors arise from a small population of stem cells, and it has been assumed that the founder cells of metastases may also be stem cells disseminated from the primary tumor to a distant metastatic site. This hypothesis is supported by the similarities between the properties of CTCs and cancer stem cells, which suggests that the founder cells of metastases arise from the CTC

Table 3. Multivariate Analysis for OS and DFS of Patients With Dukes' Stage B and C Cancer in Training and Validation Sets

Variables	Training Set						Validation Set					
	OS			DFS			OS			DFS		
	HR	95% CI	*P*	HR	95% CI	*P*	HR	95% CI	*P*	HR	95% CI	*P*
All Dukes' stage B												
CEA/CK/CD133	3.21	1.44 to 7.46	.005*	3.35	1.66 to 6.74	< .001*	3.75	1.25 to 12.47	.019*	3.10	1.33 to 7.24	.010*
Venous invasion	1.41	0.62 to 3.48	.418	1.30	0.64 to 2.74	.469	1.05	0.35 to 3.26	.933	1.29	0.56 to 3.13	.556
Separated Dukes' stage B												
CEA/CK/CD133	5.00	1.73 to 16.32	.003*	3.59	1.49 to 8.54	.005*	9.31	2.07 to 65.41	.003*	3.19	1.11 to 8.92	.032*
Venous invasion	1.28	0.44 to 3.90	.648	1.25	0.53 to 3.01	.605	0.91	0.21 to 3.88	.894	1.45	0.52 to 4.16	.472
Dukes' stage C												
CEA/CK/CD133	2.62	1.14 to 4.20	.024*	3.26	1.74 to 6.25	< .001*	2.47	1.06 to 6.25	.040*	2.97	1.44 to 6.40	.003*
Venous invasion	2.31	0.77 to 4.21	.087	0.72	0.35 to 1.53	.380	2.39	0.87 to 8.42	.095	1.54	0.68 to 3.96	.313
Serum CEA	1.79	0.78 to 4.49	.173	2.49	1.16 to 5.25	.020*	0.81	0.38 to 1.83	.597	2.21	1.07 to 4.88	.031*

NOTE. In all patients with Dukes' stage B cancer, six factors (tumor size, lymphatic invasion, venous invasion, histologic type, serum CEA, and CEA/CK/CD133 mRNA) were used for the univariate analysis. In the patients with Dukes' stage B cancer who were separated out, five factors (tumor size, venous invasion, histologic type, serum CEA, and CEA/CK/CD133 mRNA) were used for the univariate analysis. In groups with Dukes' stage B, CEA/CK/CD133 and venous invasion, which showed significance for overall survival and disease-free survival in the univariate analysis, were then used for the multivariate analysis. In patients with Dukes' stage C, seven factors (tumor size, depth of invasion, lymphatic invasion, venous invasion, histologic type, serum CEA, and CEA/CK/CD133 mRNA) were used for the univariate analysis. CEA/CK/CD133, venous invasion, and serum CEA, which showed significance for overall survival and disease-free survival in the univariate analysis, were then used for the multivariate analysis.
Abbreviations: OS, overall survival; DFS, disease-free survival; HR, hazard ratio; CEA, carcinoembryonic antigen; CK, cytokeratin.
*$P < .05$.

population. It has been reported that stem cell markers are frequently overexpressed in the CTCs of patients with metastatic breast cancer, and most CTCs have stem cell phenotypes that are nonproliferating and resistant to chemotherapy.[25-28] These facts suggest that a new marker is necessary for the detection of cytokeratin downregulated aggressive CTCs, which may include cancer stem cells. The surface markers of colon cancer stem cells, CD133, CD44, CD166, Musashi-1, CD29, CD24, leucine-rich repeat-containing G-protein-coupled receptor 5, and aldehyde dehydrogenase 1 molecules have been reported,[29-32] and the CD133 molecule has emerged as the key marker for enriching cancer stem cells in many types of cancer.[14,15,32] In contrast, other publications have suggested that CD44 may be a more specific marker for colon cancer stem cells.[33] It is known that CD44 is expressed not only in cancer stem cells but also in a variety of other cells including blood cells.[33] Because our study did not separate the cancer cells from blood cells, it was difficult to measure the CD44 that originated in the cancer cells. Therefore, we selected CD133 for the genetic marker of cancer stem cells. CD133 belongs to the prominin family of proteins, the function of which remains unknown. It has a novel isoform that follows the detection of a small exon of 27 nucleotides by alternative mRNA splicing. Conversely, CD133 is known as a marker of endothelial progenitor cells, which are capable of expanding more than 1,000-fold and initiating the premetastatic niche.[34-36] Although the present assays are not designed to differentiate CD133-positive cancer stem cells from endothelial progenitor cells, we assume that cytokeratin-negative aggressive CTCs may be included in the CD133-expressing cells.

There is a fair amount of single-institution data available on the prognostic significance of CTCs in patients with CRC.[37] However, the number of large-scale multicenter studies is limited. Although Cohen et al,[11] in an analysis of 430 patients with metastatic CTCs, reported that the number of CTCs before and during treatment is an independent predictor of survival, the prognostic value of CTCs in patients with early-stage cancer and without overt metastasis remains unclear. In contrast, we evaluated a total of 735 patients with CRC, and the prognostic value of genetic CTC markers was analyzed at each tumor stage. To examine the clinical significance of CTCs with various characteristics, we selected general markers (CEA and CK) and the cancer stem cell marker (CD133) as the genetic markers of CTCs. Interestingly, our data demonstrated that CEA/CK/CD133, but not CEA/CK, is an independent prognostic factor that surpasses other existing clinicopathologic risk factors in patients with Dukes' stage B CRC. In contrast, not only CEA/CK/CD133 but also CEA/CK showed prognostic value in patients with Dukes' stage C cancer. As for the reason for the different properties of CTCs, we speculate that general marker-positive CTCs in Dukes' stage B cancer may be less aggressive than those of Dukes' stage C, which are capable of lymph node metastasis. To date, the determination of patients with Dukes' B who are at high risk for recurrence is difficult, and efforts to find a tool to select them are ongoing. Because of the limitations of the PCR method, it was difficult to examine whether individual cells expressed all markers or not. We speculate that CTCs that include the CD133-positive cells may enable identification of a certain subgroup that includes aggressive cancer stem-like cell properties and that this may increase their prognostic value in patients with Dukes' stage B cancer. With respect to the reason that the CD133 single marker did not show prognostic value in patients with Dukes' stage B and C cancer, we speculate that it may be a result of the small sample size or the potential limitations of CD133 as a cancer stem cell marker. To the best of our knowledge, this is the first multicenter validation study to clarify the prognostic value of CEA/CK/CD133 multimarkers in patients with Dukes' stage B and C cancer. However, these multimarkers did not show prognostic value in patients with Dukes' stage A cancer, as indicated by the low number of patients with recurrence.

In this study, we demonstrated that a multigenetic marker that includes the cancer stem cell in PB could be a useful tool for the determination of patients with Dukes' stage B and C who are at high risk for recurrence and poor prognosis. We hope that this information brings forth new possibilities for the clinical application of CTCs in patients with CRC.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest.

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Glossary Terms

Cancer stem cells: A cancer cell that has the potential to transfer disease or to form tumors after transplantation. Cancer stemlike cells have the potential to self-renew (see self-renewal), forming additional tumorigenic cancer cells of similar phenotype, and to give rise to phenotypically diverse cancer cells with more limited potential.

CD133: CD133, encoded by the Prominin-1 gene, is used as a marker for stem cells of normal tissues such as neural and hematopoietic stem cells, but also as a marker for tumor stem-like cells of distinct origin such as brain tumors and breast and colon cancer.

CTC (circulating tumor cell): Demonstration of isolated tumor cell circulation/ dissemination in the peripheral blood.

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ORIGINAL ARTICLE

Feasibility study of adjuvant chemotherapy with S-1 plus cisplatin for gastric cancer

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Abstract
Purpose To evaluate the feasibility of S-1 plus cisplatin as adjuvant chemotherapy for stage III gastric cancer after curative resection.
Methods Japanese patients with stage III gastric cancer who underwent gastrectomy with D2 lymph node resection were enrolled. Treatment consisted of 3 cycles of S-1 (80 mg/m^2/day, b.i.d.) for 21 days followed by a 14-day rest, and cisplatin (60 mg/m^2 iv) on day 8. After that, S-1 monotherapy was given on days 1–28 every 6 weeks until 1-year postsurgery. After protocol amendment, the first chemotherapy cycle consisted of S-1 monotherapy; cisplatin was added to cycles 2, 3, and 4, followed by S-1 monotherapy up to 1-year postsurgery. The primary endpoint was the completion rate of three cycles of S-1 plus cisplatin.

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Results A total of 63 enrolled patients have been evaluated. Grade 3/4 toxicities included neutropenia (40%), anorexia (28%), and febrile neutropenia (4%) before protocol amendment ($n = 25$), and neutropenia (37%), anorexia (8%), and febrile neutropenia (3%) after amendment implementation ($n = 38$). Excluding ineligible cases, treatment completion rates were 57% (12/21) before and 81% (30/37) after the protocol amendment.
Conclusions The amended S-1 plus cisplatin is more feasible than the original protocol because of early dose reduction of S-1 prior to cisplatin addition and greater recovery time from surgery prior to cisplatin. This treatment should be considered as a feasible experimental arm for the next postoperative adjuvant phase III trial.

Keywords Adjuvant chemotherapy · Gastric cancer · S-1 · Cisplatin

Introduction

Gastric cancer (GC) remains a major health problem with approximately 8,03,000 deaths worldwide in 2004, although the mortality rate has steadily decreased in recent years [1]. The primary treatment for GC is surgery, which is almost always curative in early GC (stage I) patients, who have a >90% 5-year survival rate. However, locally advanced (stage II–III) GC often recurs, even after curative resection is performed. Therefore, it is very important to develop adjuvant chemotherapy regimens that can improve survival in GC patients with stage II–III disease after surgical resection.

Until recently, several randomized controlled trials of postoperative adjuvant chemotherapy for GC were conducted [2–12]. Although most of them have failed to show clinical benefit in particular multi-agent anthracycline or cisplatin-based regimens, a recent meta analysis showed that postoperative adjuvant chemotherapy was associated with reduced risk of death compared with surgery alone [13].

S-1 (TS-1, Taiho Pharmaceutical Co.) is an orally active combination of tegafur (a prodrug that is converted by cells to fluorouracil), gimeracil (an inhibitor of dihydropyrimidine dehydrogenase, which degrades fluorouracil), and oteracil (inhibits the phosphorylation of fluorouracil in the gastrointestinal tract, thereby reducing the toxic gastrointestinal effects of fluorouracil) [14] approved in Japan, Korea, Singapore, and China for GC. In 2007, the Adjuvant Chemotherapy Trial of TS-1 for Gastric Cancer (ACTS-GC) trial demonstrated the efficacy of S-1 for stage II–III GC patients who underwent curative resection with D2 lymphadenectomy [15]. S-1 improved the 3-year overall survival (OS) rate from 70.1% for surgery alone to 80.1%, with a low incidence of adverse events and good compliance with treatment for 3 months in 87.4% and for 6 months in 77.9%. However, the 3-year OS rates in stage IIIA and stage IIIB patients receiving S-1 were 77.4 and 63.4%, respectively, which are less satisfactory compared with the rate for stage II (90.7%). Therefore, further investigation into more effective treatments for patients with stage III GC is urgently needed.

Meanwhile, for metastatic or recurrent GC, the phase III trial comparing S-1 alone to S-1 plus cisplatin (S-1 Plus cisplatin vs. S-1 In RCT In the Treatment for Stomach cancer; SPIRITS trial) showed that S-1 plus cisplatin resulted in a significantly higher response rate, longer progression-free survival (PFS), and longer OS [16]. Another phase III trial (the First-Line Advanced Gastric Cancer Study; FLAGS trial) showed that S-1 plus cisplatin was associated with fewer toxic effects and demonstrated noninferiority compared with infusional fluorouracil and cisplatin [17]. Therefore, S-1 plus cisplatin is now considered to be one of the standard regimens for metastatic or recurrent GC, as well as a candidate for an experimental arm in the next adjuvant chemotherapy trial.

Before comparing S-1 monotherapy with S-1 plus cisplatin in a phase III trial, we first evaluated the feasibility of S-1 plus cisplatin as adjuvant chemotherapy for stage III GC after curative resection, to confirm that S-1 plus cisplatin can safely be used.

Patients and methods

Eligibility criteria

The following eligibility criteria were employed: (1) histologically proven adenocarcinoma of the stomach; (2) ≥ D2 lymphadenectomy, with complete resection of the primary tumor (R0 surgery); (3) stage IIIA/IIIB disease (T2, N2; T3, N1–2; or T4, N0–1 [Japanese classification]); (4) ECOG performance status 0–1; (5) age 20–75 years; (6) no prior chemotherapy or radiotherapy; (7) able to be enrolled 4–8 weeks after surgery; (8) sufficient oral food intake; (9) adequate organ function (white blood cells [WBCs] $\geq$3,000/mm^3 and $\leq$1,20,000/mm^3, neutrophils $\geq$1,500/mm^3, hemoglobin $\geq$8.0 g/dl, platelets $\geq$1,00,000/mm^3, aspartate aminotransferase [AST] and alanine aminotransferase [ALT] levels $\leq$100 IU/l, total serum bilirubin $\leq$2.0 mg/dl, serum creatinine concentration $\leq$1.2 mg/dl, estimated creatinine clearance $\leq$60 ml/min, normal electrocardiogram); and (10) written informed consent obtained from the patient. Disease stage was classified according to Japanese Gastric Cancer Association guidelines [18]. The protocol was approved by the institutional review board at each participating center.

Treatment and toxicity assessment

Treatment according to the original protocol was begun 4–8 weeks after surgery with 3 cycles of S-1 plus cisplatin ("S-1+ cisplatin [SP] step") followed by S-1 monotherapy ("S-1 step") up to 1 year after surgery. In the "SP step", each cycle consisted of 40 mg/m^2 of S-1 taken orally twice daily for 21 days plus a 2-hour infusion of 60 mg/m^2 of cisplatin on day 8. Each cycle was administered at 5-week intervals. In the "S-1 step", 40 mg/m^2 of S-1 was taken orally twice daily as monotherapy for 28 days at 6-week intervals. All patients received 5-HT3 antagonists and dexamethasone on administration of cisplatin as antiemetics.

Patients were assessed before registration, on days 1, 8, and 15 during the "SP step", and every 2 weeks during the "S-1 step". The baseline assessment included physical examination and laboratory tests. Patients were monitored for adverse effects throughout the treatment period, in addition to receiving follow-up for treatment-related adverse effects. Toxicity was assessed according to the Common Terminology Criteria for Adverse Events (CTCAE), version 3.0.

For adverse effects, the subsequent chemotherapy cycle was delayed until patient recovery, which included the following parameters: WBCs $\geq$3,000/mm^3, neutrophils $\geq$1,500/mm^3, hemoglobin $\geq$8.0 g/dl, platelets >75,000/mm^3, AST or ALT levels $\leq$100 IU/l, total serum bilirubin level $\leq$2.0 mg/dl, and serum creatinine concentration <1.5 mg/dl. Nonhematological toxicities, excluding stomatitis, alopecia, pigmentation changes, nail changes, and watery eyes, were required to be grade 0/1. Cisplatin administration was delayed and administered within 1 day of recovery of the following parameters: WBCs $\geq$3,000/mm^3, neutrophils $\geq$1,500/mm^3, platelets >75,000/mm^3, and serum creatinine <1.5 mg/dl. Both S-1 and cisplatin doses were reduced in the event of grade 4 leukopenia or neutropenia, grade 3/4 thrombocytopenia, serum creatinine $\geq$1.5 mg/dl, or other drug-related nonhematological grade 3/4 toxicities. For level −1 dose reduction, S-1 was reduced from 120 to 100 mg/day, from 100 to 80 mg/day, or from 80 to 50 mg/day, while cisplatin was reduced from 60 to 50 mg/m^2. Dose reduction was permitted twice. When dose-limiting toxicities as described previously occurred again at level −2 (S-1 reduced from 100 to 80 mg/day or from 80 to 50 mg/day [if the −1 level of S-1 was already 50 mg, the patient was withdrawn from the study]; cisplatin administration reduced from 50 to 40 mg/m^2), the patient was withdrawn from the study. A patient was also withdrawn from the study whenever the beginning of the subsequent cycle was delayed by toxicity for more than 3 weeks. When cisplatin administration was delayed beyond day 15, the cisplatin portion of the cycle was skipped.

Protocol amendment

During enrollment, some toxicity was reported during the first cycle of SP, especially neutropenia and anorexia. To minimize patient risk, the Data and Safety Monitoring Committee recommended that patient enrollment be halted and that an interim analysis be conducted using the first 25 registered cases (see "Results"). After the analysis, we decided to amend the protocol.

Treatment according to the amended protocol was begun 4–6 weeks after surgery as in the ACTS-GC trial, and consisted of the following: (1) The first cycle of chemotherapy consisted of S-1 monotherapy, and cisplatin was added to cycles 2, 3, and 4. After that, S-1 monotherapy was administered up to 1 year after surgery; (2) The dose of S-1 in the first SP cycle was reduced in case of severe toxicity during the first cycle of S-1 monotherapy; (3) The criterion for delaying cisplatin administration was changed from a neutrophil count of <1,500/mm^3 to <1,200/mm^3; (4) Dexamethasone was recommended for treatment-induced nausea with 20 mg on day 8 (the day of cisplatin administration) and 16 mg on days 9 and 10.

Statistical analysis

The primary endpoint was the rate of completion of 3 cycles of S-1 plus cisplatin; secondary endpoints were the rate of completion of 2 cycles of S-1 plus cisplatin, the proportion of patients receiving treatment according to protocol, and adverse events. Treatment completion was defined as administration of S-1 for more than 14 days in each cycle plus administration of cisplatin. Completion rate of S-1 plus cisplatin was evaluated in all eligible patients. Toxicity was evaluated among patients who received more than one cycle of S-1 plus cisplatin.

In the present trial, the rate of treatment completion was expected to be lower than compliance in the ACTS-GC trial because of the addition of cisplatin. Moreover, if the rate of treatment completion using 3 cycles of S-1 plus cisplatin were lower than 50%, this regimen would be considered inappropriate for adjuvant therapy and would not be evaluated in a phase III trial. Assuming a null hypothesis of 50% for the rate of completion of 3 cycles and an alternative hypothesis of 70%, and using a 1-sided alpha of 0.1 and a statistical power of 0.1, it is necessary to enroll a minimum of 44 patients. Therefore, the target enrollment was 50 patients, in order to make accommodations for ineligible patients.

After protocol amendment, a minimum of 33 patients is needed for a 1-sided alpha of 0.1 and a statistical power of 0.2. Therefore, 38 more patients were added to allow for ineligible patients. Statistical analysis was performed independently for patients enrolled before and after amendment.

Table 1 Patient characteristics

Characteristic	Original ($n = 25$)	Amended ($n = 38$)
Median age, years (range)	60 (47–72)	62 (40–74)
Gender		
Male	16	25
Female	9	13
PS (ECOG)		
0	17	26
1	8	12
Pathological type		
Intestinal	14	5
Diffuse	11	33
Type of gastrectomy		
Total	8	13
Distal	16	25
Proximal	1	0
T stage		
pT1	2	0
pT2	8	9
pT3	14	28
pT4	1	1
N stage[a]		
pN0	1	0
pN1	10	8
pN2	14	30
Cancer stage[a]		
IB	1[b]	0
II	2[b]	0
IIIA	17	16
IIIB	5	21
IV	0	1[b]

Original before protocol amendment, *Amended* after protocol amendment, *PS* performance status, *ECOG* Eastern Cooperative Oncology Group

[a] Japanese classification; [b] excluded after enrollment

Table 2 Toxicities

Toxicities	Original ($n = 25$)				Amended ($n = 38$)			
	All		Grade 3/4		All		Grade 3/4	
	n	(%)	*n*	(%)	*n*	(%)	*n*	(%)
(A) Hematological toxicities								
Leucopenia	19	(76)	1	(4)	26	(68)	2	(5)
Neutropenia	20	(80)	10	(40)	30	(79)	14	(37)
Anemia	23	(92)	5	(20)	35	(92)	3	(8)
Thrombocytopenia	10	(40)	1	(4)	17	(45)	1	(3)
Febrile Neutropenia	1	(4)	1	(4)	1	(3)	1	(3)
(B) Nonhematological toxicities								
Anorexia	23	(92)	7	(28)	34	(89)	3	(8)
Nausea	17	(68)	2	(8)	31	(82)	1	(3)
Vomitting	7	(28)	0	(0)	8	(21)	0	(0)
Diarrhea	13	(52)	0	(0)	24	(63)	1	(3)
Fatigue	17	(68)	0	(0)	34	(89)	2	(5)
Stomatitis	2	(8)	0	(0)	8	(21)	0	(0)
AST	5	(20)	0	(0)	10	(40)	0	(0)
ALT	5	(20)	0	(0)	8	(36)	0	(0)
Total bilirubin	6	(30)	0	(0)	22	(22)	0	(0)
Creatinine	5	(20)	0	(0)	11	(10)	0	(0)

Original before protocol amendment, *Amended* after protocol amendment, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase

Results

Patient characteristics

From August 2007 to July 2009, 63 patients (25 patients in the original protocol/38 patients in the amended protocol) were accrued from 5 Japanese hospitals. To date, all 63 patients have finished the "SP step" and have been evaluated. Clinical characteristics are summarized in Table 1. The median age was 60/62 (original/amended protocol) years (range, 47–72/40–74 years), and the following types of resection were performed: total gastrectomy ($n = 8/13$), distal gastrectomy ($n = 16/25$), and proximal gastrectomy ($n = 1/0$). In the original protocol, 17 patients had stage IIIA disease and 5 had stage IIIB disease; whereas 16 had stage IIIA and 21 had stage IIIB disease in the amended protocol. After enrollment, 4 patients were deemed ineligible during the original protocol because of confirmed stage II disease ($n = 2$), stage IB disease ($n = 1$), and cancer other than GC ($n = 1$), and 1 patient was considered ineligible during the amended protocol because of pathological stage IV ($n = 1$) disease.

Toxicity

A total of 202 cycles from the 63 cases were assessable for toxicity (Table 2). Under the original protocol ($n = 25$), neutropenia was the most common hematological toxicity, with grade 3/4 neutropenia observed in 10 patients (40%). Additional grade 3/4 hematological toxicities included anemia in 5 patients (20%), and leucopenia, thrombocytopenia, and febrile neutropenia in 1 patient (4%) each. Grade 3/4 anorexia was the most frequent nonhematological toxicity ($n = 7$ [28%]), followed by nausea ($n = 2$ [8%]). There was no grade 3/4 creatinine elevation seen.

Under the amended protocol ($n = 38$), the frequency of grade 3/4 neutropenia was similar to the original; it was seen in 14 patients (37%). Grade 3/4 anemia decreased to 3 patients (8%), and the frequencies of grade 3/4 leukopenia ($n = 2$