TABLE 3 Summary of Results for Multivariate Analyses

Factor	Hazard ratio	*p* value
Liver metastasis	13.5	0.0007
Spread beyond the bowel wall	2.95	0.0054

dance, contributing to the greater part of the tumor size. In the literature, there is no clear agreement as to the minimum percentage of extracellular mucin required to define a carcinoma as mucinous (1-4,18,19). Since the WHO classification provides uniform, simple guidelines that are particularly useful clinically, it was employed in the present study (7).

The prognostic value of various histologic and grade-related parameters for CMC has remained unclear (20), but Jass and coworkers suggested that at least nine morphologic parameters (in addition to stage) had significant prognostic relevance from their univariate analysis (13). Among these, lymphocytic infiltration, tubular configuration and pattern of growth had independent prognostic value on multivariate analysis. In contrast, Leon *et al.* found that TNM staging was the only parameter with independent prognostic importance (21).

The main purpose of this study was to determine whether signet-ring cells exert an influence on prognosis which reflects their amount. There are several reports suggesting that SRCC show a worse prognosis than other mucinous carcinomas and typical non-mucinous adenocarcinomas (3,4). However, some authors have reported no clinical differences and there is a possibility that the poor prognosis may be due to a delay in diagnosis (22-25). In our series, the proportion occupied by signet-ring cells was not a significant indicator of poor prognosis. SRCC tend to be discovered at an advanced stage, although this is also the case for mucinous carcinomas as a whole.

Metastases from mucinous carcinomas and SRCC tend to develop in the lymph nodes and peritoneal surfaces rather than the liver (5). In our series, lymph node involvement was strongly related to prognosis on univariate analysis but was not an independent factor on multivariate analysis. Peritoneal dissemination was not related to prognosis on univariate analysis. They were independent of the amount of signet-ring cells using the χ^2 test.

A second aim was to identify clinical and morphologic parameters that may be of prognostic relevance in patients with CMC undergoing curative operation. Four variables (liver metastasis, lymph node involvement, vessel involvement, spread beyond the bowel wall) were significantly related to prognosis on univariate analysis. However, using multiregression models, only liver metastasis and spread beyond the bowel wall were independent prognostic factors and thus these appear to be the most important for predicting clinical outcome. This finding seems to be almost the same for ordinary non-mucinous carcinomas (1,24,26).

This may allow us to determine the plan of adjuvant therapy and follow-up. Our study indicated that patients who have liver metastasis, even if the tumors are completely resected macroscopically, only have a poor prognosis. Six such patients all died within 13 months. Spread beyond the bowel wall also has significant importance. Adjuvant chemotherapy using intraperitoneal injection may play a positive role for patients with tumors perforating the visceral peritoneum, because peritoneal dissemination was here found to be more frequent (8 patients) than other patterns of recurrence, including local recurrence (2 patients), liver metastasis (3 patients), and distant metastasis (2 patients).

In conclusion, the present study reaffirmed the importance of liver metastasis and spread beyond the bowel wall along with staging and grading for CMC with curative surgery. This appears to be of extreme practical importance in defining the subgroups of patients who are at different risk of recurrence and who could be treated more or less intensively. Future studies should assess the prognostic significance of various biologic markers within each Dukes' class.

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A Pilot Phase II Study of Capecitabine in Advanced or Recurrent Colorectal Cancer

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Background: A pilot phase II study was conducted to evaluate the Japanese intermittent regimen of capecitabine in patients with advanced/recurrent colorectal cancer.
Methods: Twenty-two patients received oral capecitabine in a dose of 828 mg/m^2 twice daily for 3 weeks every 4 weeks.
Results: In the 20 patients evaluable for efficacy, the overall response rate was 25.0% (95% CI, 8.7–49.1%), rising to 33.0% in the subset of patients previously untreated for metastatic disease (n = 9). A further nine patients had stable disease. The median duration of response was 7.0 months. Five patients (22.7%) experienced grade 3/4 treatment-related adverse events, the most common being a bullous rash observed in two patients (9.1%).
Conclusions: The 3 weeks out of 4 intermittent regimen of capecitabine demonstrated good antitumor activity and tolerability in patients with advanced/refractory colorectal cancer, providing a clear rationale for conducting a larger phase II study in patients with advanced disease.

Key words: capecitabine (Xeloda®) – colorectal cancer – phase II study

INTRODUCTION

5-Fluorouracil (5-FU), discovered by Heidelberger et al. in 1957 (1), is widely used for the treatment of gastrointestinal and breast cancers in Japan. However, the elimination half-life of 5-FU is short (2), and its efficacy varies according to dose and regimen. Continuous infusional regimens and biomodulation with leucovorin (LV) have been previously attempted to improve the antitumor efficacy of 5-FU (3–6). While both these approaches have led to superior response rates, survival benefits have been modest, and several studies and meta-analyses have failed to identify any clinically significant advantage (7,8). Compounding these issues is the fact that regimens based on continuous infusions of 5-FU impose significant inconvenience, higher costs and complications associated with indwelling central venous access lines and pumps. Consequently, patients receiving therapy for late-stage disease prefer oral rather than I.V. chemotherapy but are unwilling to accept a lower response rate or a shorter duration of response to their preferred choice of oral chemotherapy (9–11).

Capecitabine (Xeloda®) is an oral fluoropyrimidine carbamate that delivers 5-FU predominantly to tumor cells. The drug is rapidly and extensively absorbed through the gut as an intact molecule and is then metabolized to 5-FU in three steps (12–14). Firstly, it is converted to 5′-deoxy-5-fluorocytidine (5′-DFCR) by carboxylesterase (primarily in the liver). Secondly, it is converted to 5′-deoxy-5-fluorouridine (5′-DFUR) by cytidine deaminase (in tumor cells and in the liver). Finally, it is converted to 5-FU by thymidine phosphorylase (TP), which is significantly more active in the tumor tissue than in the adjacent healthy tissue (15). The increasing specificity for tumor cells that occurs with each successive conversion step potentially reduces the systemic 5-FU exposure while increasing the 5-FU dose within the tumor tissue. Preclinical studies have shown that capecitabine has superior antitumor efficacy compared with other 5-FU derivatives, including 5′-DFUR (16).

Based on the above preclinical data, a number of clinical studies on breast cancer were initiated in 1994. Since then, capecitabine has been approved in over 80 countries worldwide (including Japan, USA and the EU) as a monotherapy for the treatment of advanced or metastatic breast cancer patients who have failed previous anthracycline and taxane chemotherapy. In addition, the combination of capecitabine and docetaxel has been approved for the treatment of patients with advanced or metastatic breast cancer after failure of cytotoxic chemo-

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therapy including anthracycline (or when further anthracycline therapy is not possible). Capecitabine is also approved for the first-line treatment of patients with metastatic colorectal cancer.

In Japan, a phase I study conducted between November 1994 and March 1996 indicated that the maximum tolerated dose (MTD) of capecitabine administered continuously in cancer patients was 1255 mg/m^2 twice daily (17). Because of the occurrence of skin disorders following continuous treatment, an intermittent treatment regimen of 828 mg/m^2 administered twice daily for 3 weeks followed by a 1-week rest period was recommended for phase II studies. This dose/schedule, which has been evaluated in a number of Japanese phase I/II trials in advanced breast, gastric and colorectal cancer, differs from the more dose-intensive internationally approved intermittent capecitabine regimen, where patients receive a higher dose of 1250 mg/m^2 twice daily for 2 weeks followed by a 1-week rest period. However, it is important to note that the total dose of capecitabine given over six cycles of treatment is similar in both the Japanese and Western schedules (208 656 mg and 210 000 mg, respectively). The open-labeled, multicenter trial presented in this study was conducted to evaluate the efficacy and safety of the Japanese intermittent capecitabine regimen in patients with advanced/recurrent colorectal cancer.

SUBJECTS AND METHODS

Study Design

An open-labeled trial was conducted to evaluate the efficacy and safety of capecitabine (828 mg/m^2 twice daily) administered according to the intermittent schedule of 3 weeks of treatment followed by a 1-week rest period per cycle. The trial was conducted in accordance with the Good Clinical Practice for Trials on Drugs (GCP), announced on October 2, 1989, by the Pharmaceutical Affairs Bureau (Notification No. 874). The institutional review board of each participating center approved the study, and all patients provided written informed consent before enrollment.

Patients

Eligible patients had to have histologically confirmed colorectal cancer with measurable or assessable lesions according to the General Rules for Clinical and Pathological Studies on Cancer of the Colon, Rectum and Anus (5th Edition) (18). Eligibility criteria also included an ECOG performance status (PS) of 0–2, an expected survival of ≥3 months and an age at enrollment between 20 and 74 years. Patients were required to have adequate renal, hepatic and hematological parameters (leukocytes 4000–12 000 cells/mm^3; platelets ≥100 000 cells/mm^3; hemoglobin concentration ≥9.0 g/dl; GOT [AST], GPT [ALT] and alkaline phosphatase ≤2.5 times the upper limit of normal [ULN] and total bilirubin and creatinine <1.5 × ULN). Patients had to have received no more than one prior chemotherapy regimen (excluding adjuvant chemotherapy completed ≥6 months before enrollment) and no radiotherapy for the lesions evaluated in the study. In addition, no patient was to have evidence of carry-over effects or toxicity associated with prior chemotherapeutic regimens (washout period of 4 weeks or more; 2 weeks or more for antimetabolites and immunotherapy).

Patients with the following conditions were excluded from the study: a history of drug hypersensitivity; ascites or pleural effusions requiring treatment; clinically severe complications such as non-malignant liver, renal or lung disease or diabetes mellitus; electrocardiographic abnormalities; a possibility of pregnancy; peripheral neurologic signs and symptoms not associated with cancer; brain metastases; active peptic ulcer requiring treatment; ascites or pleural effusions as the only assessable lesions; evidence of HIV infection or a history of prior chemotherapy with an unapproved drug.

Planned Sample Size

We adopted a two-step method with interim evaluation. An independent review committee was scheduled to perform an interim evaluation when the number of patients suitable for statistical analysis reached 20. The study was to be discontinued if four or more patients responded either completely or partially, and a late phase II study conducted in a larger population of patients. Alternatively, if less than four patients responded to treatment, a target number of 35 patients had to be enrolled to evaluate efficacy. The target number of patients was set at 35, and the interim evaluation planned with 20 patients was based on Fleming's two-step method (19). The statistical power was 86%, meaning that with an expected response rate of 20%, the lower margin of efficacy and one-sided α-level were both 5%.

Dosage and Dose Modifications

The dose of capecitabine was determined according to the patient's body surface area based on the recommended phase I dose of 828 mg/m^2 twice daily (Table 1). Capecitabine was administered orally after breakfast and evening meals for 3 weeks followed by a 1-week rest period (no treatment), unless patients developed progressive disease (PD). Patients who did not develop PD during the first course of treatment could receive more courses of capecitabine. Other anti-cancer therapies such as immunotherapy, endocrine therapy and systemic steroid therapy, were prohibited during the course of the study.

In the event of patients developing drug-related grade 3 adverse events (excluding anorexia, nausea, vomiting, alopecia, malaise or skin reactions) or laboratory abnormalities (excluding grade 3/4 leucopenia, granulocytopenia and lymphopenia with a fever persisting for 3 days or less) or in patients in whom the investigator judged continuation of treatment unfeasible, treatment could be delayed for up to 4 weeks to allow patient recovery. If the investigator considered that continuation of treatment at the same dose would be intolerable due to adverse events, irrespective of grade, the administered dose could be reduced to approximately 75% of the initial dose. Treatment was permanently discontinued in patients who

Table 1. Determination of capecitabine dose according to patient's body surface area

Body surface area (m^2)	Recommended dose* (mg, twice daily)
<1.1	800
1.1 to <1.3	1000
1.3 to <1.5	1150
1.5 to <1.7	1300
≥1.7	1500

*Based on recommended dose of 828 mg/m^2 twice daily.

developed drug-related grade 4 adverse events or who could not tolerate treatment, even at a reduced dose.

Study Assessment

Demographic characteristics, the presence of symptoms, laboratory values, electrocardiograms and tumor characteristics were assessed before treatment. If available, tumor markers were recorded as an index of response to treatment. For evaluation of response, tumors were assessed by CT, MRI or X-ray every fourth week of each course of treatment. Laboratory examinations were performed every second and fourth week of each course, and electrocardiograms were taken after completion of treatment. Symptoms were followed throughout treatment, and patients were monitored for 4 weeks after completion of treatment. All empty drug boxes and tablets were collected at the end of the study to evaluate treatment compliance.

Evaluation of Response and Safety

Complete response (CR), partial response (PR), no change (NC) and PD were defined by the investigator according to The General Rules for Clinical and Pathological Studies on Cancer of the Colon, Rectum and Anus (5th Edition) (18). These criteria differ slightly from the WHO criteria since five evaluations of response were employed: CR, PR, MR (minor response), NC and PD. However, in the present study, MR and NC were grouped together as NC so that the tumor response criteria could be compared with other studies using the WHO criteria. Responses were confirmed 4 weeks after the initial classification, and an independent response evaluation committee was responsible for evaluating and confirming tumor responses at the end of the study. Although time to disease progression (TTP) was not evaluated during the study, survival follow-up data were collected, and the median overall survival was determined according to the Kaplan–Meier method.

Abnormal findings were assessed according to the National Cancer Institute of Canada Common Toxicity Criteria (NCIC-CTC) Grading System (20). Adverse events not listed on the NCIC-CTC grading system were graded as mild (grade 1), moderate (grade 2), severe (grade 3) or life threatening (grade 4). Hand–foot syndrome (HFS or palmar–plantar erythrodysesthesia) was classified as grade 1, 2 or 3 (21).

Statistical Methods

Patients with a CR or PR were considered responders, and the response rate was calculated. The 95% confidence interval (CI) of the response rate was calculated by the exact method, based on a binomial distribution of data. The duration of response and the number of days until the onset of response were expressed by the minimum value, median value and maximum value. Overall survival was defined as the number of days from study enrollment until death and was calculated by the Kaplan–Meier method. Time to disease progression (TTP) was not recorded during the study. Safety was evaluated in all patients who received capecitabine treatment. Adverse event frequencies were recorded.

RESULTS

Patient Characteristics

Twenty-two patients were enrolled between August 1996 and December 1997 and received capecitabine (intent-to-treat [ITT] population). All patients were included in the safety analysis, although two were excluded from the efficacy evaluation: one patient with simultaneous colorectal and gastric cancer did not meet the eligibility criteria; the other patient's tumor was not adequately assessed at the beginning of the treatment. The baseline demographic characteristics of the ITT population are shown in Table 2. The median age was 60 years (range, 46–72 years). The majority, 13 patients (59.1%), had cancer of the rectum and the remaining nine patients (40.9%) had colon cancer. The lungs and the liver were the most common sites of metastases. All patients had undergone prior surgical resection, and 12 (54.5%) had received prior chemotherapy, including eight treated with adjuvant chemotherapy. All of these had received 5-FU derivatives and four had received 5-FU-based combination chemotherapy. None of the patients had received prior radiotherapy.

Treatment Duration

The median duration of treatment was 3.9 months (range, 1.0–13.6 months and 1–12 courses). Although treatment was scheduled for two or more courses, one patient withdrew at the completion of the first course because of PD; a second patient withdrew during the second course because of adverse events (bowel obstruction, anorexia, nausea and vomiting). Two other patients also met the protocol criteria for withdrawal (grade 4 raised alkaline phosphatase and hyperglycemia). However, because the investigator considered that these were not true grade 4 events and had little or no clinical impact on the patient, both the patients continued on the study.

Of the 20 patients who received two or more courses of capecitabine, 16 (72.7%) were eventually withdrawn due to PD, two (9.1%) were transferred to a maintenance study, one (4.5%) was withdrawn because of adverse events and one (4.5%) went off therapy for other reasons. Patients with CR, PR or SD (stable disease) were eligible to receive capecitabine

Table 2. Baseline demographic characteristics (intent-to-treat population)

	No. of patients
Number of patients enrolled	22 (100%)
Age, median (range)	60.0 (46–72 years)
Sex	
Male	16 (72.7%)
Female	6 (27.3%)
Primary sites	
Colon	9 (40.9%)
Rectum	13 (59.1%)
Performance status*	
0	17 (77.3%)
1	4 (18.2%)
2	1 (4.5%)
Previous treatment	
Surgery	22 (100%)
Chemotherapy	12 (54.5%)
5-FU derivatives	8 (36.4%)
5-FU + other	4 (18.2%)
Evaluable disease sites	
Lung	16 (72.7%)
Liver	13 (59.1%)
Skin	4 (18.2%)
Lymph nodes	2 (9.1%)
Bone	2 (9.1%)
Ascites	1 (4.5%)

*World Health Organization grading.

in a maintenance study designed to observe the safety of long-term capecitabine treatment. In the present study, compliance with capecitabine therapy (received dose versus planned dose in each patient) was measured at 84% or more during the study period, and there were no episodes of clinically relevant non-compliance.

Efficacy

The response to capecitabine in the 20 evaluable patients assessed by the response evaluation committee is shown in Table 3. Five patients had PR, although no CRs were reported. Of the remaining patients, nine had NC and six had PD. The overall response rate of 25.0% (95% CI, 8.7–49.1%) was superior to the expected response rate of 20.0% and was higher (33.3%) in the subset of patients previously untreated for metastatic disease ($n = 9$). Furthermore, a higher response rate (28.6%) was obtained in the subset of patients who were previously untreated ($n = 9$) or had completed adjuvant chemotherapy before enrollment ($n = 5$). Patients with lung metastases at baseline had a response rate of 28.6% (one CR and three PRs

Table 3. Tumor responses in evaluable patients (overall and according to site of metastasis)

	No. of patients	CR	PR	NC	PD	NE	Response rate (%)
Overall	20	0	5	9	6	0	25.0*
Site of metastasis							
Lung	14	1	3	8	2	0	28.9
Liver	11	0	3	4	4	0	27.3
Skin	4	0	0	3	1	0	0
Lymph nodes	2	0	0	2	0	0	0
Bone	2	0	0	1	1	0	0
Ascites	1	0	0	1	0	0	0

*95% CI: 8.7–49.1%.

in 14 patients), while that of 11 patients with liver metastases was 27.3% (three PRs) (Table 3).

In the five patients with PR, the median time to onset of response was 0.9 months (range, 0.9–4.6 months) and the median duration of response was 7.0 months (range, 6.4–14.0 months). Median overall survival was 13.3 months (95% CI, 9.2–18.0 months) (Fig. 1).

Adverse Events

All adverse events, excluding those clearly unrelated to the study drug, were defined as treatment-related adverse events. Twenty patients (90.9%) experienced at least one adverse event during the study, the majority of which were not higher than grade 1. The most common adverse events (all grades) were as follows: hand–foot syndrome in seven patients (31.8%); increase in total bilirubin in six patients (27.3%); nausea, anorexia and decreased erythrocyte count in five patients each (22.7%); and vomiting, diarrhea, glycosuria, raised alkaline phosphatase, decreased hematocrit and thrombocytopenia in four patients each (18.2%, Table 4).

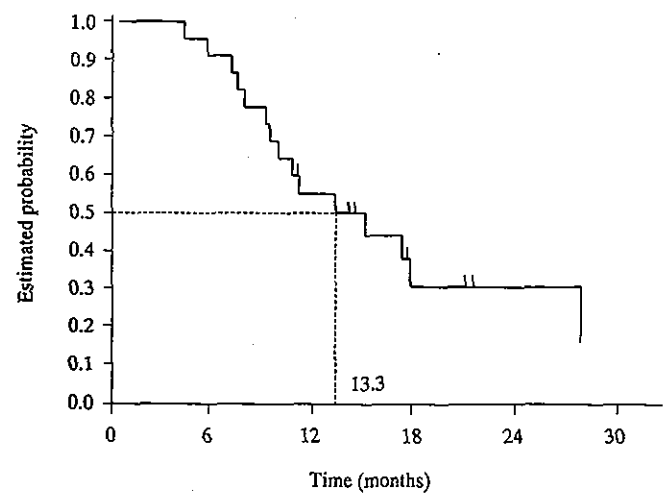

Figure 1. Overall survival.

Table 4. Summary of treatment-related adverse events (occurring in ≥10% of the intent-to-treat population)

Body system/event	Grade*				No. of patients	Total (%)
	1	2	3	4		
No. of patients	5	10	3	2	20	90.9
Skin/appendages						
Hand–foot syndrome	4	1	2	–	7	31.8
Gastrointestinal system						
Nausea	1	4	–	–	5	22.7
Vomiting	3	1	–	–	4	18.2
Diarrhea	3	1	–	–	4	18.2
Anorexia	3	2	–	–	5	22.7
Liver/biliary system						
GOT increased	3	–	–	–	3	13.6
GPT increased	3	–	–	–	3	13.6
Hyperbilirubinemia	–	5	1	–	6	27.3
Metabolic/nutritional system						
Glycosuria	2	2	–	–	4	18.2
Alkaline phosphatase raised	4	–	–	–	4	18.2
Hyperglycemia	–	1	1	1	3	13.6
Blood						
Erythrocytes decreased	4	1	–	–	5	22.7
Hemoglobin decreased	2	1	–	–	3	13.6
Hematocrit decreased	3	1	–	–	4	18.2
Leucopenia	3	–	–	–	3	13.6
Lymphopenia	1	1	1	–	3	13.6
Thrombocytopenia	4	–	–	–	4	18.2

*NCIC-CTC grade.

Overall, five patients (22.7%) experienced grade 3/4 treatment-related adverse events (Table 4). The most common grade 3/4 drug-related adverse events were hand–foot syndrome and hyperglycemia in two patients each (9.1%); bowel obstruction, increase in total bilirubin, lymphopenia and prolonged activated partial thromboplastin time (APTT) were also reported in one patient each. While two grade 4 drug-related adverse events (prolonged APTT and hyperglycemia in one patient each) were reported, there were no treatment-related deaths during the study. Skin disorders, which were dose limiting in an earlier phase I study (17), were observed in eight patients (36.4%), although only two grade 3 and no grade 4 skin-related adverse events were observed.

Temporary interruptions of treatment, decreases in dosage, or termination of treatment were required in nine patients (40.9%). The most common reasons for interruptions/discontinuation were vomiting, nausea and hand–foot syndrome in three patients each (13.6%). Bowel obstruction with hospitalization was listed as a serious drug-related adverse event in one patient, although the patient recovered within 13 days and discontinued the study. This event was considered to be related to study treatment because none of the other potential causes (intestinal accretion due to surgery, aggravation of intestinal motility due cold-induced dehydration or alteration of intestinal blood circulation due to sclerosis of the arteries related to diabetes mellitus) were apparent.

DISCUSSION

Capecitabine has demonstrated consistently high single-agent activity and a favorable safety profile in taxane and anthracycline pre-treated metastatic breast cancer (21–24) and improved overall survival when added to docetaxel in the anthracycline-failure setting (25). In addition, in randomized phase III trials, comparing the efficacy and tolerability of 3-weekly intermittent capecitabine with i.v. bolus 5-FU/LV as first-line treatment of advanced colorectal cancer, capecitabine was more active than 5-FU/LV in the induction of tumor response and at least equivalent in terms of TTP and overall survival (26). Furthermore, a combined analysis of these randomized phase III studies in colorectal cancer revealed that capecitabine offers a clinically meaningful advantage over 5-FU/LV in terms of safety (27).

In the current phase II trial using the 4-weekly Japanese intermittent capecitabine regimen, the overall response rate was 25.0% as second-line treatment, rising to 33.0% as first-line treatment. In comparison, the response rate of intravenous 5-FU/LV therapy in the first-line treatment setting is recognized to be in the range of 17–33% based on the results of four randomized studies (6,26,28,29). These findings regarding capecitabine and 5-FU/LV compare very favorably with those reported previously for 5′-DFUR in a multicenter phase II study, where the response rate in 76 previously treated colorectal cancer patients was only 9.2% (30). Interpretation of overall survival data in such a small group of patients is difficult due to selection bias. However, the median survival time for patients receiving capecitabine in the present study is comparable to those previously reported for 5-FU/LV (12.6–14.3 months) and 5′-DFUR (15.4 months) (6,26,28–30); a larger phase II trial is required to confirm these findings. In addition to other trials, a trial of this type has since been conducted in patients with advanced breast cancer, and findings are expected to be published later in 2004.

In terms of safety, grade 3/4 adverse events such as diarrhea and neutropenia have been reported relatively frequently following 5-FU/LV therapy in the studies mentioned above. In the present study, five patients (22.7%) had grade 3/4 drug-related adverse events, the most common being grade 3 hand–foot syndrome in two patients (9.1%). Grade 4 drug-related adverse events occurred in only two patients, including prolonged APTT and hyperglycemia in one patient each. These findings suggest that the oral capecitabine regimen tested in the current study might be better tolerated compared with intravenous 5-FU/LV and have similar tolerability to 5′-DFUR (30), although a larger phase II trial is required to confirm these findings.

It is also interesting to compare the results of the current study with the responses reported in phase II and III studies of the internationally approved intermittent capecitabine regimen (1250 mg/m^2 twice daily for 2 weeks every 3 weeks). In a phase II study, first-line treatment of 34 colorectal cancer patients with capecitabine was associated with a response rate of 24% (31). The response rate of capecitabine vs 5-FU/LV in the large phase III studies conducted in the EU and in the USA was 26% vs 17% (26). Based on our limited data, the Japanese intermittent regimen appears to be associated with a lower incidence of grade 3/4 adverse events than the standard 3-weekly intermittent capecitabine regimen (27): grade 3 HFS (4.5% vs 17%); grade 3/4 diarrhea (0% vs 13%); grade 3/4 hyperbilirubinemia (4.5% vs 23%). In addition, the rate of dose reductions because of adverse events was lower (9%) compared with the EU and the USA phase III studies (34%) (26). This type of cross-study comparison is feasible because the total dose of capecitabine given over six cycles is similar in both the Japanese and Western schedules (208 656 mg vs. 210 000 mg, respectively), and the difference in tumor response assessment times (every 4 weeks vs every 6 weeks) is unlikely to have a major impact on response rates. However, the usual limitations of cross-study comparisons should be taken into account when interpreting the current results.

Previous studies have shown that in patients with metastatic colorectal cancer, oral capecitabine provides a more convenient alternative to the commonly used protracted i.v. infusions of 5-FU that are known to impose significant inconvenience, costs and complications associated with indwelling central venous access lines and pumps. In terms of tolerability, capecitabine has a different adverse event profile compared with 5-FU and its derivatives, including a lower frequency of commonly observed adverse events such as myelosuppression and gastrointestinal disorders, the latter of which tends to impact the patients' quality of life. Consequently, capecitabine provides an effective, convenient and well-tolerated therapy for use in the outpatient setting. The results from our small phase II study indicate that the Japanese intermittent capecitabine regimen is active and well tolerated in patients with advanced/refractory colorectal cancer. Nevertheless, these promising findings require confirmation in larger phase II/III trials before any firm conclusions can be arrived at.

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Case report

Rectal cancer associated with chronic lymphocytic leukemia

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It has been reported that chronic lymphocytic leukemia (CLL) often occurs concomitantly with other malignant neoplasms. However, because CLL is rare in Japan, there are only a limited number of reports of the occurrence of malignant neoplasia in Japanese patients with CLL. We report here the simultaneous occurrence of rectal cancer and CLL in a 57-year-old man. Because the clinical stage of CLL was Rai system I, we decided, in accordance with the National Cancer Institute–Sponsored Working Group guidelines, to monitor him without therapy for CLL until evidence of disease progression, and we performed abdominoperineal resection of the rectum for the cancer. The small rectal tumor was associated with aggressive lymphangiosis carcinomatosa, and multiple nodal metastases were observed in the pool of CLL cells. He died of rectal cancer 7 months after the operation, and autopsy revealed extensive metastases of the cancer. Cellular and humoral immunity is often impaired in patients with CLL, and the defective immunity in this patient may have had an etiological role in the development and rapid progression of the cancer. In the follow-up of CLL patients, we must always be aware of the possible existence of a second malignant disease. Particular attention should be paid to those with defective immunity, and screening should be performed, especially for pulmonary and gastrointestinal malignancies.

Key words: rectal cancer, chronic lymphocytic leukemia, synchronous cancer

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Introduction

Chronic lymphocytic leukemia (CLL) is a rare form of leukemia in Japan, though it is the most common form in Western countries.[1–5] Several studies from Western countries have indicated a significantly increased risk of subsequent neoplasms in patients with CLL.[6,7] Though the precise etiology remains unclear, it has been suggested that concurrent defective immunity may play a central role in the development of subsequent neoplasms.[6,7] Because of the rarity of CLL in Japan, there are only a limited number of reports of the occurrence of additional malignancy in Japanese patients with CLL. To supplement existing information on the simultaneous occurrence of CLL and other malignant neoplasms, we report here a case of rectal cancer associated with CLL.

Case report

A 57-year-old man noticed a right cervical mass in 1999. It gradually enlarged, and he noticed another mass, on the left side of his neck, in 2001. He consulted a doctor in September 2001, and physical examination revealed bilateral enlargement of the superficial cervical lymph nodes. Malignant lymphoma was suspected on biopsy, and colonoscopic examination performed for screening revealed a synchronous rectal cancer. In October he was referred to the Department of Hematology of Gunma Prefectural Cancer Center for detailed investigation.

He had undergone surgery for a herniated disk at the age of 42 years, and there was nothing of note in his family history. On presentation to our hospital, multiple enlarged nodes were palpable on both sides of his neck, in the right axilla, and in the bilateral inguinal regions. Digital examination revealed a hard tumor on the anterior wall of the rectum, 7 cm from the anal verge.

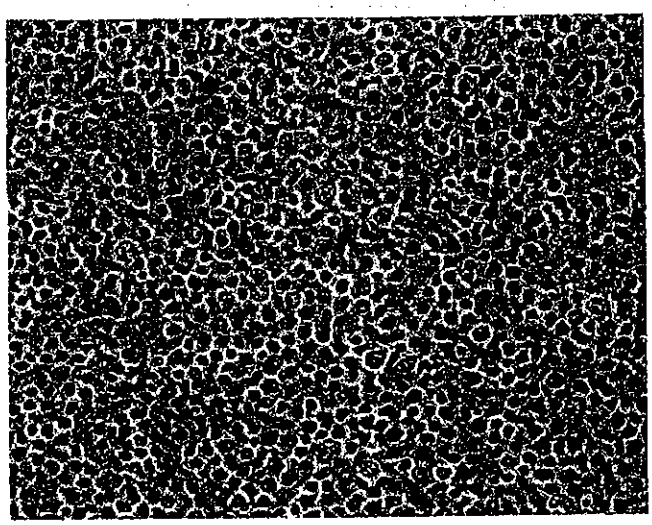

Fig. 1. Biopsy specimen, showing diffuse infiltration of mature lymphocytes. H&E, ×400

Re-examination of the biopsy specimen revealed a picture consistent with CLL (Fig. 1). Laboratory findings showed a hemoglobin concentration of 13.9 g/dl, white cell count of 15.8 × 10⁹/l with 79.0% lymphocytes; platelet count of 154 × 10⁹/l; IgG, 582.5 mg/dl (normal range [NR], 800.0–1800.0 mg/dl); IgA, 73.5 mg/dl (NR, 90.0–450.0 mg/dl); and IgM, 12.9 mg/dl (NR, 60.0–250.0 mg/dl). A bone marrow aspirate smear showed that 76.4% of all nucleated cells were lymphocytes. The predominant population of lymphocytes shared B-cell markers (CD19 and CD20) with the CD5 antigen, and the B cells were monoclonal with regard to the expression of IgM, κ. These data met the diagnostic criteria proposed by the National Cancer Institute–Sponsored Working Group (NCI-WG).[8] Barium enema showed a rectal tumor on the anterior wall of the middle of the rectum, with thickened folds and large nodules around it (Fig. 2). Colonoscopic examination revealed an ulcerated rectal tumor 7 cm from the anal verge (which was adenocarcinoma histologically) and also revealed large nodules with a smooth surface around the tumor (Fig. 3). Computed tomography (CT) scans of the neck, chest, and abdomen revealed enlarged nodes in the perirectal and right obturator spaces, around the aorta, behind the portal vein, in the mediastinum, and in the superficial and deep cervical regions. There was no sign suggestive of distant metastasis of cancer cells. These findings indicated that the clinical stage of CLL was Rai system I and Binet system B. We decided, in accordance with the NCI-WG guidelines,[8] to monitor him without therapy for CLL until evidence of disease progression, and we performed abdominoperineal resection of the rectum for the rectal cancer in November. The surgical specimen showed an ulcerated rectal tumor with enlarged folds and nodules caused by lymphangiosis

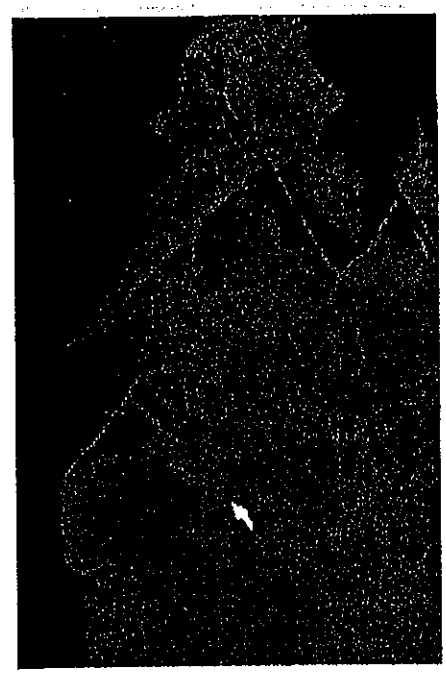

Fig. 2. Barium enema (lateral view), showing a middle rectal tumor (*arrow*) on the anterior wall, with thickened folds and large nodules around it

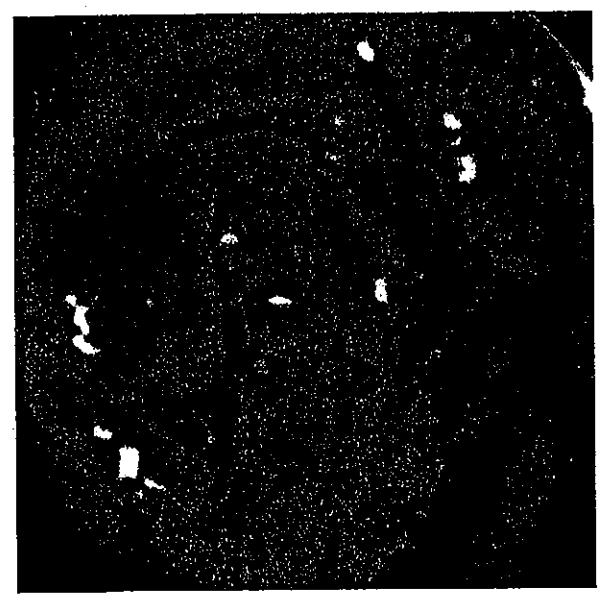

Fig. 3. Colonoscopic examination, showing an ulcerated rectal tumor and nodular change of the mucosa with smooth surface

carcinomatosa (Fig. 4). A total of 27 nodes were detected in the specimen, and diffuse invasion of CLL cells was observed in all of them. Metastatic carcinoma nests were observed in 19 nodes, in the pool of CLL cells. Two enlarged obturator nodes also included carcinoma

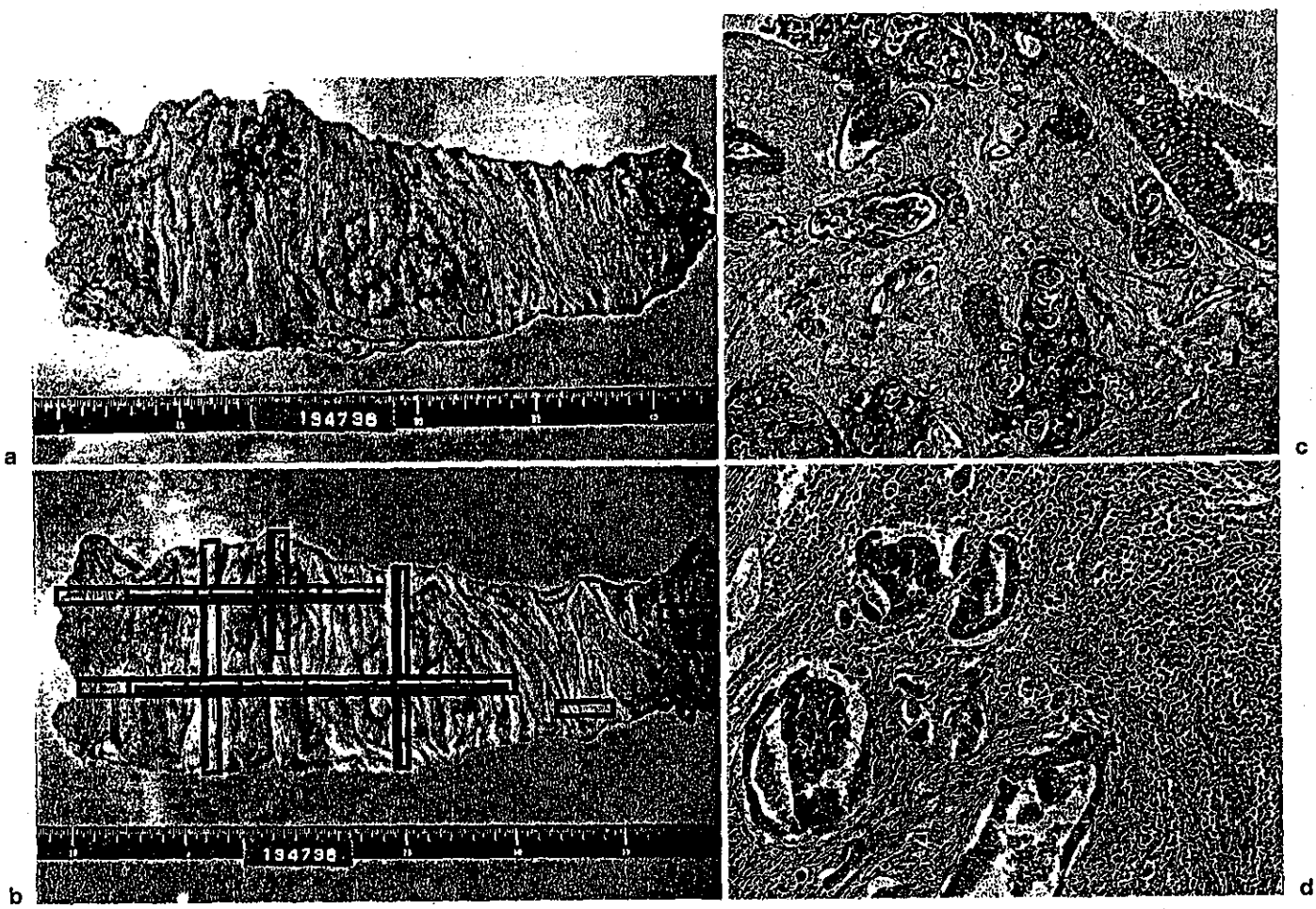

Fig. 4. **a** Macroscopic view of specimen removed at surgery. *Arrow* indicates an ulcerated rectal tumor. **b** Scheme showing infiltration of cancer cells and leukemia cells. The range of infiltration of both cell types is indicated by *gray bars*. **c** Microscopic view of submucosal layer of a thickened fold. Cancer cells have infiltrated laterally and vertically, by way of lymphatics. They appear to float in the expanded lymph vessels or block them. The submucosal layer is markedly edematous because of congestion with lymph. **d** Chronic lymphocytic leukemia (CLL) cells appear to gather around cancer cells in the subserosal layer. **c** H&E, ×12.5; **d** H&E, ×100

nests in the large pool of CLL cells. Adjuvant chemotherapy was administered, using fluorouracil and leucovorin.

It was interesting that all of the enlarged superficial nodes were markedly decreased in size after the operation. However, the patient developed difficulty in breathing because of right pleural effusion, and cytological examination of the effusion revealed numerous CLL cells. Low-dose cyclophosphamide (50 mg per day) and prednisolone (5 mg per day) were administered, and he had no complaints until May 2002, when he was readmitted with shortness of breath. Chest X-ray revealed bilateral pleural effusions and reticular shadows on the whole lung fields. Cytological examination of the effusion revealed numerous cancer cells, and he died 1 month later. Autopsy revealed bilateral pleuritis carcinomatosa with cancer metastases to the bilateral lungs, thyroid, liver, and right adrenal gland. Diffuse infiltration of cancer cells was observed in the cervical, mediastinal, and abdominal lymph nodes, and small numbers of CLL cells were also found in these nodes peripherally.

Discussion

CLL is a rare form of leukemia in Japan, though it is the most common form in Western countries.[1–5] It accounted for 3% of all leukemias according to a Japanese nationwide survey in 1978.[1] A recent study reported that is comprised 6%, of all leukemias, suggesting an increase, partly because of the detection of asymptomatic patients by nationwide regular health check-ups.[2] The annual incidence of CLL was estimated to be 0.27–0.48 per 100 000 in Japan,[2,3] one-tenth to one-fifth of that in Western countries, where CLL accounts for 30% of

all leukemias, and the incidence was estimated to be 2.7 per 100000.[4,5]

CLL occasionally occurs concomitantly with other malignant neoplasms, and previous reports have indicated a modest but significant increase in the risk of subsequent neoplasms in CLL patients.[6,7] In a large study including 4869 patients with CLL diagnosed from 1935 through 1971, subsequent neoplasms were observed in 4.8% of them, and the relative risk (RR) was estimated to be 1.1, with a 95% confidence interval (CI) of 1.0–1.3.[6] In a recent study including 16367 CLL patients registered between 1973 and 1996, subsequent neoplasms were observed in 11.1% of them (RR, 1.20; 95% CI, 1.15–1.26).[7] Though significant excesses were found in both studies, particularly for sarcomas, including Kaposi sarcoma (RR, 5.3 and 5.09, respectively) and malignant melanoma (RR, 6.7 and 3.18, respectively), the most common neoplasms with an elevated RR were cancers of the lung (RR, 1.5 and 1.66, respectively), colon (RR, 1.0 and 1.13, respectively), and rectum (RR, 1.6 and 1.09, respectively).[6,7] In Japan, cancers were observed in 5.2% of CLL patients, and the most common neoplasm was gastric cancer, followed by cancers of the lung and large bowel.[9,10] Only 30 cases of malignant neoplasms associated with CLL have been reported in Japan, including 4 cases of colorectal cancer; 3 of sigmoid colon cancer, and 1 of rectal cancer.[11,12] This is the second reported case of rectal cancer associated with CLL.

Though the precise etiology of the development of additional malignancy is not clear, it has been suggested that defective immunity in CLL patients may have an etiological role,[6,7] from the observation that a similar array of tumors, particularly lung cancer and sarcomas, develop following therapeutic immunosuppression in transplant recipients.[13] Though CLL is a B-cell proliferation in most cases,[2,14] patients with CLL often display defective cellular immunity.[15–19] The CD5-positive neoplastic B cells often show decreased expression, not only of immunoglobulins but also of important glycoproteins on the cell surface. Specifically, qualitative or quantitative decreases in major histocompatibility complex molecules or critical activation ligands such as CD80 on the neoplastic B cells directly impairs their ability to function as antigen-presenting cells, resulting in their inability to provide the essential costimulatory signals required for T-cell activation.[15,16] In addition, several cytoskeletal abnormalities in neoplastic B cells not only cause reduced antigen presentation but also prevent effective B-cell-T-cell conjugate formation.[17] Reduced antigen presentation by neoplastic B cells, as well as defective B-cell-T cell interaction, results in T-cell anergy. Furthermore, because natural killer (NK) cells mediate cell killing by antibody-dependent cell-mediated cytotoxicity (ADCC), the opportunity for ADCC to occur in vivo is greatly reduced in CLL patients with hypogammaglobulinemia, which can alter the potential benefits of NK cells in the host's control of tumor growth.[18,19] Thus, B-cell CLL is often accompanied by a multitude of immune abnormalities. Though we did not examine cellular immunity, concurrent hypogammaglobulinemia in our patient may have caused dysfunction of T cells and NK cells, and this, in turn, could have been associated with the development of the cancer. It has been demonstrated that CLL patients with an additional malignancy more often show low immunoglobulin levels than those patients without it,[20] and that T-cell abnormality is more prominent in CLL patients with hypogammaglobulinemia.[21]

As the present patient showed an aggressive course of rectal cancer, the defective immunity may also have been associated with the rapid progression of the second malignancy. It has been shown, in mice, that highly immunogenic tumors exhibit more aggressive behavior in the presence of immunosuppression.[22] While Greene et al.[6] reported clustering of lung cancer and sarcoma among CLL patients who died early in the course of their disease,[6] case reports show an unusually aggressive course of additional malignancies other than lung cancer and sarcoma. For example, a sigmoid colon cancer infiltrating the muscular layer in a CLL patient with a low level of IgM and depressed lymphocyte response to phytohemagglutinin showed extensive metastases to the lungs and liver.[11] Skin cancers in CLL patients are also known to be unusually aggressive.[23,24]

The reason that the superficial nodes in our patient decreased in size after operation is not clear. One possible explanation is that cytokines such as granulocyte colony-stimulating factor might have been produced in the tumor as a paraneoplastic syndrome.[25]

Because CLL patients have an increased risk of additional malignancy, physicians who deal with them must always be aware of the possible existence of a second malignant disease. Particular attention should be paid to those with defective immunity, and screening should be performed, especially for pulmonary and gastrointestinal malignancies.

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MUTATIONS OF *BRAF* ARE ASSOCIATED WITH EXTENSIVE *hMLH1* PROMOTER METHYLATION IN SPORADIC COLORECTAL CARCINOMAS

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Activating mutations of *BRAF* have been frequently observed in microsatellite unstable (MSI^+) colorectal carcinomas (CRCs), in which mutations of *BRAF* and *KRAS* are mutually exclusive. Previously, we reported that hypermethylation of *hMLH1* might play an important role in the tumorigenesis of right-sided sporadic CRCs with MSI showing less frequency of *KRAS/TP53* alteration. Therefore, we have assumed that *BRAF* mutations might be highly associated with *hMLH1* methylation status rather than MSI status. In this study, mutations of *BRAF* and *KRAS* and their relationship with MSI and *hMLH1* methylation status were examined in 140 resected specimens of CRC. The methylation status was classified into 3 types: full methylation (FM), partial methylation (PM) and nonmethylation (NM). Only FM closely linked to reduced expression of hMLH1 protein. *BRAF* mutations were found in 16 cases (11%), all leading to the production of $BRAF^{V599E}$. As for MSI status, *BRAF* mutations were found in 43% of MSI^+ and 4% of MSI^- cases ($p < 0.0001$). Among the MSI^+ individuals, *BRAF* mutations were more frequent in cases with hMLH1 deficiency (58%) than those with hMSH2 deficiency (0%; $p = 0.02$). Moreover, they were found in 69% of FM, 4% of PM and 4% of NM, revealing a striking difference between FM and the other 2 groups (FM *vs.* PM or NM; $p < 0.0001$). These findings suggest that BRAF activation may participate in the carcinogenesis of sporadic CRCs with *hMLH1* hypermethylation in the proximal colon, independently of KRAS activation.

Key words: *sporadic colorectal cancer;* BRAF *mutation;* hMLH1 *hypermethylation; microsatellite instability*

In the development of colorectal cancer (CRC), it is now widely accepted that some forms of genetic instability lead to the sequential accumulation of genetic alterations and consequently develop carcinomas.[1] RAS activation in the MAP kinase cascade is supposed to constitute a part of the primary events in colorectal carcinogenesis, and the *KRAS* gene mutations have been found in about 30–40% cases of sporadic CRCs.[2–4]

Recently, activating *BRAF* mutations have been found almost invariably in melanoma cells and sometimes in other types of carcinoma, including CRCs,[5–7] implying a function of *BRAF* as a protooncogene. The *RAF* genes are members of MAPK pathway, encoding serine/threonine kinases that integrate the upstream input signals.[8,9] Once recruited at the cell membrane by GTP-loaded RAS, RAF becomes activated and subsequently phosphorylates the downstream kinases, MEKs, which eventually induce transcriptional activation of the target genes.[9]

More recently, frequent *BRAF* mutations and infrequent *KRAS* mutations have been reported in DNA-mismatch repair (MMR)-deficient CRCs.[10] Inactivation of MMR genes incurs instability of genomic microsatellite sequence (microsatellite instability, or MSI), which is found in the majority of patients with hereditary nonpolyposis colorectal cancer syndrome (HNPCC) and in 10–15% of cases of sporadic CRCs.[11–13] Moreover, it was also reported that 70–90% of sporadic CRCs with MSI (MSI^+ CRCs) are associated with hypermethylation of *hMLH1*, one of DNA-MMR genes, and have distinct clinical and pathologic characteristics, *i.e.*, occurrence in older females, location in the proximal colon and histopathology of mucious or poor differentiation.[14–20]

We have previously examined the methylation status of *hMLH1* gene in sporadic CRCs by use of 5 sets of primer spanning the whole CpG sites within its promoter region and have classified the methylation status into 3 subtypes: full methylation, partial methylation and nonmethylation.[21,22] We reported that an extensive methylation, or full methylation, of *hMLH1* promoter was found in about 80% of MSI^+ CRC cases and was highly associated with loss of expression of its gene product. Interestingly, this type of CRC cells are rarely associated with *KRAS* mutations and loss of heterozygosity (LOH) of *TP53* gene.[22] It is therefore possible that extensive methylation of *hMLH1* promoter region may contribute to the carcinogenesis of the right-sided sporadic CRCs, independently of *KRAS/p53* alterations.

From these results, 2 questions may arise. First, does the activation of BRAF, instead of KRAS, take part in the carcinogenesis of CRCs with extensive *hMLH1* methylation? Second, if so, does the BRAF activation have any relationship with the CRCs with partial methylation, although most of which are microsatellite stable (MSI^-), maintain MMR gene expression and show a relatively high incidence of KRAS and p53 alterations?[22]

Additionally, in the melanoma cells, high frequency of mutations of *β-catenin* and *BRAF* has been recognized.[23] Some researchers previously reported that *β-catenin* mutations were more common in MSI^+ CRCs than in MSI^- ones.[19,23–25] However, it has not been elucidated yet whether there are any relationship between the mutations of *β-catenin* and *BRAF* in the CRCs with *hMLH1* hypermethylation.

In this study, we have investigated the frequency of *BRAF* mutation and its relationship with *KRAS* and *β-catenin* mutations in a large consecutive series of sporadic CRCs in regard to both MSI status and degrees of *hMLH1* methylation.

Abbreviations: CRC, colorectal cancer; FM, full methylation; HNPCC, hereditary nonpolyposis colorectal cancer syndrome; LOH, loss of heterozygosity; MAPK, mitogen-activated protein kinase; MAPKKK, mitogen-activated protein kinase kinase kinases; MEK, mitogen-activated protein/extracellular signal-regulated kinase kinase; MGMT, O^6-methylguanine DNA methyltransferase; MMR, mismatch repair; MSI, microsatellite instability; NM, nonmethylation; PM, partial methylation.

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MATERIAL AND METHODS

Tumor samples

Tumor samples were obtained from 140 sporadic CRC patients who underwent surgical treatment at the Jichi Medical School Hospital. None of the patients had first-degree relatives with CRC. Informed consents were obtained from all patients, and the ethics committee of the Jichi Medical School approved this study (#02-01). We selected these cases from approximately 380 consecutive series of CRCs previously analyzed for MSI status.[21,26] All the MSI^+ cases were reconfirmed for the MSI status by pentaplex PCR method, whereas MSI^- CRCs were selected so that the gender and tumor site were balanced between the MSI^+ and MSI^- groups (MSI^+, $n = 28$; MSI^-, $n = 112$). The patients were 69 men and 71 women, and their age ranged from 19 to 86 years with a mean of 63 years.

DNA extraction

Genomic DNA was extracted from fresh-frozen samples of tumor by use of QIAamp DNA Mini Kit (Qiagen, Chatsworth, CA) according to the manufacturer's protocol.

BRAF *mutation analysis*

BRAF mutations were analyzed in exons 11 and 15. These exons were chosen because all reported *BRAF* mutations occurred at these regions. PCR was performed with 2–5 ng of genomic DNA as a template by using the same PCR primer as reported previously.[5] PCR condition was as follows: 94°C for 9 min, followed by 35 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 2 min. The PCR products were purified using a QIAquick spin purification kit (Qiagen), and the purified PCR products were sequenced with BigDye Terminator Cycle Sequencing Ready Reaction kits (PE Applied Biosystems, Foster City, CA), all according to the manufacturers' instructions. Sequencing was performed in both directions using forward and reverse PCR primers. The purified products were run on an ABI 310 PRISM Genetic Analyzer (PE Applied Biosystems). The data were collected and analyzed using the Applied Biosystems sequencing analysis software.

MSI status analysis

MSI was analyzed by using 9 microsatellite repeat loci (3 markers were dinucleotide repeats and 6 were mononucleotide repeats) as described previously.[21] MSI status was stratified as follows according to the criteria of the National Cancer Institute (NCI) workshop.[27] High-frequency MSI (MSI-H) was defined as the alterations of microsatellite repeat were found in more than 40% of examined markers or in 2 or more NCI-recommended markers. Low-frequency MSI (MSI-L) was defined as the alterations in less than 40% or only one NCI-recommended marker. If no alterations of any examined markers were found, tumors were defined as microsatellite stable (MSS). In this study, we defined MSI-H as MSI-positive (MSI^+), and both MSI-L and MSS as MSI-negative (MSI^-), because only the MSI-H phenotype in sporadic CRCs is associated with true MMR defects and distinctive clinicopathologic features.[28,29] For the precision of MSI status, we reexamined all MSI^+ samples by pentaplex PCR method using 5 quasimonomorphic mononucleotide repeats, because this method has been reported to be simpler to use and show higher sensitivity and specificity.[30]

Analysis of methylation status of hMLH1 *promoter region*

Analysis of methylation status of *hMLH1* gene was performed by Na-bisulfite treatment and PCR single-strand conformation polymorphism (SSCP) analysis (BiPS) as described previously.[21] In brief, 5 sets of primers comprising the whole CpG sites within the *hMLH1* promoter region were prepared (Fig. 1), and methylated and unmethylated DNA amplicons were separated through SSCP analysis. When the bands showed mobility shifts, they were cut from the gels and subsequently sequenced directly by use of an ABI 310 PRISM Genetic Analyzer. Primer sequences and PCR conditions were utilized as reported previously.[21] The methylation patterns were defined as full methylation if all the CpG sites within the promoter regions showed methylation; as partial methylation if some CpG sites in the upstream region showed methylation; and as nonmethylation if no CpG sites in the region showed methylation.

KRAS *mutation analysis*

KRAS mutations were analyzed by direct sequencing at codons 12 and 13 of *KRAS* by using its genomic DNA. First, a flanking PCR product of 179 bp was amplified (annealing temperature was 58°C) using the primers 5′-AGGCCTGCTGAAAATGACTGAATA-3′ (sense) and 5′-CTGTATCAAAGAATGGTCCTGCAC-3′ (antisense). The resulting fragment was then used as a template to amplify a 114 bp fragment, including codons 12 and 13 using the primers 5′-AAAATGACTGAATATAAACTTGTGG-3′ (sense) and 5′-CTCTATTGTTGGATCATATTCGTC-3′ (antisense; annealing temperature was 50°C). The PCR product was sequenced by the same method as in the *BRAF* mutation analysis.

β-catenin *mutation analysis*

Mutations in *β-catenin* were analyzed by direct sequencing at its exon 3, in which the majority of mutation hot spots were included. The PCR primers were 5′-GATTTGATGGAGTTGGACATGG-3′ (sense) and 5′-TGTTCTTGAGTGAAGGACTGAG-3′ (antisense; annealing temperature was 63°C). The direct sequencing of the PCR product was performed by the same method as in the *BRAF* mutation analysis.

Immunohistochemical analysis

Immunohistochemical analysis for both hMLH1 and hMSH2 expression was performed on all MSI^+ tumor samples as described previously.[26]

Statistical analysis

Statistical analyses for variable results were performed by Fisher's exact test and Student's *t*-test. Probability values below 0.05 were considered to be statistically significant (StatView J 5.0 software, Abacus Concepts, Berkeley, CA).

RESULTS

Clinicopathologic features of patients with BRAF *mutations*

We identified 16 patients whose CRCs showed *BRAF* mutations (Table I). All the mutations resulted in a V599E substitution in the

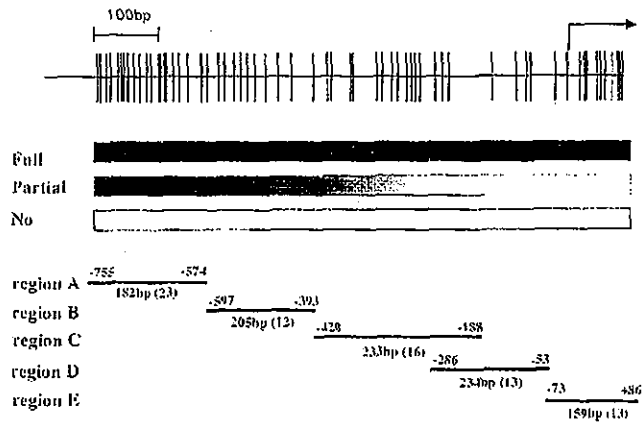

FIGURE 1 – Top: Schematic presentation of the *MLH1* promoter region. Middle: Full methylation (all CpG sites in regions A–E show methylation), partial methylation (some CpG sites in upstream region show methylation) and nonmethylation (no CpG site shows methylation). Bottom: Design of the PCR primers and the PCR products for regions A–E. Their positions relative to the adenine residue at the start codon and the size of the amplified DNA fragments are shown. Numbers in parenthesis indicate the number of CpG sites within each region.

BRAF protein (Fig. 2). None of the cases had *BRAF* mutations in the normal colonic mucosa positioned far away from the cancer area, implying that the *BRAF* mutations should be a somatic event. The mean age of cancer onset in the patients with *BRAF* mutations was older than those without *BRAF* mutations, although the difference was not statistically significant: 73.1 ± 10.5 years compared with 62.5 ± 12.5 years ($p = 0.06$, Student's *t*-test; Table II). Gender distribution was also different between these 2 groups, with females comprising 75% (12/16) of the *BRAF* mutation group and 48% (59/124) of the nonmutation group ($p = 0.06$, Fisher's exact test; Table II). The tumor with *BRAF* mutation cases was more frequently located in the proximal colon (94%; 15/16) than that with nonmutation ones (37%; 46/124; $p < 0.0001$, Fisher's exact test; Table II).

BRAF *mutations and MSI status*

BRAF mutations were found in 43% (12/28) of MSI^+ CRCs and 4% (4/112) of MSI^- CRCs ($p < 0.0001$, Fisher's exact test; Table III).

BRAF *mutations and MMR protein expression*

BRAF mutations were more common in the tumors showing reduced hMLH1 protein expression (58%; 11/19) than those showing reduced hMSH2 expression (0%; 0/6; $p = 0.02$, Fisher's exact test; Table IV).

BRAF *mutations and* hMLH1 *promoter methylation status*

BRAF mutations were found in 69% (11/16) of full methylation, 4% (2/45) of partial methylation and 4% (3/79) of nonmethylation (Table V). The ratio of *BRAF* mutations was statistically significant between full and partial as well as between full and none ($p < 0.0001$, Fisher's exact test).

KRAS *mutations*

KRAS mutations were identified in 38 cases. Two cases with *KRAS* mutations were in MSI^+ (7%; 2/28) and 36 cases were in MSI^- (32%; 36/112; $p = 0.008$, Fisher's exact test; Table III). Regarding the methylation status, *KRAS* mutations were not found

TABLE I – ALL CRC CASES WITH *BRAF* MUTATIONS

Patient no.	Age (yr)	Gender	Site	MSI	*hMLH1* methylation	*BRAF* amino acid	*KRAS*	β-*catenin*	Dukes' stage	Histologic grade
225	83	F	P	+	Full	V599E	Wild	Wild	C	Well
263	86	F	P	+	Full	V599E	Wild	Wild	B	Moderate
268	85	F	P	+	Full	V599E	Wild	Wild	B	Poor
280	83	F	P	+	Full	V599E	Wild	Wild	C	Well
305	74	M	P	+	Full	V599E	Wild	Wild	B	Poor
318	76	F	P	+	Full	V599E	Wild	Wild	B	Well
336	68	M	P	+	Full	V599E	Wild	Wild	B	Mucinous
413	69	F	P	+	Full	V599E	Wild	Wild	B	Well
416	76	F	P	+	Full	V599E	Wild	Wild	B	Mucinous
479	74	F	P	+	Full	V599E	Wild	Wild	B	Moderate
507	64	M	P	+	Full	V599E	Wild	Wild	A	Moderate
274	81	M	D	−	Partial	V599E	Wild	Wild	B	Moderate
328	52	F	P	−	Partial	V599E	Wild	Wild	B	Moderate
293	70	F	P	−	Non	V599E	Wild	Wild	D	Mucinous
384	77	F	P	+	Non	V599E	Wild	Wild	A	Poor
509	51	F	P	−	Non	V599E	Wild	Wild	C	Poor

P, proximal colon; D, distal colon; Full, full methylation; Partial, partial methylation; Non, nonmethylation; Well, well-differentiated adenocarcinoma; Mod, moderately differentiated adenocarcinoma; Poor, poorly differentiated adenocarcinoma; Muc, mucinous carcinoma.

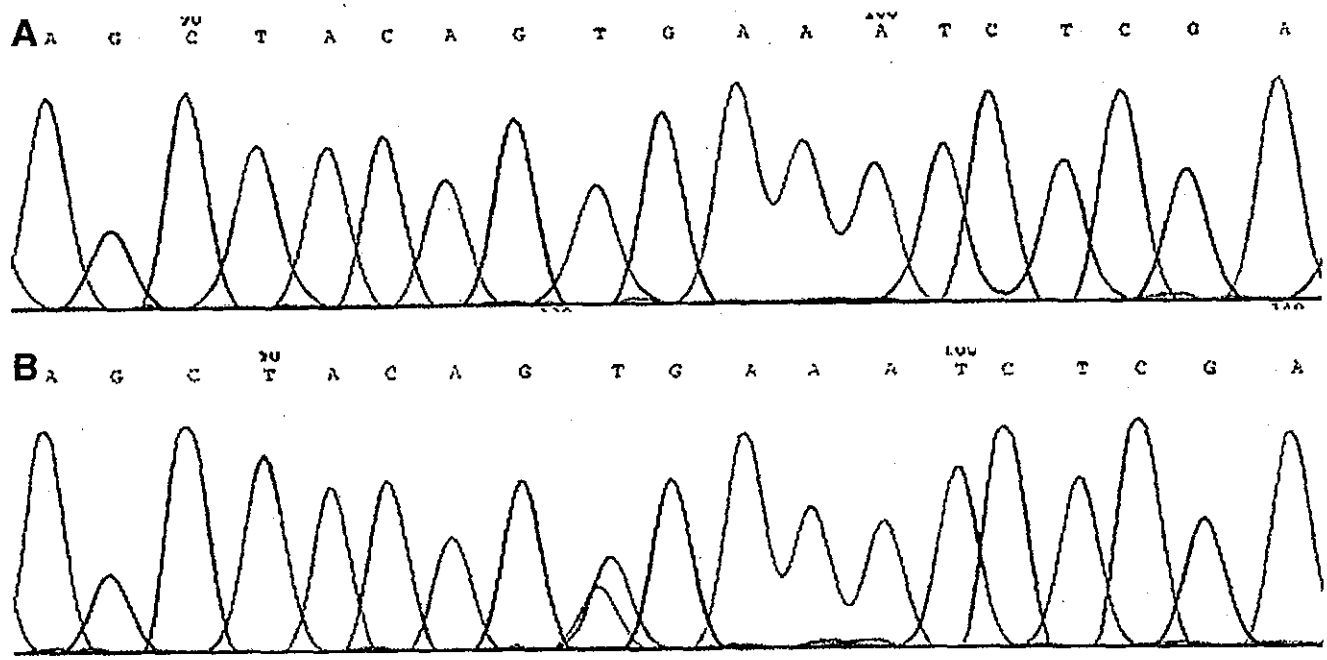

FIGURE 2 – (*a*) Representative sequence chromatographs from *BRAF* exon 15 showing wild type. (*b*) T1796A transversion resulting in a V599E substitution.

TABLE II – CLINICOPATHOLOGIC FEATURES OF *BRAF* MUTATION CASES

	BRAF mutation (n = 16) (%)	Non-*BRAF* mutation (n = 124) (%)	P-value
Age (yr)	73.1 ± 10.5	62.5 ± 12.5	0.06
Gender			0.06
Male	4 (25)	65 (52)	
Female	12 (75)	59 (48)	
Tumor site			< 0.0001
Proximal	15 (94)	46 (37)	
Distal	1 (6)	78 (63)	
Dukes' stage			0.07
A/B	12 (75)	59 (50)	
C/D	4 (25)	59 (50)	
Histologic grade			0.0008
Well/moderate	9 (56)	109 (92)	
Poor/mucinous	7 (44)	10 (8)	

Well, well-differentiated adenocarcinoma; moderate, moderately differentiated adenocarcinoma; Poor, poorly differentiated adenocarcinoma; mucinous, mucinous carcinoma.

TABLE III – MSI STATUS AND MUTATIONS OF *BRAF*, *KRAS* AND β-*CATENIN*

MSI status	*BRAF* mutation (%)	*KRAS* mutation (%)	β-*catenin* mutation (%)
MSI$^+$	42.9 (12/28)[a]	7.1 (2/28)[b]	7.1 (2/28)
MSI$^-$	3.6 (4/112)[a]	32.1 (36/112)[b]	2.7 (3/112)
Total	11.4 (16/140)	27.1 (38/140)	3.6 (5/140)

[a]Fisher's exact test, $p < 0.0001$.–[b]Fisher's exact test, $p = 0.008$.

TABLE IV – *BRAF* MUTATION AND MMR PROTEIN EXPRESSION

	Number of cases	*BRAF* mutation (%)
hMLH1-deficient	19	57.9 (11/19)[a]
hMSH2-deficient	6	0 (0/6)[a]

[a]Fisher's exact test, $p = 0.02$.

TABLE V – *hMLH1* METHYLATION STATUS AND MUTATIONS OF *BRAF*, *KRAS* AND β-*CATENIN*

hMLH1 methylation status	*BRAF* mutation (%)	*KRAS* mutation (%)	β-*catenin* mutation (%)
Full methylation	68.8 (11/16)[a,b]	0 (0/16)[c,d]	0 (0/16)
Partial methylation	4.4 (2/45)[a]	37.8 (17/45)[c]	0 (0/45)
Nonmethylation	3.8 (3/79)[b]	26.6 (21/79)[d]	6.3 (5/79)
Total	11.4 (16/140)	27.1 (38/140)	3.6 (5/140)

[a]Fisher's exact test, $P < 0.0001$.–[b]Fisher's exact test, $P < 0.0001$.–[c]Fisher's exact test, $P = 0.003$.–[d]Fisher's exact test, $P = 0.02$.

(0%; 0/16) in any of the full methylation cases, but were found in 17 of the partial methylation patients (38%; 17/45) and 21 of the nonmethylation group (26.6%; 21/79), which was consistent with our previous results[21] (Table V). The ratio of *KRAS* mutations was significantly different between full and partial and between full and none cases ($p = 0.003$ and 0.02, respectively, Fisher's exact test). None of the cases with *BRAF* mutations exhibited *KRAS* mutations simultaneously.

β-catenin *mutations*

β-catenin mutations were found in 5 cases. Two cases were in MSI$^+$ (7%; 2/28) and 3 cases were in MSI$^-$ (3%; 3/112; $p = 0.26$, Fisher's exact test; Table III). However, none of the cases with full or partial methylation showed *β-catenin* mutations, and there were no cases exhibiting both *BRAF* and *β-catenin* mutations simultaneously (Table V).

DISCUSSIONS

The MAPK pathway plays a crucial role in the signal transduction of many hormones, growth factors and differentiation factors.[31,32] At the level of MAPKKKs, several RAF family members exist, that is, ARAF, BRAF and RAF1 with divergent tissue specificity and upstream regulation.[33] The 3 proteins are thought to have uneven ability to activate MEK, and BRAF has been identified as the major MEK activator.[32]

Recently, *BRAF* activating mutations were observed in some proportion of human carcinomas, especially in melanoma, lung cancer, as well as colon cancer.[5–7] *BRAF* gene was therefore supposed to be a novel protooncogene that might contribute to the tumorigenesis in these types of transformed cells. Interestingly, the mutational spots of *BRAF* gene cluster within the activation segment (exon 15) and the G-loop (exon 11) of the kinase domain, which are highly conserved among serine/threonine kinases throughout evolution.[5] Activating mutations in these hot spots are supposed to increase its kinase activity and subsequently urge to phosphorylate the downstream kinase, MEK. V599 is the major site of point mutations in the BRAF protein and V599E acidic substitution has been commonly found in melanoma, colon cancer and ovarian cancer cells.[5] Intriguingly, the tumors with BRAF^{V599E} showed no *KRAS* mutations simultaneously, although non-V599E cases were sometimes coincident with *KRAS* mutations.[5,10,34] It has been hypothesized therefore that V599E might mimic the phosphorylation of T598 of BRAF that constitutes the natural activation mechanism of this protein. Because of its potent kinase activity, BRAF with this type of mutation might have no need to depend on RAS for the initiation of the MAP kinase pathway activation.

In our series of sporadic CRCs, 28 cases showed MSI$^+$, in which 19 were with hMLH1 deficiency and 6 were with hMSH2 deficiency. *BRAF* mutations were more frequent in MSI$^+$ CRCs than in MSI$^-$ CRCs (43%, 12/28 *vs.* 4%, 4/112; $p < 0.0001$). This result was nearly consistent with that in the previous report.[10] Interestingly, *BRAF* mutations were more frequent in hMLH1-deficient cases than hMSH2-deficient ones (58%, 11/19 *vs.* 0%, 0/6; $p = 0.02$). It has been widely accepted that MSI in sporadic CRCs commonly results from epigenetic silencing of *hMLH1* gene, secondary to its promoter methylation, and 70–90% of MSI$^+$ CRCs indeed show hypermethylation of the *hMLH1* gene.[15,21,35] Moreover, extensive methylation of *hMLH1* promoter is closely correlated with hMLH1 inactivation.[21] Therefore, we have examined the frequency of *BRAF* mutations with regard to the methylation status of *hMLH1* promoter region. Amazingly, *BRAF* mutations were extremely frequent in the cases with full methylation compared to those without full methylation (69%, 11/16 *vs.* 4%, 5/124; $p < 0.0001$). As generally seen in the cases with *hMLH1* methylation, the CRCs with *BRAF* mutations were more frequent in older females, commonly located in the proximal colon, and showed the histopathology of mucinous or poor differentiation. Our data suggest that the activating mutation of *BRAF* may be highly associated with an extensive methylation of *hMLH1* gene.

In a recent study, we proposed that the shift of methylation status from partial to full might be critical in the tumorigenesis of right-sided sporadic CRCs with MSI, because more than half of the cases with full methylation showed partial methylation in their normal mucosa far from the tumor.[21] However, the cancers with partial methylation not yet reaching full methylation showed distinct clinical and biologic features from those with full methylation, with relatively high frequency in the alterations of *KRAS*/*p53*.[22] In this study, *BRAF* mutations were less frequent in the cases with partial methylation compared to those with full methylation (4%, 2/45 *vs.* 69%, 11/16; $p < 0.0001$). We state that partial methylation is not generally the true pathogenic methylation status of *hMLH1* gene.

In our study, all the mutations of *BRAF* resulted in V599E substitutions (T-to-A transversion at nucleotide 1796). Rajago-

palan *et al.*[10] reported that all but one of the 15 *BRAF* mutations in MMR-deficient cases resulted in V599E. O^6-methylguanine DNA methyltransferase (MGMT) is a DNA repair protein and MGMT epigenetic inactivation by its promoter hypermethylation is supposed to cause G-to-A transition mutation in *KRAS* and G:C-to-A:T transition mutation in *p53*. Indeed, 71% cases with *KRAS* and *p53* mutations showed hypermethylation of *MGMT*.[36,37] Therefore, it might be possible that inactivation of an anonymous DNA-repair gene by promoter hypermethylation has an association with A-to-T transition mutation in *BRAF* gene.

Yuen *et al.*[34] have reported that there are many similarities between the phenotypic patterns of CRCs with *KRAS* and *BRAF* mutations. However, they showed that the cases with *BRAF* mutations differ from those with *KRAS* mutations in the Dukes' stage. Consistent with their results, the cases with *BRAF* mutations in our study were more common with Dukes' A/B grades than with Dukes' C/D, although the difference was not statistically significant ($p = 0.07$, Fisher's exact test). Moreover, the patients with *BRAF* mutations were approximately 13 years older than those with *KRAS* mutations (data not shown). Therefore, we speculate that the CRCs with *BRAF* mutations may belong to a clinical entity distinct from one of CRCs with *KRAS* mutations.

In melanoma cells as well as MSI^+ CRC cells, high frequency of *β-catenin* and *BRAF* mutations have been reported.[19,23–25] In this study, *β-catenin* mutations were uncommon in both MSI^+ (7%; 2/28) and MSI^- (3%; 3/112), as reported previously by Jass *et al.*[29] Moreover, none of our cases with full or partial methylation showed *β-catenin* mutations. There were no cases harboring *BRAF* and *β-catenin* mutations simultaneously, implying that *β-catenin* mutation may have no association with *hMLH1* hypermethylation with regard to CRC carcinogenesis.

We previously proposed that extensive methylation of *hMLH1* promoter might play a crucial role in tumorigenesis in the proximal colon.[21] In this study, we additionally demonstrated that the activating mutations of *BRAF* might take part in the carcinogenesis of sporadic CRCs with *hMLH1* hypermethylation in the proximal colon, independently of KRAS activation. However, one question remains to be addressed. At which stage does BRAF activation contribute to malignant transformation of colon epithelial cells? Hyperplastic polyps and serrated adenomas in the right-sided colon show high frequency of *hMLH1* hypermethylation, and these lesions have been presumed to be premalignant lesions of right-sided CRCs with MSI.[29] It would be interesting to examine whether the majority of such hyperplastic polyps and serrated adenomas already have *BRAF* mutations. If most of the CRCs with extensive methylation are associated with $BRAF^{V599E}$, such subtype would be a good target for novel anticancer drugs acting on the MAPK pathway.[38,39]

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