been reached. Continuous infusion of fluorouracil was once considered the strongest candidate for standard first-line therapy (1). However, Phase II studies of S-1 (1 M tegafur–0.4 M gimestat–1 M otastat potassium) have reported response rates of 44–49% (2,3). A subsequent study in which S-1 was combined with cisplatin reported a response rate of 74%, exceeding that achieved with S-1 monotherapy (4) and suggesting that combined treatment further improves survival. In Japan, the Japan Clinical Oncology Group (JCOG) 9912 study was conducted to examine the non-inferiority of S-1 to a continuous infusion of fluorouracil. The results verified the non-inferiority of S-1, associated with a median survival time (MST) of 11.4 months when compared with 10.8 months for fluorouracil (5). The SPIRITS trial, a randomized controlled study comparing S-1 monotherapy with a combination of S-1 and cisplatin, showed that MST was significantly longer with S-1 plus cisplatin (13 months) than with S-1 monotherapy (11 months; hazard ratio 0.77, $P = 0.04$) (6). The MST in patients who received S-1 in these two studies was similar, suggesting that the results were reproducible. On the basis of these two studies, a combination of S-1 and cisplatin was considered a new standard therapy for the first-line treatment of advanced or recurrent gastric cancer.

To date, various fluorouracil-based regimens have been used as first-line therapy. However, options for second-line therapy in patients with fluorouracil-resistant tumors remain to be fully explored. Besides fluorouracil, drugs such as irinotecan hydrochloride (CPT-11) (7) and taxanes (8–12) are effective against advanced or recurrent gastric cancer. Among these drugs, paclitaxel has been shown to be effective for ovarian cancer (13), non-small cell lung cancer (14,15) and breast cancer (16). Paclitaxel has produced good results, with response rates of 23% overall and 26% in previously treated patients and an MST of 340 days in Phase II trials of patients with unresectable, advanced or recurrent gastric cancer (9).

Various dosages, treatment schedules and methods for the administration of paclitaxel have been studied. Conventional regimens in which paclitaxel is given at 3-week interval (triweekly) are associated with a high incidence of peripheral neuropathy. However, the risk of such toxicity may be reduced by weekly treatment with paclitaxel. Rosenberg et al. conducted a controlled study comparing weekly paclitaxel with triweekly paclitaxel in patients with ovarian cancer. They concluded that the weekly regimen had fewer side effects and was safer than the triweekly regimen, with no difference in effectiveness (17). In patients with gastric cancer, weekly paclitaxel is often used, producing response rates of 16–25.8%, an MST of 222–234 days and a progression-free survival (PFS) of 78–99 days (18–20). In breast cancer, paclitaxel has been reported to have good tolerance and response rates, with higher convenience than weekly treatment (21).

We previously performed a Phase I trial (dose-finding study) of paclitaxel every 2 weeks (biweekly) in patients with advanced or recurrent gastric cancer (22). The recommended dose was estimated to be 140 mg/m^2. Dose-limiting toxicity included peripheral neuropathy, anorexia and fatigue. The present Phase II trial was designed to evaluate the safety and effectiveness of paclitaxel given biweekly in the recommended dose to patients with advanced or recurrence gastric cancer resistant to fluoropyrimidine derivatives.

PATIENTS AND METHODS

Eligibility

Eligible patients had to have (i) a histologically proved diagnosis of gastric cancer; (ii) an Eastern Cooperative Oncology Group (ECOG) performance status (PS) of 0–2; (iii) at least one measurable target lesion; (iv) a history of treatment with 5-FU, with resistant or recurrent disease (up to one regimen with a treatment-free interval of 2 weeks or longer); (v) adequate main organ functions, i.e. white cell count: $\geq$4000/mm^3, ANC: $\geq$2000/mm^3, platelets: $\geq$100 000/mm^3, hemoglobin: $\geq$8.0 g/dl (>2 weeks after blood transfusion), aspartate aminotransferase and alanine aminotransferase: less than twice the upper limit of normal (except for patients with hepatic metastasis), serum total bilirubin: $\leq$1.5 mg/dl, serum creatinine: $\leq$1.5 mg/dl and a normal electrocardiogram (no abnormalities requiring treatment); (vi) a life expectancy of >2 months; (vii) no severe concurrent disease or other active primary cancers; (viii) an age between 20 and 80 years; and (ix) the ability to give written informed consent to participate in this study.

Patients were excluded from the study if they had one or more of the following conditions: (i) severe concurrent disease, including heart conditions such as ischemic heart disease or arrhythmias severe enough to require treatment (excluding left ventricular hypertrophy associated with hypertension, mild left ventricular overload or right bundle branch block); a history of myocardial infarction within the past 6 months; liver cirrhosis; dyspnea due to conditions such as interstitial pneumonia or pulmonary fibrosis, requiring oxygen inhalation; fresh gastrointestinal bleeding requiring repeated transfusions; psychiatric disorders treated with antipsychotic agents or requiring such treatment; uncontrolled diabetes mellitus; clinical evidence of ileus or subileus; (ii) a history of severe drug hypersensitivity; (iii) a history of hypersensitivity to preparations containing polyoxyethylene castor oil (e.g. cyclosporine preparations) or hardened castor oil (e.g. injectable vitamin preparations); (iv) acute inflammatory disease; (v) peripheral neuropathy caused by previous treatment; (vi) confirmed or suspected pregnancy or breastfeeding; (vii) another active malignant disease potentially affecting survival or adverse events; (viii) symptomatic brain metastasis; and (ix) any other condition considered by the investigator to preclude

participation in the present study. Patients confirmed to meet all eligibility criteria were enrolled at a central patient registration center.

Treatment

Enrolled patients received paclitaxel 140 mg/m^2 intravenously on days 1 and 15 of a 4-week cycle. Treatment was continued for at least two cycles or until the onset of disease progression or severe toxicity. As premedication, patients received 8–20 mg of dexamethasone intravenously, 50 mg of diphenhydramine orally and 50 mg of ranitidine intravenously at least 30 min before paclitaxel to prevent hypersensitivity reactions.

The next cycle of treatment was postponed until recovery to the standard value if patients had any of the following findings: (i) a white cell count of $<3000/mm^3$ or a platelet count of $<75\,000/mm^3$); (ii) a fever of 38°C or higher; (iii) a PS of 3; and (iv) non-hematological toxicity of Grade 3 or higher. If any of the following adverse events occurred after treatment, the dose for the next cycle was reduced in decrements of 20 mg/m^2 from the initial dose of 140 mg/m^2: hematological toxicity of Grade 4, non-hematological toxicity of Grade 3 or higher or peripheral neuropathy of Grade 2 or higher. The minimum permissible dose was 100 mg/m^2.

Follow-up Evaluation

Before entering the study, all patients gave a detailed medical history, underwent a complete physical examination, completed blood cell count, and serum chemistry, abdominal computed tomography, weight, height and ECOG PS were recorded. Physical examination, a symptom evaluation and routine blood tests and biochemistry blood examination were monitored on every 2 weeks during the treatment. The objective response was evaluated every 2 months. Complete response, partial response (PR), stable disease (SD), progression disease (PD) or not evaluated (NE) was defined according to RECIST. Toxicity was evaluated every 2 weeks according to Common Terminology Criteria for Adverse Events (CTCAE), version 3.0.

Statistical Analysis

The target sample size was 40 patients. Given that the threshold response rate would be 10% and expected response rate would be 25%, the required sample size was estimated to be 40 patients under the conditions of $\alpha = 0.05$ and power = 0.8.

PFS was measured from the date of enrollment to the first objective evidence of PD or the date of death, whichever came first. Overall survival was measured from the date of enrollment to the date of death. PFS and overall survival were estimated by the Kaplan–Meier method. Adverse events were evaluated according to the CTCAE, version 3.0, for each patient.

Table 1. Patients characteristics

Variable	No. of patients	%
No. included	40	
Sex		
Male	31	77.5
Female	9	22.5
Age (median), years	63 (48–77)	
Performance status (ECOG)		
0	22	55.0
1	13	32.5
2	5	12.5
Site of metastasis		
Liver	18	45.0
Lung	1	2.5
Abdominal lymph nodes	28	70.0
Cervical lymph nodes	2	5.0
Peritoneum	2	5.0
Other	5	12.5
Previous chemotherapy		
S-1	32	80.0
S-1 + others[a]	5	12.5
5-FU	1	2.5
UFT-E	1	2.5
Capecitabine + cisplatin	1	2.5
Histology		
Intestinal	15	37.5
Diffuse	25	62.5

ECOG, Eastern Cooperative Oncology Group; S-1, 1 M tegafur–0.4 M gimestat–1 M otastat potassium; 5-FU, 5-fluorouracil; UFT-E, uracil-tegafur enterogranules.
[a]Others: four patients received cisplatin and one received irinotecan.

RESULTS

Patients Characteristics

The present study was conducted after the protocol had been approved by the Institutional Review Board of each participating center. A total of 41 patients were enrolled in the study between June 2005 and April 2007. Although all 41 patients met the entry criteria, one did not receive paclitaxel because of an acute deterioration of condition after enrollment. Safety and efficacy were assessed in the other 40 patients. The patients characteristics are shown in Table 1. The median age was 63 years (range: 48–77); 31 patients were men and 9 were women. Thirty-five patients (87.5%) had a relatively good performance status of 0 or 1. Histologically, 15 patients had intestinal type tumors and

Table 2. Antitumor activity

Response	No. of patients ($n = 40$)	%
Complete response	0	0.0
Partial response	7	17.5
Stable disease	21	52.5
Progressive disease	10	25.0
Not evaluable	2	5.0
Overall response rate	7	17.5
Disease control rate	28	70.0

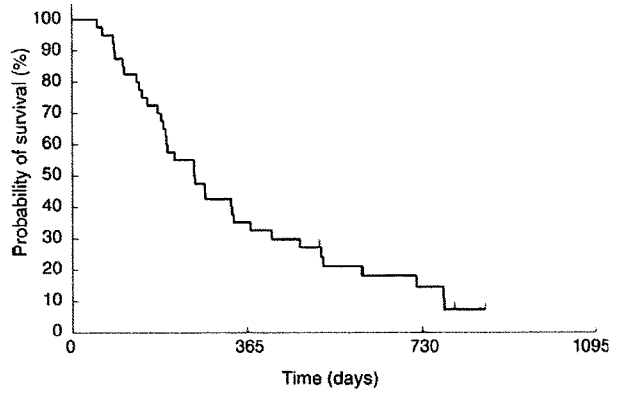

Figure 1. Analysis of overall survival.

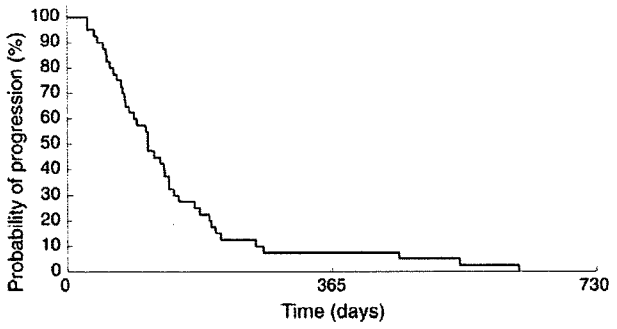

Figure 2. Analysis of progression-free survival.

25 had diffuse type tumors. First-line chemotherapy was S-1 monotherapy in 32 patients (80.0%), S-1 plus cisplatin (4 patients) or irinotecan (1 patient) in 5 (12.5%), fluorouracil in 1 (2.5%), uracil-tegafur enterogranules (UFT-E) in 1 (2.5%) and capecitabine plus cisplatin in 1 (2.5%). Eighteen patients (45.0%) had liver metastasis, 1 (2.5%) had lung metastasis, 28 (70.0%) had abdominal lymph node metastasis, 2 (5.0%) had cervical lymph node metastasis, 2 (5.0%) had peritoneal metastasis and 5 (12.5%) had metastasis to other organs.

Table 3. Toxicity according to CTCAE, ver. 3.0 ($n = 40$)

Toxicity	Grade				Grade 3/4(%)
	1	2	3	4	
Leukopenia	12	14	2	1	7.5
Neutropenia	0	14	8	3	27.5
Anemia	14	18	3	2	12.5
Thrombopenia	3	1	0	0	0.0
Fatigue	10	2	0	0	0.0
Anorexia	10	4	0	0	0.0
Nausea/vomiting	6	2	0	0	0.0
Stomatitis	7	0	0	0	0.0
Diarrhea	4	2	1	0	2.5
Motor neuropathy	4	2	0	0	0.0
Sensory neuropathy	16	9	1	0	2.5
Muscle pain	7	1	0	0	0.0
Joint pain	2	0	0	0	0.0
Alopecia	23	4	—	—	—
Allergy	2	0	0	0	0.0

CTCAE, Common Terminology Criteria for Adverse Events.

Treatment Summary

The median number of treatment courses received by the patients was 3.5 (range: 1–14), and the median duration of treatment was 92.5 days (range: 1–429). The dose of paclitaxel was reduced in seven patients (17.5%) because of hematological toxicity such as neutropenia (six patients), or neutropenia, diarrhea and peripheral neuropathy (one patient). Treatment was delayed in nine patients (22.5%). The reasons for delaying the treatment were the patients' request in six patients, abdominal pain in one, suspected pneumonia in one, and nausea, vomiting and anorexia in one. The reasons for termination of treatment were progression of primary disease in 32 patients, adverse events in 7 (infection 3 patients, neuropathy 3 patients and allergy 1 patient) and the patients' request in 1.

Efficacy

On intention-to-treat analysis, 7 patients (17.5%) had a PR, 21 (52.5%) had SD, 10 (25.0%) had PD and 2 (5.0%) were NE. The overall response rate was 17.5% [95% confidence interval (CI): 7.3–32.8%]. The disease control rate (PR + SD) was 70.0% (28 patients; Table 2). The median duration of follow-up was 8.5 months. Median overall survival was 254 days (Fig. 1) and median PFS was 111 days (Fig. 2).

Table 4. Comparison of paclitaxel according to regimen in patients with advanced and recurrent gastric cancer

Reports	Triweekly			Weekly			Biweekly
	Ohtsu et al. (8)	Yamada et al. (9)	Yamaguchi et al. (10)	Emi et al. (18)	Arai et al. (19)	Kodera et al. (20)	Present study
Number of patients	15	60	32	68	45	45	40
Dose (mg/m^2)	210	210	210	80	80	80	140
Prior chemotherapy							
(+)	13	32	21	54	45	45	40
(−)	2	28	11	14	0	0	0
Toxicity							
Hematologic (G3–4)							
WBC	33.3	10.0	59	13.2	8.9	18	7.5
ANC	80.0	36.7	88	20.1	13.3	16	27.5
Non-hematologic							
Neuropathy (any)	80.0	75.0	59.4	14.7	53.3	—	65.0
G3–4	0	1.7	0	0	4.4	2	2.5
Muscle/joint pain	40.0/33.3	45.0/38.3	40.6/53.1	10.3	—		20.0/5.0
Response rate (%)	20	23	28	17.6	25.8	16	17.5
Disease control rate (%)	73.3	53	65.6	55.8	61.3	48	70.0
PFS	—	—	—	—	99[a]	78	111
OS	—	340	234	222	226	234	254

WBC, white blood cell count; ANC, absolute neutrophil count; PFS, progression-free survival; OS, overall survival.
[a]Time to progression.

Toxicity

Toxicity is summarized in Table 3. The major hematological toxic effects of Grade 3 or 4 were neutropenia (27.5%) and anemia (12.5%). The major non-hematological toxic effects of Grade 3 were diarrhea in 1 patient (2.5%) and sensory neuropathy in 1 (2.5%). Sensory neuropathy occurred in 26 patients (65.0%), although 16 (61.5%) had Grade 1 reactions. Other toxic effects included alopecia (67.5%), anorexia (35.0%), fatigue (30.0%) and motor neuropathy (15.0%). There were no episodes of hepatic or cardiac toxicity and no treatment-related death. Paclitaxel was thus well tolerated.

DISCUSSION

We conducted a Phase II study of biweekly paclitaxel as the second-line therapy in patients with advanced or recurrent gastric cancer that was resistant to fluoropyrimidine derivatives. Efficacy and safety could be evaluated in 40 of the 41 patients enrolled. The response rate was 17.5%, median overall survival was 254 days and median PFS was 111 days. The main adverse events were neutropenia and anemia.

Paclitaxel has usually been administered triweekly to treat ovarian cancer, non-small cell lung cancer and breast cancer. Recently, however, the concept of 'dose-dense' therapy has led to the administration of paclitaxel in smaller divided doses, expanding treatment possibilities (23). A randomized controlled trial comparing weekly with triweekly paclitaxel in patients with metastatic breast cancer reported that weekly paclitaxel was associated with better PFS, but a higher incidence of motor and sensory neurotoxicity (24). Another controlled study compared triweekly paclitaxel as standard therapy with the weekly paclitaxel, weekly docetaxel (another taxane) and triweekly docetaxel in the postoperative adjuvant treatment of early stage breast cancer. The results showed that weekly paclitaxel was more therapeutically useful than the other treatments (25). Another study in women with ovarian cancer showed that weekly paclitaxel plus carboplatin was associated with significantly longer PFS (27.9 months) than conventional triweekly paclitaxel plus carboplatin (17.1 months). The rate of survival at 2 years was higher for weekly treatment (83.6%) than conventional triweekly treatment (77.7%), demonstrating that weekly paclitaxel was more therapeutically useful (26). These studies in women with breast cancer and ovarian cancer thus showed that overall survival and PFS were longer for weekly paclitaxel than for triweekly paclitaxel. These studies have reported that the incidence of neuropathy, a common toxic effect of paclitaxel, is lower with weekly treatment.

In Japan, weekly paclitaxel is used more often than triweekly paclitaxel in patients with gastric cancer (Table 4). The response rate for weekly paclitaxel ranges from 16% to

25.8%, with an MST of 222–234 days and a PFS of 78–99 days (18–20). Weekly paclitaxel is reported to have less hematologic toxicity and neurotoxicity than triweekly paclitaxel (8–10). Biweekly paclitaxel is equivalent to weekly paclitaxel in terms of response rate, PFS, overall survival and toxicity, with lower toxicity than triweekly paclitaxel. As for biweekly paclitaxel, the lower bound value of 95% CI of the response rate fell below the threshold of the hypothesis in our study. The disease control rate with biweekly paclitaxel was 70.0%, which is higher than that with weekly paclitaxel (48–61.3%).

The incidence of muscle pain and joint pain (25.0%) was lower than that with triweekly paclitaxel (40–53%) and higher than that with weekly paclitaxel (10.3%). The response rate (17.5%), MST (254 days) and PFS (111 days) in the present study compare favorably with the previous results obtained with weekly paclitaxel, as described above. It seems that biweekly paclitaxel is one of the useful chemotherapies in practice.

Our results suggest that biweekly paclitaxel is as effective as weekly paclitaxel in patients with advanced or recurrent gastric cancer, with the additional benefit of reducing the number of hospital visits. Biweekly paclitaxel may thus offer important advantages with respect to healthcare costs and patients' quality of life when compared with weekly paclitaxel, with equivalent efficacy and safety. The results of controlled studies comparing biweekly paclitaxel with weekly paclitaxel are awaited.

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Conflict of interest statement

None declared.

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Phase II study of S-1 plus leucovorin in patients with metastatic colorectal cancer

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Background: S-1, a novel oral fluoropyrimidine, is well tolerated in patients with metastatic colorectal cancer (mCRC). The response rate of S-1 for colorectal cancer is high, ranging from 35% to 40%. This study aimed to evaluate the safety and efficacy of S-1 combined with oral leucovorin (LV) to enhance antitumor activity in chemotherapy-naive patients with mCRC.

Patients and methods: S-1 was given orally twice daily for two consecutive weeks at a daily dose of 80–120 mg, followed by a 2-week rest period, within a 4-week cycle. LV was given orally twice a day at a daily dose of 50 mg, simultaneously with S-1.

Results: Of the 56 patients with previously untreated mCRC, 32 (57%) had partial responses. The median follow-up period was 27.2 months. The median time to progression was 6.7 months (95% confidence interval 5.4–7.9). The median survival time was 24.3 months. There was no treatment-related death or grade 4 toxicity. The most common grade 3 toxic effects were diarrhea (32%), anorexia (21%), stomatitis (20%), and neutropenia (14%).

Conclusion: S-1 combined with LV therapy demonstrated promising efficacy and acceptable safety in chemotherapy-naive patients with mCRC without the concurrent use of irinotecan, oxaliplatin, or molecular-targeted drugs.

Key words: colorectal cancer, leucovorin, LV, phase II, S-1

original article

introduction

Recently, the development of irinotecan and oxaliplatin in combination with 5-fluorouracil (5-FU)-based regimens has led to significant improvement of survival in patients with metastatic colorectal cancer (mCRC). Various phase III studies of first-line chemotherapy have reported combination therapy with i.v. 5-FU/leucovorin (5-FU/LV) plus oxaliplatin (FOLFOX regimen) or 5-FU/LV plus irinotecan (FOLFIRI regimen) as a standard regimen for mCRC [1–4]. Recent clinical trials have examined whether oral fluoropyrimidines such as uracil–tegafur (UFT)/LV and capecitabine could be a replacement for i.v. 5-FU/LV. A combination of capecitabine and oxaliplatin (XELOX regimen) was found not to be inferior to FOLFOX in terms of progression-free survival (PFS) [5]. The standard treatment of mCRC is consequently shifting from 5-FU/LV-based regimens, which require central venous access, to more convenient oral-based care.

S-1 is a capsule preparation combining FT, an oral 5-FU derivative, with gimeracil (CDHP) and oteracil potassium (Oxo) at a molar ratio of 1.0 : 0.4 : 1.0. CDHP reversibly inhibits the activity of dihydropyrimidine dehydrogenase (DPD), a metabolizing enzyme of 5-FU. Oxo inhibits the activity of orotate phosphoribosyltransferase and is distributed in high concentrations in the gastrointestinal (GI) tract, where it suppresses GI disorders caused by 5-FU.

In Japan, S-1 was approved for the treatment of gastric cancer in 1999 and was subsequently approved for the treatment of colorectal cancer (CRC), head and neck cancer, non-small-cell lung cancer, inoperable or recurrent breast cancer, pancreatic cancer, and biliary tract cancer. Recently, several phase III studies have established S-1 as a standard treatment of gastric cancer, including postoperative adjuvant chemotherapy [6–8]. Two phase II studies of S-1 were conducted in patients with mCRC. Single-agent S-1 was shown to be very effective, with high response rates (36% and 40%) and good median survival times (MSTs) (12 months) for at

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the time these studies were conducted given the lack of second-line therapies available [9, 10].

LV is known to enhance the efficacy of 5-FU by inhibiting thymidylate synthase. A meta-analysis consisting of >3000 patients' clinical data revealed that LV improves response rates and overall survival (OS) when combined with 5-FU, as compared with 5-FU alone [11]. Oral UFT/LV has been shown to be as effective as i.v. 5-FU/LV (Mayo regimen), with significantly favorable safety profile against metastatic disease [12, 13]. In an adjuvant setting, oral UFT/LV regimen was demonstrated to be as effective as i.v. 5-FU/LV (Roswell Park Memorial Institute regimen) in patients with curatively resected stage II/III colon cancer[14]. On the other hand, addition of oral LV to another fluoropyrimidine, capecitabine, leads to increased GI toxicity or hand–foot skin reaction, with no enhancement of response [15].

In a phase I study of oral LV plus S-1 in patients with mCRC, recommended treatment schedule with fixed dose of S-1 and LV was determined. S-1 and LV were administered twice a day at a daily dose of 80–120 mg for S-1, a conventional dose of S-1, and 25 mg for LV. The dose (schedule)-limiting toxic effects (DLTs) were mainly GI symptoms such as grade 3 stomatitis/pharyngitis, nausea, diarrhea or ileus, and exanthema. The response rate was 67% (10 of 15). The recommended treatment schedule was 2 weeks of administration followed by 2 weeks of rest [16]. To evaluate the safety and efficacy of a combination of S-1 and LV (S-1/LV regimen) given in the recommended schedule, we conducted a phase II study in chemotherapy-naïve patients with mCRC.

patients and methods

patient selection

Eligible patients had histologically confirmed CRC; have at least one measurable lesion; adequate oral intake; aged 20–74; no previous treatment of metastatic disease (adjuvant chemotherapy was allowed if finished 180 days before enrollment); an Eastern Cooperative Oncology Group performance status of zero to two; adequate bone marrow, liver, and renal functions as follows: a serum hemoglobin concentration of ≥9.0 g/dl, a white blood cell count of 4000–12 000/mm^3, a neutrophil count of ≥2000/mm^3, a platelet count of ≥100 000/mm^3, a serum total bilirubin concentration of ≤1.5 mg/dl, serum aspartate aminotransferase and alanine aminotransferase concentrations of ≤100 IU/l, a serum alkaline phosphatase level of less than twice the upper limit of the normal institutional level (ULN), and a serum creatinine level of less than ULN; and written informed consent. Patients were excluded from this study if they had a contraindication for S-1; a history of serious hypersensitivity to LV; an active infection; serious concomitant diseases or conditions (intestinal obstruction, pulmonary fibrosis, heart failure, renal failure, liver failure, etc.); severe ascites or pleural effusion; extensive bone metastasis; brain metastasis or symptoms of brain metastasis; diarrhea (watery stools); or another synchronous cancer. We also excluded patients participating in other clinical studies; women who were pregnant, nursing infants, possibly pregnant, or planning to become pregnant; and men who were intending to conceive children.

treatment plan

S-1 (capsules containing 20 or 25 mg of FT) and LV (25-mg tablets) were provided by Taiho Pharmaceutical Co., Ltd, Tokyo, Japan. The dose of S-1 was determined according to body surface area as follows: <1.25 m^2, 40 mg; 1.25–1.50 m^2, 50 mg; and ≥1.50 m^2, 60 mg. LV was given at a fixed dose of 25 mg each time. S-1 and LV were given together orally twice a day for two consecutive weeks, followed by 2 weeks rest. This 4-week cycle was repeated until the onset of disease progression or unacceptable adverse events. No pretreatment was allowed. The dose of S-1 could be decreased by one level in the event of the following toxicity: grade 4 leucopaenia or thrombocytopaenia; grade 4 non-hematologic toxicity; or grade 3 diarrhea, stomatitis, skin conditions, or febrile neutropaenia that did not resolve with symptomatic treatment. The dose of LV could not be decreased.

toxicity and response criteria

Laboratory and clinical examinations were carried out within 15 days before enrollment, every 1 week during the first course of treatment and every 2 weeks from the second course onward. Tumors were evaluated on the basis of computed tomographic scans and serum carcinoembryonic antigen levels within 30 days before enrollment and every 4–6 weeks after the start of treatment. In the assessment of the best overall response, a complete response (CR: the disappearance of all lesions and normalization of tumor marker level) or partial response (PR: at least a 30% decrease in the sum of the longest diameter of all measured lesions taking as reference the baseline sum longest diameter) had to continue for at least 4 weeks and to be confirmed. A best overall response of stable disease (SD: neither sufficient shrinkage to quality for PR nor sufficient increase to qualify for PD taking as reference the smallest sum longest diameter since the treatment started) required no evidence of progressive disease (PD: at least a 20% increase in the sum of the longest diameter of all measured lesions taking as reference the smallest sum longest diameter recorded since the treatment started or the appearence of new lesion) for at least 6 weeks after the start of treatment. Response to S-1/LV treatment was externally reviewed and analyzed. Tumors were assessed according to RECIST criteria. Toxicity was evaluated according to the Common Terminology Criteria for Adverse Events (version 3.0).

statistical analysis

The response rate in previous phase II studies of S-1 alone in patients with CRC was 33% [42 of 129; 95% confidence interval (CI) 25–41]. Therefore, the threshold rate of response to the S-1/LV regimen was set at 30%, and the expected response rate was estimated to be 50%, which was ~20 percentage points higher than the response rate for S-1 alone. Assuming that the response rate follows a binomial distribution, we calculated the number of patients required to obtain the expected response rate (given a threshold response rate of 30%), with a one-sided test, a significance level of 2.5% ($\alpha/2 = 2.5\%$), and a statistical power ($1-\beta$) of 80%. We estimated that a target sample size of 54 patients would be needed to reject the null hypothesis with a power of 80%.

The Kaplan–Meier method was used to estimate time to progression (TTP), time to treatment failure (TTF), and OS. All data obtained until the completion of the study period were included in the safety analyses. Clinical cut-off date for this study was 25 June 2008.

The study was approved by the institutional review board at each participating center. For the duration of the study, an independent data-monitoring committee monitored safety. The study was undertaken in accordance with the Helsinki Declaration and Japanese Good Clinical Practice Guidelines.

results

patient characteristics

From October 2005 through June 2006, a total of 56 patients were enrolled from 12 hospitals: all were eligible. Patient characteristics are described in Table 1. A total of 406 courses of

Table 1. Patient characteristics

Characteristics	N = 56	
	n	%
Gender		
Male	30	54
Female	26	46
Age, years		
Median	62	
Range	32–72	
ECOG performance status		
0	53	95
1	3	5
2	0	0
Primary site		
Colon	32	57
Rectum	24	43
Histologic grading		
Well differentiated	20	36
Moderately differentiated	29	52
Poorly differentiated	5	9
Mucinous	2	4
Site of metastases		
Liver	39	70
Lung	26	46
Lymph nodes	24	43
Peritoneum	3	5
Other	7	13
No. of sites evaluated		
1	24	43
2	20	36
3	8	14
4	2	4
≥5	2	4
Prior adjuvant therapy		
Yes	10	18
No	46	82
Hemoglobin (g/dl)		
Median	12.50	
Range	9.0–16.8	
Alkaline phosphatase (IU/l)		
Median	280.0	
Range	137–1408	

ECOG, Eastern Cooperative Oncology Group.

Table 2. Tumor response

	N = 56	
	n	%
Complete response	0	0
Partial response	32	57
Stable disease	16	29
Progressive disease	8	14
Not evaluable	0	0
Overall response rate (%)	32	57
95% CI	43.2–70.3	
Time to progression, months		
Median	6.7	
95% CI	5.4–7.9	

Tumor response was externally assessed according to the RECIST criteria. CI, confidence interval.

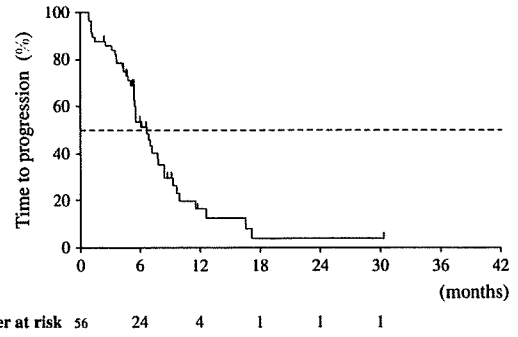

Figure 1. Kaplan–Meier curve of time to progression.

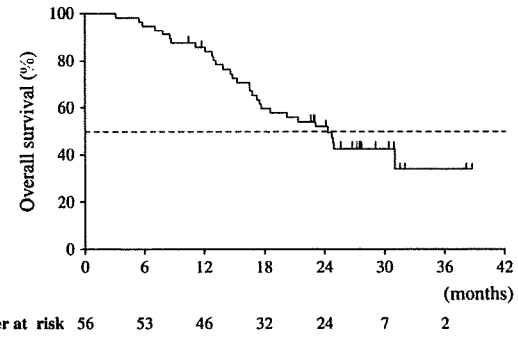

Figure 2. Kaplan–Meier curve of overall survival.

the study treatment cycles were delivered to patients. The median number of treatment courses was 6 (range 1–26). The median treatment period was 5.1 months (range 0.3–29.4). The median relative dose intensity was 81% (range 43–109) for S-1 and 93% (range 49–113) for LV.

response to therapy

The response rate, which was the primary end point of this study, was evaluated in all 56 patients. No patient had a complete response, but 32 had PRs, 16 had stable disease, and 8 had progressive disease. The response rate was 57% (95% CI 43–70) (Table 2). The median time to response was 1.9 months (range 0.9–5.3).

With a median follow-up time of 27.2 months, the median TTP was 6.7 months (95% CI 5.4–7.9) (Figure 1). The median TTF was 6.0 months (95% CI 5.4–7.8). The MST was 24.3 months (95% CI 17.5–XXX; upper bound of 95% CI was not estimable) (Figure 2) with the survival rate of 86% at 1 year and 52% at 2 years. Second-line treatment, including curative or palliative surgery, was given to 52 (93%) of the 56 patients, among whom 36% received oxaliplatin-based chemotherapy and 41% received irinotecan-based chemotherapy (Table 3).

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Table 3. Further treatment after study chemotherapy

	N = 56	
	n	%
Oxaliplatin based	20	36
Irinotecan based	23	41
Surgery		
Curative	3	5
Palliative	2	4
None	4	7
Other	4	7

Table 4. Hematological and non-hematological adverse events

	N = 56		
	All grade (%)	Grade 3 (%)	Grade 4 (%)
Leucopaenia	31 (55)	0	0
Neutropaenia	36 (64)	8 (14)	0
Anemia	35 (63)	2 (4)	0
Thrombocytopaenia	14 (25)	1 (2)	0
AST	17 (30)	0	0
ALT	20 (36)	1 (2)	0
Bilirubinaemia	25 (45)	1 (2)	0
Nausea	42 (75)	1 (2)	0
Vomiting	20 (36)	1 (2)	0
Stomatitis	49 (88)	11 (20)	0
Abdominal pain	18 (32)	0	0
Diarrhea	46 (82)	18 (32)	0
Fatigue	48 (86)	0	0
Anorexia	48 (86)	12 (21)	0
Weight loss	21 (38)	1 (2)	0
Rash	33 (59)	1 (2)	0
Skin exfoliation	21 (38)	0	0
Hand–foot syndrome	4 (7)	0	0
Pigmentation disorder	50 (89)	0	0
Lacrimation increased	18 (32)	1 (2)	0
Dysgeusia	31 (55)	0	0

Numbers are patients who reported events. Severity was graded according to the CTCAE, version 3.0.
AST, aspartate aminotransferase; ALT, alanine aminotransferase; CTCAE, Common Terminology Criteria for Adverse Events.

safety assessment

The most frequent common adverse events are shown in Table 2. Grade 3 toxicity occurred in 35 patients (55%). There was no grade 4 toxicity. Grade 3 toxic effects with an incidence of ≥10% were given to diarrhea (32%), anorexia (21%), stomatitis (20%), and neutropaenia (14%) (Table 4).

The dose had to be decreased at least once in 33 patients (59%). The median number of treatment courses until the dose of S-1 was initially decreased was 2 (range 1–4). The main reasons for dose reductions were diarrhea, stomatitis, and rash. Rest periods were prolonged in 53.6% of the patients, mainly because of diarrhea, stomatitis, and rash, similar to the reasons for dose reductions.

The median times to the onset of diarrhea, stomatitis, and rash were 15 days (range 1–169), 12 days (range 3–201), and 8 days (range 3–148), respectively. The median times to the worst grade of these toxic effects were 20 days (range 1–769), 14 days (range 5–237), and 10 days (range 3–148), respectively. The median times from the worst grade to the resolution of these toxic effects were 7 days (range 1–29), 10 days (range 2–79), and 10 days (range 1–70), respectively.

The reasons for the withdrawal of treatment were mainly disease progression (86%). Withdrawal due to toxic effects was rare (4%) and there were no treatment-related deaths.

discussion

This phase II trial was conducted to evaluate the response rate of the S-1/LV regimen in patients with previously untreated mCRC. All 56 enrolled patients were eligible. S-1/LV regimen yielded promising results, without combination with oxaliplatin, irinotecan, or molecular-target agent as first-line treatment. The response rate, the primary end point of this trial, was 57%. With a median follow-up time of 27.2 months, the median TTP was 6.7 months, the MST was 24.3 months, and survival rates were 86% at 1 year and 52% at 2 years. In previous phase II studies of single-agent S-1, the response rate was 35%–40%, the median TTP was 5.3 months, and the MST was 12 months. In these studies, S-1 was given for 4 weeks, followed by 2 weeks of rest [9, 10]. In our study, the S-1 combined with LV was clearly more effective than S-1 alone, despite a shorter treatment period (2 versus 4 weeks). The antitumor activity of 5-FU is thought to involve the following mechanism: 5-fluoro-2′-deoxyuridine-5′-monophosphate, a metabolite of 5-FU, forms a ternary complex with thymidylate synthase and 5,10-methylenete terahydrofolate, a metabolite of LV. This complex inhibits thymidylate synthase, thereby blocking DNA synthesis [17]. In our study, enhancement of the antitumor activity of S-1 by oral LV is ascribed to this mechanism.

UFT is a derivative of 5-FU which is the same as S-1 and is a compounding oral agent of FT and uracil. *In vitro*, CDHP has been shown to inhibit DPD activity 180-fold higher than uracil [18]. In the previous pharmacokinetic (PK) studies, there was difference in PK profile about 5-FU between S-1 and UFT. Compared with UFT, S-1 showed longer maximum plasma concentration time (T_{max}) (3.5 versus 1.1 h), lower maximum plasma concentration (C_{max}) (128.5 versus 265 ng/ml) and longer half-time ($T_{1/2}$) (1.9 versus 0.34 h). The area under the curve (AUC) of 5-FU were 723.9 ng·h/ml for S-1 ($AUC_{0-14\ h}$) and 338 ng·h/ml for UFT ($AUC_{0-8\ h}$) [19, 20]. In this study, S-1/LV regimen demonstrated higher response rate and longer TTP compared with previously reported UFT/LV [12, 13]. Although these comparisons are limited in value, it was considered that these differences were due to the difference in the inhibitory effect of DPD.

In phase III studies of 5-FU/LV reported in the past decade or so, response rates were 10%–30%, with a PFS/TTP of 4.5–6.0 months. Response rates with FOLFOX or FOLFIRI range from 30% to 55%, with a PFS/TTP of 7.0–8.5 months [21, 22]. XELOX regimen showed response rates of 48% and a PFS of 7.1 months [23]. Although there is limitation to

compare due to the differences in study population between this study (Asians) and referred studies (Western countries), the S-1/LV regimen has one of the highest response rates, despite the absence of oxaliplatin and irinotecan, among currently available regimens not including molecular-targeted drugs such as bevacizumab. The efficacy profiles of S-1 seemed to be generally better in Asian studies than that of others; however, it remained still unclear whether this difference was due to ethnic difference or good selected study population.

Recent clinical trials in patients with mCRC have not only compared treatment regimens but also examined strategies for subsequent treatment. The capecitabine, irinotecan, and oxaliplatin (CAIRO) trial compared sequential chemotherapy (first-line capecitabine, second-line irinotecan, and third-line capecitabine plus oxaliplatin) with combination chemotherapy (first-line capecitabine plus irinotecan and second-line capecitabine plus oxaliplatin). OS did not differ significantly between sequential chemotherapy and combination chemotherapy [hazard ratio (HR) = 0.92; 95% CI 0.79–1.08; $P = 0.33$ by the log-rank test]. Sequential therapy was thus considered a valid treatment option for mCRC [24]. The 5-FU, oxaliplatin, and irinotecan: use and sequencing (FOCUS) trial, compared three different strategies of sequential and combination chemotherapy in patients with unresectable mCRC: single-agent 5-FU (given with levofolinate), followed by single-agent irinotecan (strategy A, control group); 5-FU, followed by combination chemotherapy (strategy B); and combination chemotherapy from the outset (strategy C). Compared with strategy A, strategy B did not significantly prolong survival (HR = 0.94; 95% CI 0.84–1.05; $P = 0.24$ by the log-rank test), whereas strategy C did (HR = 0.88; 95% CI 0.79–0.98, $P = 0.02$ by the log-rank test). There was no significant difference in survival between strategy B and strategy C (HR = 1.06; 90% CI 0.97–1.17). The FOCUS trial concluded that maximum tolerable treatment should be used as first line in the noncurative setting, and the staged approach of initial single-agent treatment upgraded to combination was not inferior to first-line combination therapy. This was an alternative option for discussion with patients [25]. At present, sequential therapy is recognized as a useful alternative for combined therapy.

After the study treatment, 93% of the patients in our study were given subsequent therapy. Of the 56 patients, 36% received oxaliplatin-based chemotherapy, 41% received irinotecan-based chemotherapy, and 5% underwent curative surgery. At the time of starting this trial, neither the FOLFOX nor the FOLFIRI regimen and no bevacizumab was approved in Japan. Grothey et al. [26] found that 5-FU, oxaliplatin, and irinotecan contributed to prolonged survival. In fact, Tournigand et al. [21] reported that survival exceeded 20 months in patients with mCRC who were enrolled in a crossover study of FOLFOX and FOLFIRI. The good survival in our study seems to be attributed to three reasons. First, the relatively recent approval of the FOLFOX and FOLFIRI regimens for use in Japan increased options for subsequent treatment. Secondly, the S-1/LV regimen, an intensive treatment that does not include oxaliplatin or irinotecan, was given as first-line therapy. Thirdly, the S-1/LV regimen was associated with a low rate of treatment withdrawal due to toxicity and a high rate of subsequent therapy; consequently, a high proportion of patients were able to receive sequential chemotherapy.

As for safety, there was no grade 4 toxicity or treatment-related mortality in our study. Common non-hematologic toxic effects included pigmentation, stomatitis, anorexia, fatigue, diarrhea, nausea, rash, and taste disorders. The incidences of grade 3 diarrhea, anorexia, and stomatitis were 32%, 21%, and 20%, respectively. Although these rates are higher than those reported for single-agent S-1 or standard chemotherapy, these toxic effects did not raise treatment discontinuation. The median number of courses until the first decrease in the dose of S-1 was 2 (range 1–4). The dose was decreased in 33 patients (59%). The main reasons for decreases in dose were stomatitis (11 patients), diarrhea (11 patients), and rash (nine patients). Mucositis characterized by stomatitis and diarrhea was considered the DLT of the S-1/LV regimen. Observed DLT was shifted from hematological toxicity to GI toxicity when S-1 was administered with LV. The median time to the onset of the worst grade of diarrhea and stomatitis was 14–20 days after the start of treatment. These toxic effects resolved after 7–10 days. Our experience indicates that toxicity associated with the S-1/LV regimen is manageable by appropriately reducing the dose of S-1 or by extending the rest period between treatment courses. So the S-1/LV regimen was generally well tolerated, with an acceptable toxicity profile.

Both S-1 and LV are administered orally, so this regimen does not require a central venous port. Patients therefore have to spend less time on follow-up visits, and the convenience of oral administration makes the S-1/LV regimen extremely useful clinically. Another advantage is the low incidence of hand–foot syndrome, the most common toxicity of capecitabine, another oral fluoropyrimidine.

In phase I/II studies of S-1 combined with oxaliplatin (SOX regimen), S-1 was given for 2 weeks at the conventional dose in Japan, similar to our S-1/LV regimen, followed by 1 week of rest. Oxaliplatin (130 mg/m^2) was given on day 1, within a 3-week cycle. The SOX regimen was very effective, with a response rate of 50% and a median PFS of 6.4 months. The most common toxicity of grade 3 or higher was thrombocytopaenia, typically associated with oxaliplatin [27]. Since the DLT of S-1/LV regimen was mucositis such as diarrhea and stomatitis, the combination with oxaliplatin, the toxicity profile of which does not overlap with that of S-1/LV, may be more appropriate than irinotecan for the treatment of metastatic disease requiring intensive chemotherapy. The preliminary results of a phase I study evaluating the S-1/LV regimen plus oxaliplatin have been reported. S-1/LV was given for 1 week followed by 1 week of rest, and oxaliplatin was given every 2 weeks (SOL regimen). The S-1/LV regimen was administered at the standard dose in Japan; the recommended dose of oxaliplatin was determined to be 85 mg/m^2. In that phase I study, five (83%) of the six patients who received the recommended dose of S-1, LV, and oxaliplatin had PRs. DLTs (grade 3 diarrhea and grade 3 hypertension) occurred in one of the six patients [28]. As for combinations of S-1 and irinotecan, a phase III study is going on to compare survival between the FOLFIRI regimen and S-1 plus irinotecan (IRIS

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regimen), given as second-line treatment. The results are scheduled to be available in the near future.

Our results indicate that the S-1/LV regimen is a promising treatment of mCRC. On the basis of these preliminary data, further clinical trials of S-1/LV-based chemotherapy are going on. After the completion of these trials, phase III studies are promptly required to validate the clinical usefulness of S-1/LV-based chemotherapy.

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Microsatellite instability-low colorectal cancer acquires a *KRAS* mutation during the progression from Dukes' A to Dukes' B

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The classification of colorectal cancer (CRC) by microsatellite instability (MSI) status is important for effective clinical management. In fact, microsatellite instability-high (MSI-H) cancer has distinctive clinicopathological and molecular features. However, microsatellite instability-low (MSI-L) cancer is not clearly defined. The objective of this study was to further clarify the characteristics of MSI-L CRC. A consecutive series of 940 primary CRCs were subdivided into three groups according to the level of MSI and analyzed the clinicopathological features and genetic changes in the *KRAS*, *BRAF* and *p53* mutation and the loss of heterozygosity (LOH) of adenomatous polyposis coli (*APC*) gene and methylation status of the *O*^{6}-methylguanine-DNA methyltransferase (*MGMT*) and *MLH1* promoter. Of the 940 CRCs, 5.9% were MSI-H, 7.1% were MSI-L and 87% were microsatellite stable (MSS). *KRAS* and *BRAF* mutations were detected in 39.4 and 4.6% of the CRCs, respectively. The frequency of *KRAS* mutations in MSI-H, MSI-L and MSS cancer was 30, 48 and 39%, respectively. The proportion of *KRAS* mutations in MSI-L cancer increased from 16 to 63% accompanying the progression from Dukes' A to Dukes' B. While the LOH of D5S346, which is located near the *APC* gene, and *p53* mutation was observed in 75 and 67% of MSI-L CRC at Dukes' A, respectively. These results indicated that the LOH of *APC* and *p53* mutation has already occurred by the Dukes' A lake 'suppressor pathway' but not the *KRAS* mutation in MSI-L CRCs. The genes involving MSI-L carcinogenesis are similar to MSS but the timing and frequency of the *KRAS* mutation is different.

Introduction

There are two types of genomic instability, microsatellite instability (MSI or MIN) or chromosomal instability associated with the carcinogenesis process of colorectal cancer (CRC) (1). The great majority of CRCs develop through the chromosomal instability pathway (also called the 'suppressor pathway'), which arise from adenomas and is initiated by the inactivated adenomatous polyposis coli (*APC*) gene and followed by the well-established genetic steps involved in the adenoma–carcinoma sequence (2,3). While another type of genomic instability, MSI caused by a failure of the DNA mismatch repair (MMR) system, is observed in ~10% of all CRCs (4–7). DNA MMR deficiency leads mutations in the target genes that are implicated in tumor progression such as *TGFbetaR2* (8), *IGF2R* (9), *CDX2* (10) and *BAX* (11) and it is known as the 'mutator pathway'. MSI can be subdivided into three groups, microsatellite instability-high (MSI-H), microsatellite instability-low (MSI-L) and microsatellite stable (MSS), according to the degree of instability. The recommended method to distinguish these subgroups is to analyze paired tumor and normal tissue DNAs using a panel of five microsatellite markers known as the Bethesda panel (12).

The MSI-H CRCs phenotype is more likely to occur at a proximal site, to occur in women, to be associated with a favorable prognosis (5,7,13–15) and severe inflammatory cell infiltration into the tumor tissue (16,17). A large percent of MSI-H CRC is sporadic and demonstrates somatic promoter methylation of the *hMLH1* gene (18,19), whereas a germ line mutation of the MMR genes, such as *hMSH2*, *hMLH1*, *hMSH6* and *hPMS2*, is found in the majority of MSI-H CRC without *hMLH1* promoter methylation and is known as Lynch syndrome/hereditary non-polyposis colorectal cancer (20–23). Recent morphological and molecular studies have proposed the existence of a serrated pathway, thus suggesting that serrated polyps may serve as a precursor of the MSI-positive cancers (24–26).

On the other hand, most studies have found no obvious clinicopathological or molecular differences between MSI-L and MSS cancers (27). The DNA MMR genes *hMLH1* and *hMLH2* do not appear to be implicated in the MSI-L subset (28). Some studies have reported that MSI-L is associated with cancers from individuals with germ line mutations of *hMLH6* (29), but genetic alteration of this gene is infrequent in MSI-L CRC patients. Furthermore, some researchers deny the presence of MSI-L cancers because most non-MSI-H cancers exhibit MSI-L when large numbers of microsatellite loci are tested (27,30).

Meanwhile, there is evidence indicating that the MSI-L phenotype could reflect a distinct pathway of tumor development with a different clinical behavior and different genetic and epigenetic changes. For example, a high frequency of a *KRAS* mutation (31,32) that is associated with loss of expression of the *O*^{6}-methylguanine-DNA methyltransferase (*MGMT*) gene by methylation of its promoter region (33), lower frequency of 5qLOH (31), a high frequency of *APC* mutation (34) and reduced expression of Bcl-2 protein (35) are global molecular phenotypes by which MSI-L cancers are distinguished from non-MSI-L cancers.

Therefore, MSI-L CRC is still controversial. This study investigated the genetic changes and clinicopathological features of MSI-L CRCs using a series of 940 CRCs.

Abbreviations: APC, adenomatous polyposis coli; CI, confidence interval; CRC, colorectal cancer; HR, hazard ratio; LOH, loss of heterozygosity; MGMT, *O*^{6}-methylguanine-DNA methyltransferase; MMR, mismatch repair; MSI, microsatellite instability; MSI-H, microsatellite instability-high; MSI-L, microsatellite instability-low; MSS, microsatellite stable; PCR, polymerase chain reaction.

Materials and methods

Patients and tissue samples

A consecutive series of 940 primary CRCs excised surgically at Saitama Cancer Center from January 1998 to May 2006 were investigated after obtaining the informed consent. Any patients who were treated by preoperative radiotherapy or chemotherapy were excluded. Furthermore, patients with inflammatory bowel disease or a known history of familial adenomatous polyposis were also excluded. This study was approved by the Ethics Committee of the Saitama Cancer Center.

Analysis of MSI

Primary CRCs and paired normal colorectal mucosa obtained by surgery were immediately frozen at −80°C. The genomic DNA was extracted from fresh frozen specimens using standard methods. The Bethesda five markers, BAT25, BAT26, D5S346, D2S123 and D17S250, were used to classify the MSI status of the tumors. Polymerase chain reaction (PCR) and subsequent analyses were performed as reported previously (5). CRCs were subdivided into three groups according to the degree of MSI; MSI-H if two or more of the five markers show instability, MSI-L if only one marker shows instability and MSS if absence of MSI in all five markers. MSI-positive markers were re-examined at least twice to confirm the result. Loss of heterozygosity (LOH) was defined by at least a 30% reduction in the relative intensity of one allele in the tumor in comparison with normal levels.

Analysis of KRAS, BRAF and p53 mutation

The mutations in exon 1 and 2 of the *KRAS* gene were analyzed by denaturing gradient gel electrophoresis as described previously (36).

The *BRAF* V600E mutation was examined using PCR combined with restriction enzyme digestion. DNA fragments containing exon 15 of the *BRAF* gene were amplified by PCR using the following oligonucleotide primers: *BRAF* forward primer 5'-CTGTTTTCCTTTACTTACTACACC-3' and *BRAF* reverse primer 5'-CTGTTCAAACTGATGGGACC-3'. PCR amplification was carried out with 100 ng of genomic DNA in a volume of 20 μl containing 0.2 μM deoxynucleoside triphosphate, 0.1 μM each of primers and 1 U Taq Gold. Thermal cycling was initiated with denaturation at 94°C for 10 min followed by 37 three-step cycles at 94°C for 30 s, 59°C for 30 s and 72°C for 45 s and followed by a final incubation for 7 min at 72°C. PCR products were digested with HpyCH4III at 37°C for 1 h and analyzed on 8% polyacrylamide gels.

The mutations in exon 5–8 of p53 gene were analyzed by denaturing gradient gel electrophoresis as described previously (37).

Analysis of hMLH1 and MGMT promoter methylation

hMLH1 and *MGMT* promoter methylation was analyzed in 55 of MSI-H cancer and 67 of MSI-L cancer samples. The methylation status of each gene was determined by the methods previously reported (5). The primers were *hMLH1* methylation specific, 5'-AACGAATTAATAGGAAGAGGCGGATAGCG-3' and 5'-CGTCCCTCCCTAAAACGACTACTACCC-3'; *hMLH1* unmethylation specific, 5'-TAAAAATGAATTAATAGGAAGAGTGGATAGTG-3' and 5'-AATCTCTTCATCCCTCCCTAAAACA-3'; *MGMT* methylation specific, 5'-TTTCGACGTTCGTAGGTTTTCGC-3' and 5'-GCACTCTTCCGAAAACGAAACG-3' and *MGMT* unmethylation specific, 5'-TTTGTGTTTTGATGTTTGTAGGTTTTTGT-3' and 5'-AACTCCACACTCTTCCAAAAACAAAACA-3'.

Statistical analysis

Differences were assessed using the chi-square or Fisher's exact test for categorical variable and unpaired Student's *t*-test for continuous factors. The overall survival was defined as the interval from the date of resection until the date of death from any cause, censored patients being those alive at the close of the study or lost to follow-up. Survival was measured from the date of the resection of the CRCs until death or until the censor date of 1 July 2006. The distribution of survival time was compared with the use of the log-rank test; survival distribution curves were estimated by the method of Kaplan–Meier. Multivariate analyses were performed with the use of the Cox proportion hazard model. The independent prognostic factors for survival were determined by a stepwise backward conditional selection in which the non-significant factors ($P > 0.1$) were successively rejected. All statistical analyses were performed using the StatView 5.5 program. $P < 0.05$ was considered to be statistically significant in all cases.

Results

MSI status

The Bethesda panel, BAT25, BAT26, D5S346, D2S123 and D17S250, was used to classify the MSI status of the tumors. Of the 940 CRCs, 55 (5.9%) were MSI-H, 67 (7.1%) were MSI-L and 818 (87%) were MSS (Table I). Mononucleotide marker BAT25 and BAT26 exhibited

Table I. Clinicopathological and genetic features of CRCs

	MSS, *n* (%)	MSI-L, *n* (%)	MSI-H, *n* (%)	*P* value		
				MSI-L versus MSS	MSI-H versus MSS	MSI-L versus MSI-H
Patient	818 (87.0)	67 (7.1)	55 (5.9)	0.3723	0.0011	0.0661
Men	509 (62.2)	38 (56.7)	22 (40)			
Women	309 (37.8)	29 (43.3)	33 (60)			
Mean (±SE) age	63.6 ± 10.3	63.3 ± 9.95	60.5 ± 13.4	0.785	0.035	0.199
Location				0.44	<0.0001	0.0003
Proximal	217 (26.5)	22 (32.8)	36 (65.5)			
Distal	243 (29.7)	16 (23.9)	12 (21.8)			
Rectum	358 (43.8)	29 (43.3)	7 (12.7)			
Tumor size				0.23	<0.0001	<0.0001
Mean ± standard error (mm)	45.42 ± 24.1	41.8 ± 23.0	61.35 ± 32.3			
Histologic feature				>0.999[a]	<0.0001[a]	0.0018[a]
Well differentiated	73 (8.9)	10 (14.9)	4 (7.3)			
Moderately differentiated	706 (86.3)	54 (80.6)	38 (69.1)			
Poorly differentiated	16 (2.0)	3 (4.5)	10 (18.2)			
Mucinous	21 (2.6)	0 (0)	2 (3.6)			
Others	2 (0.2)	0 (0)	1 (1.8)			
Mucinous component				0.73	<0.0001	0.001
+	84 (10.3)	6 (9.0)	18 (32.7)			
–	734 (89.7)	61 (91.0)	37 (67.3)			
Dukes' stage				0.22	0.012	0.039
A	150 (18.3)	19 (28.4)	12 (21.8)			
B	246 (30.1)	16 (23.9)	26 (47.3)			
C	246 (30.1)	20 (29.9)	13 (23.6)			
D	176 (21.5)	12 (17.9)	4 (7.3)			
Depth of tumor invasion				0.27	0.073	0.42
T1	68 (8.3)	8 (11.9)	5 (9.1)			
T2	123 (15.0)	14 (20.9)	7 (12.7)			
T3	571 (69.8)	39 (58.2)	34 (61.8)			
T4	56 (6.8)	6 (9.0)	9 (16.4)			
Extramural venous invasion				0.99	0.69	0.76
+	586 (71.6)	48 (71.6)	38 (69.1)			
–	232 (28.4)	19 (28.4)	17 (30.9)			
KRAS mutation				0.1836	0.1798	0.051
+	311 (39.5)	32 (47.8)	16 (30.2)			
–	477 (60.5)	35 (52.2)	37 (69.8)			
BRAF mutation				0.3885	<0.0001	<0.0001
+	21 (2.7)	3 (4.5)	17 (32.1)			
–	767 (97.3)	64 (95.5)	36 (67.9)			

[a]Well and moderately differentiated versus mucinous and poorly differentiated.

instability in 95% of MSI-H CRCs whereas in 1–3% of MSI-L CRCs. Therefore, BAT25 and BAT26 were identified to be the most specific and sensitive markers to detect MSI-H CRCs. The dinucleotide marker D2S123 exhibited instability not only in 95% of the MSI-H CRCs but also in 56.7% of MSI-L. D2S123 is the most sensitive but not specific for MSI-L (Figure 1A).

Clinicopathological features

The association of MSI status with the clinicopathological features in the 940 CRCs is shown in Table I. Consistent with the findings of previous studies, MSI-H cancers are observed more frequently in females, in the proximal colon and in poorly differentiated or mucinous CRCs in comparison with MSS. While some differences were observed between MSI-L and MSS cancer, with regard to the female to male ratio, the site of the tumor and the stage did not reach significance.

The prognosis was assessed based on the MSI status (Figure 1B). Since no Dukes' A patients died during the follow-up period, these patients were excluded from the overall survival analysis. In total, 155 of the 731 Dukes' B–D patients (21.2%) died during a mean follow-up period of 30.3 ± 19 months after surgery. The prognosis of patients with MSI-H tumors was significantly better than that of patients with MSS tumors (log-rank test, $P = 0.0335$). The prognosis of patient with MSI-L tumors had an intermediate tendency among the three groups (Figure 1B).

In a stepwise multivariate analysis, age {hazard ratio (HR) 1.627 [confidence interval (CI) 1.216–2.301]; $P = 0.0016$}, men sex [HR 1.429 (CI 1.019–2.004); $P = 0.0388$], low-grade pathology [HR 2.029 (CI 1.231–3.343); $P = 0.0055$], *KRAS* [HR 1.69 (CI 1.215–2.351); $P = 0.0018$], *BRAF* [HR 3.593 (CI 1.933–6.678); $P < 0.0001$] and Dukes' stage [Dukes' B versus Dukes' C: HR 1.636 (CI 0.964–2.775); $P = 0.068$ and Dukes' B versus Dukes' D: HR 10.406 (CI 6.548–16.537); $P < 0.0001$] were independent variables. However, MSI was not an independent variable.

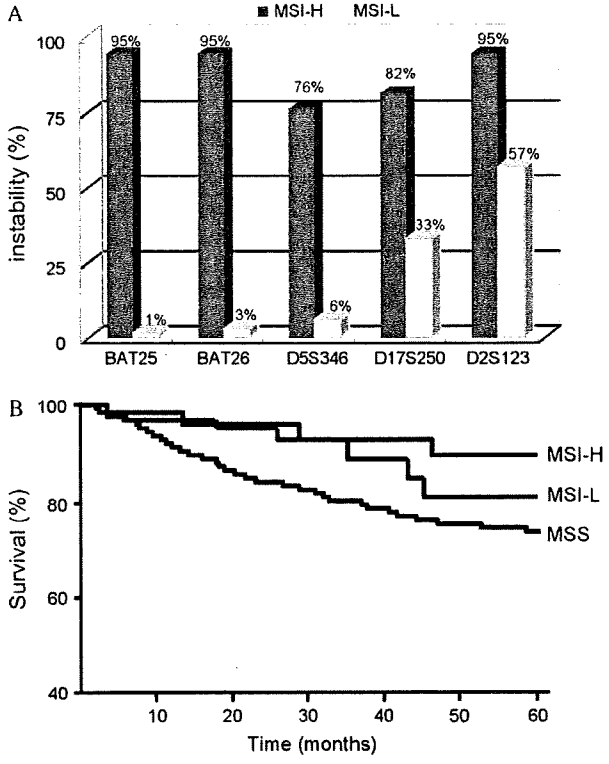

Fig. 1. **(A)** Frequency of markers demonstrated instability for MSI-H and MSI-L CRCs. **(B)** Overall survival of patients according to the MSI status. In total, 155 of the 731 Dukes' B–D patients (21.2%) died during a mean follow-up period of 30.3 ± 19 months after surgery.

Mutation analysis of the KRAS and BRAF genes

A *KRAS* mutation was detected in 39.4% and a *BRAF* V600E mutation in 4.6% of the 905 CRCs that were examined. The *BRAF* mutation was found more frequently in MSI-H cancer (32%) in comparison with MSS (3%) and MSI-L cancers (4%; $P < 0.0001$, Figure 2A). The frequency of *BRAF* mutation decreased accompanying the tumor progression in MSI-H cancer, whereas it increased in MSI-L and MSS cancers (Figure 2D–F).

The *KRAS* mutation analysis in CRCs demonstrated that MSI-L cancer showed higher frequency of the *KRAS* mutation than MSS and MSI-H cancers: MSS 39% (311/788), MSI-H 30% (16/53) and MSI-L 48% (32/67; MSI-L versus MSI-H; $P = 0.066$, MSI-L versus MSS; $P = 0.244$, MSI-H versus MSS; $P = 0.180$; Figure 2A). However, accompanying the progression from Dukes' A to Dukes' B, the frequency of the *KRAS* mutation in MSI-L cancer drastically increased from 16 to 63% (Figure 2E, MSI-L; *KRAS* mutation in Dukes A versus *KRAS* mutation in Dukes B–D, $P = 0.045$, Fisher's exact test) and was significantly higher than that in MSS or MSI-H cancers at Dukes' B–D (MSI-L versus MSS or MSI-H; $P = 0.014$, $P = 0.0394$, respectively; Figure 2C). MSI-H cancer also demonstrated an increased proportion of the *KRAS* mutation accompanying the progression from Dukes' A to Dukes' B (Figure 2F), but the number of MSI-H cases was too small to find significance (MSI-H; *KRAS* mutation in Dukes' A versus *KRAS* mutation in Dukes' B–D, $P = 0.08$, Fisher's exact test). The ratio of the *KRAS* mutation in MSI-H cancer was the same as that in MSS cancer after Dukes' B stage (Figure 2C). The ratio of tumors having either the *KRAS* or *BRAF* mutation at Dukes' B–D in MSI-H and MSI-L cancers was statistically higher than that in MSS cancer [MSS (40%) versus MSI-L (66%) or MSI-H (63%); $P = 0.0034$, $P = 0.0108$, respectively; Figure 2C].

Type of KRAS mutation

Of the 321 tumors with *KRAS* mutations, 196 (61%) were a G to A transition, 107 (33%) were a G to T transversion and 18 (6%) were a G to C transversion. The type of KRAS mutation was investigated in each MSI status. This revealed that 93% (14 of 15 tumors) of the *KRAS* mutations were a G to A transition in MSI-H cancer and there were significant differences between the types of *KRAS* mutations among the three MSI groups ($P = 0.0152$, chi-square test). The frequency of G to A transition mutations in MSI-L cancer was lower than in MSI-H but higher than in MSS cancer (Figure 2G). To investigate whether the high frequency of G to A transition mutation of *KRAS* gene in MSI-H and MSI-L cancer is involved in the inactivation of *hMLH1* or *MGMT*, the methylation status of the *hMLH1* and *MGMT* promoter was analyzed. Of the 30 MSI-L tumors with *KRAS* mutations, 13 (43%) had *MGMT* promoter methylation, whereas among 35 MSI-L tumors without *KRAS* mutations, 10 (29%) had it (Table II). Furthermore, 53% (10 of 19) of MSI-L tumors with G to A transition mutations in *KRAS* harbored *MGMT* promoter methylation, whereas 30% (three of 10) of MSI-L tumors with G to C or T transversion mutations in *KRAS* and 29% (10 of 35) of MSI-L tumors without a *KRAS* mutation showed *MGMT* promoter methylation (G to A versus G to C or T and wild-type, $P = 0.0705$; Table III).

These results suggest that G to A transition mutations in MSI-L tumors seem to correlate with *MGMT* promoter methylation ($P = 0.0705$). On the other hand, the frequency of *MGMT* methylation was observed in 38% of MSI-H tumors with *KRAS* mutation and 56% of MSI-H tumor without KRAS mutations. This result suggests that there is an inverse correlation between *KRAS* mutations and *MGMT* methylation in MSI-H tumors ($P = 0.2026$; Table II).

The frequency of *hMLH1* methylation was observed 25% of MSI-H tumors with *KRAS* mutations and 59% of MSI-H tumors without KRAS mutations. This result clearly shows a significant inverse correlation between *KRAS* mutations and *hMLH1* methylation in MSI-H tumors ($P = 0.0366$). The frequency of *hMLH1* methylation was

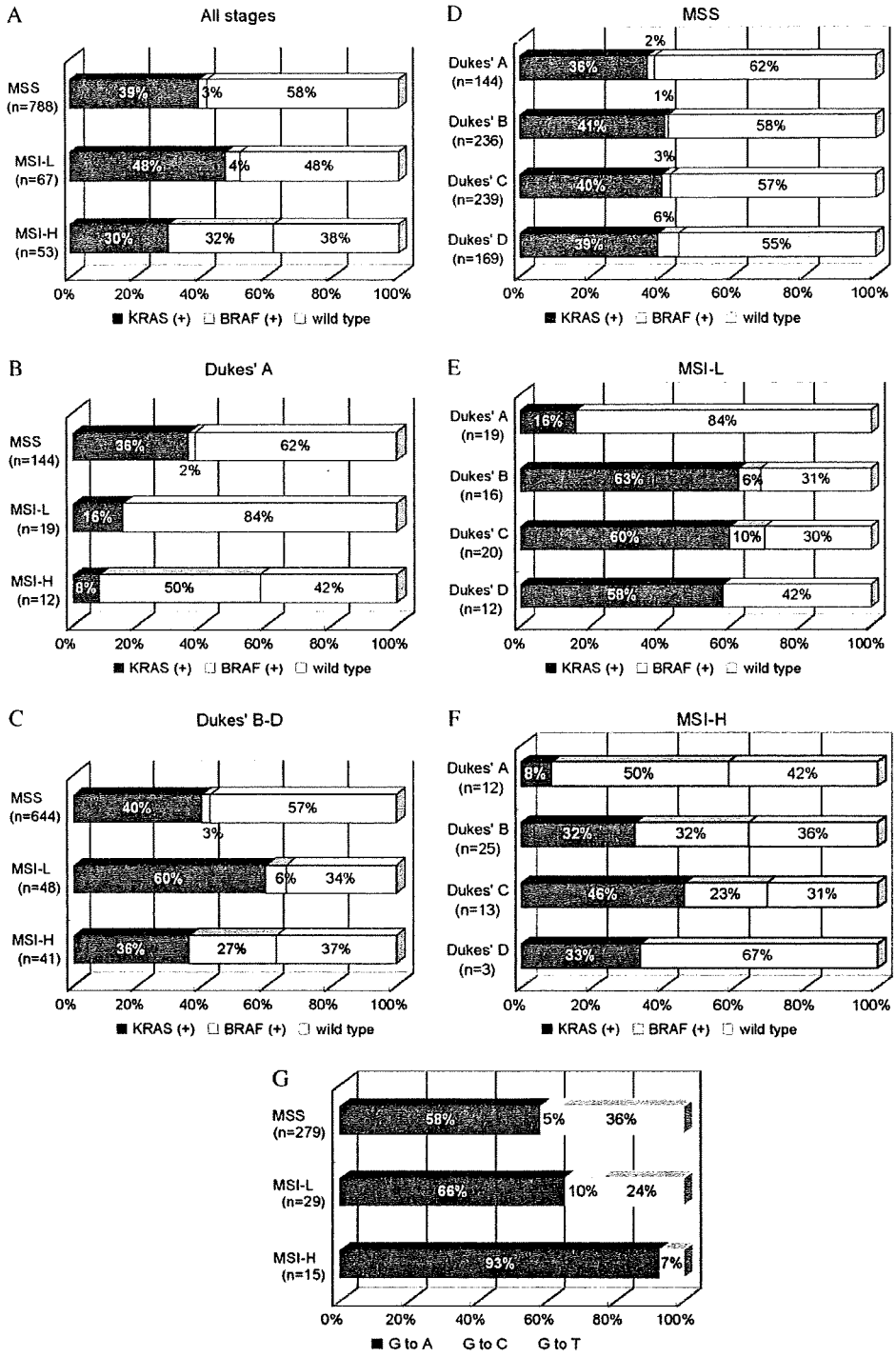

Fig. 2. *KRAS* and *BRAF* mutations in each MSI status. **(A)** *KRAS* and *BRAF* mutation of all CRCs. **(B)** *KRAS* and *BRAF* mutation of Dukes' A CRCs. **(C)** *KRAS* and *BRAF* mutation of Dukes' B–D CRCs. Frequency of *KRAS* and *BRAF* mutations at each stage according to the MSI status; **(D)** MSS, **(E)** MSI-L and **(F)** MSI-H. **(G)** Spectrum of KRAS mutations in each MSI status.

significantly higher in MSI-H tumors (50%) than in MSI-L tumor (6.7%; $P < 0.0001$; Table II).

LOH of D5S346 and p53 mutation in MSI-L CRCs

Since D5S346, one of the MSI makers, locates near the *APC* gene, MSI analysis with the Bethesda panel can also assess the LOH of *APC* gene, simultaneously. LOH of the D5S346 and *p53* mutation was detected in 75% (9/12) and 67% (12/18) of MSI-L CRC at Dukes' A, respectively (Table IV). In addition, the frequency of LOH of D5S346 and *p53* mutations in MSI-L at Dukes' B–D were 55 and 61%, respectively. These results indicate that LOH of *APC* and *p53* mutations has already occurred before Dukes' A.

Table II. Promoter methylation and KRAS mutation

		KRAS mut, *n* (%)	Wt, *n* (%)	*P* value
	MGMT			
MSI-L	M	13 (43)	10 (29)	0.2147
	U	17 (57)	25 (71)	
MSI-H	M	6 (38)	22 (56)	0.2026
	U	10 (62)	17 (44)	
	hMLH1			
MSI-L	M	2 (7)	2 (6)	>0.9999
	U	28 (93)	33 (94)	
MSI-H	M	4 (25)	23 (59)	0.0366
	U	12 (75)	16 (41)	

M, methylated; U, unmethylated; mut, mutation; Wt, wild-type.

Table III. Type of KRAS mutation according to MGMT methylation

	MGMT	KRAS mutation		Wt, *n* (%)	*P* value
		G to A, *n* (%)	G to C, T; *n* (%)		
MSI-L	M	10 (53)	3 (30)	10 (29)	0.0705
	U	9 (47)	7 (70)	25 (71)	
MSI-H	M	6 (43)	0 (0)	22 (56)	0.4339
	U	8 (57)	1 (100)	17 (44)	

M, methylated; U, unmathylated; Wt, wild type. *P* : G to A versus G to C, T + Wt.

Discussion

Molecular features

Although the *BRAF* and *KRAS* mutations are found more frequently in MSI-H and MSI-L CRC, respectively (31,34,38), the frequency of *KRAS* and *BRAF* mutation changed between each tumor stage in this study.

The development of CRC requires a multistep process characterized by the accumulation of genetic alterations. According to the well-known genetic model for colorectal tumorigenesis proposed by Fearon and Vogelstein, *KRAS* mutations occur in the early to intermediate adenomas (3). However, the frequency of *KRAS* mutations was significantly lower (16%) at Dukes' A and higher (60%) at Dukes' B–D in MSI-L CRCs. This means that most *KRAS* mutations occurred at different times in MSI-L CRC, namely, during the progression from Dukes' A to Dukes' B but not in early to intermediate adenomas. It has been reported previously that the *KRAS* mutation is found more frequently in MSI-L CRCs (31,32), but according to the current detailed study, it depends on the tumor stage. Since a large number of specimens were collected in an unbiased manner for this study, the results demonstrate representative findings of CRCs in Japan.

Meanwhile LOH of D5S346, which is located near the *APC* gene, and the *p53* mutation was observed in 75% (9/12) and 67% (12/18) of MSI-L CRC at Dukes' A, respectively. These frequencies were almost the same at Dukes' B–D in MSI-L CRC (Table IV).

Taken together, these findings indicated that LOH of *APC* and *p53* mutations have already occurred by the Dukes' A lake suppressor pathway but not the *KRAS* mutation in MSI-L CRCs.

MSI-L CRC may develop through a mild mutator pathway, which differs from the suppressor and mutator pathway and show different clinical features (31,39). However, some studies doubt the presence of the MSI-L group (27,30). In the current study, the involved genes such as LOH of *APC*, *KRAS* and *p53* mutation in MSI-L CRCs are similar to those in MSS CRCs, but at least the timing and frequency of the *KRAS* mutation is different. This may explain why the clinicopathological features of MSI-L tumors are similar to those of MSS tumors but not completely identical.

Table IV. LOH of D5S346 and p53 mutation in MSI-L CRCs

Dukes' stage	D5S346 LOH	p53 mutation
A	75% (9/12)	67% (12/18)
B	46% (6/13)	57% (12/21)
C	67% (8/12)	45% (10/22)
D	50% (3/6)	92% (12/13)

On the other hand, considering the presence of the *BRAF* mutation and methylation of the *hMLH1* promoter at the early stage in MSI-H CRC, these genetic changes should occur in the precursor of MSI-H CRC. This is not inconsistent with the concept of serrated pathways resulting from serrated polyps that were revealed in recent morphological and molecular studies (24–26,40–42).

The mechanism of the *KRAS* mutation was also analyzed and the results showed that a G to A transition mutation of *KRAS* occurs more frequently in MSI-L than MSS. Some reports demonstrated that *MGMT* inactivation by promoter methylation causes a G to A transition mutation of *KRAS* (33,43,44) and *p53* (45) and such a mutation is frequently observed in MSI-L CRCs. We attempted to determine whether or not the inactivation of *MGMT* by promoter methylation is associated with the type and frequency of *KRAS* mutation. Our results indicated that *MGMT* promoter methylation seems to affect the G to A transition and frequency of the KRAS mutation in MSI-L CRC. However, most *KRAS* mutations in MSI-H CRC show a G to A transition, *MGMT* inactivation was inversely related and the *BRAF* mutation often observed in MSI-H CRC shows a T to A transversion. Considering these results, a different mechanism might therefore be involved in mutation between MSI-L and MSI-H CRC.

Clinical feature

As mention above, the genes associated with developing MSI-L CRC are similar to the suppressor pathway but the frequency and timing of KRAS mutations is different; thus, there may be different clinical and pathological features in MSI-L.

Comparing the stage distribution for each MSI status, the distribution of Dukes' B in MSI-H CRC is significantly larger than in MSS and MSI-L CRC (7). Gyef *et al.* (15) demonstrated with a logistic analysis that MSI-H CRC is less metastatic to the regional lymph nodes and distant organs than MSS CRC, even though their depth of tumor invasion is same. The same result was observed in the current study, but MSI-L CRC did not show this characteristic. This suggests that there is a mechanism restricting the progression from Dukes' B to C in MSI-H cancer. Although the precise explanation for this mechanism is still unknown, tumor-infiltrating lymphocytes, apoptosis, proliferative activity (46,47) or a mutation of *p53* (7) may lead to the 'restraining effect'.

On the other hand, the distribution of Dukes' A in MSI-L CRC is larger than MSS. Considering the *KRAS* mutation during the progression from Dukes' A to B in MSI-L CRC, tumor progression may be hindered until the occurrence of the *KRAS* mutation in MSI-L CRC.

Various studies have reported the prognosis of each MSI status. Some investigations show that the patients with MSI-H cancer demonstrate a better prognosis and the patients with MSI-L cancer have a poorer survival than patients with MSS in stage C (48,49). Considering the high frequency of the *KRAS* mutation after Dukes' B in MSI-L cancer, the worse prognosis of such patients may therefore be reasonable (50).

Although the number of cases in the current study was not sufficient to study the prognosis at each stage, among all patients with Dukes' B to D CRCs, MSI-H patients showed significantly better survival than MSS (log-rank test, $P = 0.0335$) while MSI-L patients had a slightly better prognosis than MSS. These findings may result from the fact that the proportion of Dukes' D for each MSI status is smaller, in order, MSI-H, MSI-L and MSS.

Furthermore, MSI-L tumors in the current series developed more frequently in females and in the proximal colon than MSS tumors, although no significant difference was observed.

In this study, a series of 940 patients with CRCs suggests that MSI-H, MSI-L and MSS cancer each progress through different pathways. Further study on these themes will probably attempt to clarify not only MSI cancer but also try to elucidate the true nature of CRCs itself.

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ORIGINAL PAPER

Nucleolin as cell surface receptor for tumor necrosis factor-α inducing protein: a carcinogenic factor of *Helicobacter pylori*

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Abstract
Purpose Tumor necrosis factor-α inducing protein (Tipα) is a unique carcinogenic factor released from *Helicobacter pylori* (*H. pylori*). Tipα specifically binds to cells and is incorporated into cytosol and nucleus, where it strongly induces expression of *TNF-α* and *chemokine* genes mediated through NF-κB activation, resulting in tumor development. To elucidate mechanism of action of Tipα, we studied a binding protein of Tipα in gastric epithelial cells.
Methods Tipα binding protein was found in cell lysates of mouse gastric cancer cell line MGT-40 by FLAG-pull down assay and identified to be cell surface nucleolin by flow cytometry using anti-nucleolin antibody. Incorporation of Tipα into the cells was determined by Western blotting and expression of *TNF-α* gene was quantified by RT-PCR.
Results Nucleolin was co-precipitated with Tipα-FLAG, but not with del-Tipα-FLAG (an inactive mutant). After treatment with Tipα-FLAG, incorporated Tipα was co-immunoprecipitated with endogenous nucleolin using anti-nucleolin antibody. The direct binding of Tipα to recombinant His-tagged nucleolin fragment (284–710) was also confirmed. Although nucleolin is an abundant non-ribosomal protein of the nucleolus, we found that nucleolin is present on the cell surface of MGT-40 cells. Pretreatment with anti-nucleolin antibody enhanced Tipα-incorporation into the cells through nucleolin internalization. In addition, pretreatment with tunicamycin, an inhibitor of N-glycosylation, decreased the amounts of cell surface nucleolin and inhibited both internalization of Tipα and expression of *TNF-α* gene.
Conclusions All the results indicate that nucleolin acts as a receptor for Tipα and shuttles Tipα from cell surface to cytosol and nuclei. These findings provide a new mechanistic insight into gastric cancer development with Tipα.

Keywords Gastric cancer · TNF-α · NF-κB · *Helicobacter pylori* · Tumor promotion

Abbreviations

H. pylori	*Helicobacter pylori*
Tipα	TNF-α inducing protein
CagA	Cytotoxin associated antigen
LC–MS	Liquid chromatography–mass spectrometry
RBD	RNA binding domain
NF-κB	Nuclear factor-kappa B
NEMO	NF-κB essential modulator

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Introduction

Helicobacter pylori (*H. pylori*) is a gram-negative bacterium that colonizes in the mucosa of human stomach, resulting in induction of chronic gastritis, peptic ulcer, and stomach cancer (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans 1994, Peek and Blaser 2002). Key criteria of these clinical outcomes are the severity and persistence of inflammation caused by *H. pylori*-infection, associated with strong induction of inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukine-1 (IL-1) and chemokines (El-Omar et al. 2000; Peek 2008; Snaith and El-Omar 2008). It is well accepted that inflammatory cytokines contribute to maintain cancer microenvironment (Balkwill 2009; El-Omar et al. 2003), and among the inflammatory cytokines, TNF-α plays a master role as an endogenous tumor promoter in carcinogenesis (Balkwill 2009; Moore et al. 1999; Suganuma et al. 1999). Moreover, TNF-α released from the cells acts as an instigator of a cytokine network sequence, from TNF-α to IL-1 and IL-6 and back to TNF-α, maintaining inflammation in the process of tumor promotion (Suganuma et al. 2002).

To extend the concept, a new gene, *TNF-α inducing protein* (*Tipα*) gene, was cloned from the genome of *H. pylori* strain 26695. Tipα directly induces *TNF-α* gene expression in gastric epithelial cells (Suganuma et al. 2005, 2006, 2008). The unique features of Tipα protein are as follows: (1) *H. pylori* lacking *Tipα* gene reduced the colonization levels of *H. pylori* in the stomach of mice (Godlewska et al. 2008); (2) Vaccination with Tipα significantly reduced colonization of *H. pylori* in mice associated with high levels of Tipα-specific antibody (Inoue et al. 2009); (3) Tipα protein is secreted from *H. pylori* but not mediated through Type IV secretion system (Suganuma et al. 2005); and (4) clinical isolates of *H. pylori* obtained from gastric cancer patients secreted Tipα protein in larger amounts than did *H. pylori* from patients with simple gastritis (Suganuma et al. 2008), strongly suggesting that Tipα plays an important role in *H. pylori*-induced inflammation and cancer development in human stomach (Balkwill 2009). All these features are different from those of other virulence factors, such as the cag pathogenicity island (cagPAI), CagA (cytotoxin associated antigen) and VacA (vacuolating cytotoxin A).

Members of the *Tipα* gene family include *Tipα* itself, *H. pylori-membrane protein 1* (*HP-MP1*), and *jph0543*, and these do not have any obvious homologues in other species (Suganuma et al. 2005; Yoshida et al. 1999). Tipα protein consists of 172 amino acids with a molecular weight of 19 kDa, and it forms a homodimer via two disulfide bonds with two cysteine residues in the N-terminal region. We previously reported that homodimer formation of Tipα is essential for induction of *TNF-α* gene expression in gastric epithelial cells (Suganuma et al. 2008) and also for transformation of Bhas 42 (v-H-*ras* transfected BALB/3T3) cells (Suganuma et al. 2005). To extend our experiments, we made two inactive Tipα mutants: a deletion mutant of Tipα (del-Tipα) that deleted six amino acids including two cysteine residues from native Tipα, and C5A/C7A double mutant (C5A/C7A-Tipα), two cysteine residues of Tipα are replaced by two alanines. The two mutated Tipα proteins induced *TNF-α* gene expression less strongly than native Tipα did (Suganuma et al. 2008). The crystal structures of del-Tipα and truncated forms of Tipα were recently reported by three independent groups, which revealed that they take dimerized forms, although they do not have the full length of protein (Jang et al. 2009; Tosi et al. 2009; Tsuge et al. 2009). If so, it is understandable that del-Tipα has weak activity.

We also found that fluorescence-labeled Tipα specifically binds to the surface of MGT-40 cells and enters into the cytosol and nuclei, whereas del-Tipα and C5A/C7A-Tipα bind weakly to the cells (Suganuma et al. 2008). In the light of this evidence, we think that homodimers of Tipα can easily bind to a specific receptor molecule on the cell surface of gastric epithelial cells. We identified nucleolin as a specific receptor of Tipα on the cell surface using pull-down assay with anti-FLAG antibody against FLAG-tagged Tipα protein. Nucleolin is a well-known major non-ribosomal protein consisting of 710 amino acids in nucleolus, and it has three different structural domains: an N-terminal domain containing highly acidic residues, a central domain containing four RNA recognition motifs, and a C-terminal domain containing Arg-Gly-Gly (RGG) repeats (Ginisty et al. 1999). Nucleolin is known to have multi-functions, including chromatin remodeling, DNA recombination, DNA replication, RNA transcription by RNA polymerase I and II, rRNA processing, mRNA stabilization, cytokinesis and apoptosis (Ginisty et al. 1999; Storck et al. 2007). Furthermore, recent evidence indicates that nucleolin is present on the surface of a wide range of cancer cells and some other types of the cells, and acts as receptors for several molecules (Hirano et al. 2005; Hoja-Lukowicz et al. 2009; Hovanessian et al. 2000; Legrand et al. 2004; Reyes-Reyes and Akiyama 2008). To investigate the specific interaction of nucleolin with Tipα, we conducted experiments to characterize localization of nucleolin, and studied the internalization of Tipα and subsequent induction of *TNF-α* gene expression. This paper reports for the first time that nucleolin is clearly involved in the carcinogenic process of *H. pylori* as a major cellular receptor of Tipα protein.