histological or cytological examination for which standard treatments have failed or no standard treatment exists; (2) age, from 20 to 74 years; (3) Eastern Cooperative Oncology Group performance status score of no more than 2; (4) a life expectancy of more than 12 weeks; (5) no chemotherapy within 3 weeks before participating in the study; (6) measurable or evaluable disease according to the Response Evaluation Criteria in Solid Tumors (RECIST), version 1.0; (7) adequate liver function (bilirubin levels $\leqslant 1.5\,mg\,dl^{-1}$ and transaminase levels $\leqslant 2.5$ times the upper limit of normal or $\leqslant 5$ times in the case of liver metastases); (8) adequate renal function (creatinine levels $\leqslant 1.5\,mg\,dl^{-1}$); and (9) adequate bone marrow function (absolute neutrophil counts $\geqslant 2000$ cells per $mm^3$, platelet counts $\geqslant 100\,000$ cells per $mm^3$, and haemoglobin levels $\geqslant 9.0\,g\,dl^{-1}$). Patients with central nervous system metastasis, those with a history of extensive radiation therapy within the past 6 weeks, and pregnant women were excluded from this study.

### Pre-treatment evaluation and study procedures

Baseline evaluations, including computed tomography, chest radiography, and electrocardiography, were performed within 4 weeks before treatment, and medical histories, physical examinations, and laboratory tests were performed within 1 week before treatment.

Patients were considered to be evaluable if they were given TAS-102 at least once. Toxicity was evaluated according to the National Cancer Institute's Common Terminology Criteria for Adverse Events, version 3.0. The study protocol was approved by independent ethics committees and the government authorities. All patients provided written informed consent. This trial was conducted in accordance with the Declaration of Helsinki (October 1996; JapicCTI-No.: JapicCTI-111545).

### Study design, DLT, and MTD

This study was conducted using a conventional 3+3 dose-escalation study design. The starting dose of $30\,mg\,m^{-2}$ per day was selected on the basis of the results of a previous study performed in the United States (Green *et al*, 2006). If a DLT occurred at a particular dose level, additional three patients were enroled at the same dose level. If two or more DLTs occurred at a dose level, the next lower dose level was determined to be the MTD. Intra-patient dose escalation was not allowed.

A DLT was defined as the occurrence of any of the following toxicities during the first course of TAS-102 administration. Non-haematological toxicity (excluding nausea/vomiting) of grade 3 or more, nausea/vomiting of grade 3 or more uncontrolled by aggressive anti-emetic support, grade 4 neutropenia lasting 5 days or more, febrile ($\geqslant 38.5\,°C$) neutropenia of grade 3 or more, grade 4 thrombocytopenia, or unresolved toxicities causing more than a 2-week delay of the next scheduled dose.

Before the next cycle of therapy could be initiated, patients were required to have a non-haematological toxicity of grade $\leqslant 1$, excluding alopecia, a platelet count $>100\,000$ cells per $\mu l$, and a granulocyte count $>1500$ cells per $\mu l$. For patients who developed grade 3 or 4 toxicities, the TAS-102 dose was reduced by 10 mg per day for the next cycle to a minimum dose of $30\,mg\,m^{-2}$ per day.

### Study treatment

The actual dose of TAS-102 for each patient was set to the nearest dose by a 10-mg increment. The 28-day cycle of treatment involved administration of TAS-102 twice a day within 1 h after a meal for 5 days a week for 2 weeks, followed by a 2-week rest. This treatment cycle was repeated every 4 weeks until disease progression or an unacceptable toxicity was observed. During cycle 1, the prophylactic administration of granulocyte-colony stimulating factor (G-CSF) or antibiotics was prohibited.

### Pharmacokinetic analysis

Pharmacokinetic studies were performed during cycle 1. Blood samples were collected at 0, 0.25, 0.5, 1, 2, 4, 6, 8, and 10 h after TAS-102 administration on days 1 and 12. A urine sample was collected on day 1 before administration, and from 0 to 10 h after administration. The concentrations of TFT, TPI, and an inactive form of TAS-102 (FTY) in the plasma, and TFT and TPI concentrations in the urine were measured at the Toray Research Center, Inc. (Tokyo, Japan) using a validated liquid chromatography-coupled tandem mass spectrometry method.

Pharmacokinetic parameters of TFT and TPI in the plasma were determined by non-compartmental methods (WinNonlin v. 5.2; Pharsight, Mountain View, CA, USA). The maximum plasma concentrations ($C_{max}$) and time to maximum plasma concentrations ($T_{max}$) were determined from the highest concentration, and the time of at which it was observed. The area under the concentration-time curve ($AUC_{0-10}$) was calculated using the linear trapezoidal method from 0 to 10 h, and $AUC_{inf}$ was calculated using the linear trapezoidal rule from time zero to the time of the last quantifiable concentration, which was followed by extrapolation to infinity. The elimination half-life ($t_{1/2}$) was estimated from ln(2)/Ke, where the terminal phase elimination rate constant (Ke) was estimated using log-linear regression during the terminal phase. The oral clearance (CL/F) was the body weight-normalised dose ($mg\,kg^{-1}$) divided by the $AUC_{inf}$ determined on day 1.

The volume of distribution (Vd/F) was CL/F divided by Ke. The cumulative excretion of TFT and TPI in the urine (Ae) was the total amount of urinary excretion of each compound over the 10-h period after administration divided by the administered dose amount (% of dose).

### Statistics

The number of patients in each cohort was based on a standard 3+3 design for dose-escalation studies. We planned to enrol 21 patients to assess the safety and tolerability of TAS-102, depending on the observed toxicities.

The DLT analysis set consisted of patients who had completed the first cycle in the DLT evaluation period. All patients who received TAS-102 at least once were included in the safety analysis. Pharmacokinetic analyses were performed on patients who received the scheduled dosage of TAS-102 and recorded dosing and sampling times correctly (PK analysis set). The efficacy analysis set consisted of patients with measurable disease at baseline. Descriptive statistics were provided for all endpoints by cohort. Continuous measurements were summarised with the central tendency (mean or median) and variability (s.d. or s.e.m.). Categorical data were summarised using frequency counts and percentages of patients. Correlations between the percentage decrease in neutrophil count and the log-transformed $C_{max}$ or $AUC_{inf}$ values of TFT on day 12 in course 1 were investigated by linear regression analyses (SAS v8.02 software, SAS Institute, Inc., Cary, NC, USA). The per cent change of neutrophil count was (pre-treatment count − nadir)/(pre-treatment count) × 100, where the nadir of the neutrophil count was the minimum value in course 1.

## RESULTS

### Patient characteristics

Between March 2006 and July 2007, a total of 21 patients were enroled (Table 1). The median age of the patients was 59 years (range, 38–68 years). The performance status of all patients was 0–2. The median number of prior chemotherapies was 3. Of the 21 patients, 18 had colorectal cancer. All patients had progressive disease during prior 5-FU treatment. All patients with colorectal

**Table 1** Patient characteristics

| Characteristics | *n* (%) |
|---|---|
| *Sex* | |
| Male | 14 (66.7) |
| Female | 7 (33.3) |
| *Age* | |
| Median (range) | 59 (38–68) |
| *ECOG PS* | |
| 0 | 16 (76.2) |
| 1 | 4 (19.0) |
| 2 | 1 (4.8) |
| *Primary lesion* | |
| Colon/rectum | 12 (57.1)/6 (28.6) |
| Gastric | 1 (4.8) |
| Gastric + prostate | 1 (4.8) |
| Oesophagus | 1 (4.8) |
| *Histological type* | |
| Well differentiated | 6 (28.6) |
| Moderately differentiated | 12 (57.1) |
| Poorly differentiated | 2 (9.5) |
| Squamous cell | 1 (4.8) |
| *Number of prior therapy* | |
| Median (range) | 3 (2–6) |
| ⩽2 | 6 (28.6) |
| ⩾3 | 15 (71.4) |
| *Prior therapy* | |
| Include fluropyrimidine, irinotecan, oxaliplatin | 18 (85.7) |
| Other | 3 (14.3) |

Abbreviation: ECOG PS = Eastern Cooperative Oncology Group performance status.

cancer were refractory to the three conventional cytotoxic agents, 5-FU, irinotecan, and oxaliplatin, and the three patients were refractory to anti-epidermal growth factor receptor or vascular endothelial growth factor monoclonal antibody.

### TAS-102 administration

We administered 66 treatment courses. All patients received at least one dose of TAS-102. The median TAS-102 treatment duration was 68 days (30 mg m$^{-2}$ per day cohort, 29 days; 40 mg m$^{-2}$ per day cohort, 40 days; 50 mg m$^{-2}$ per day cohort, 47 days; 60 mg m$^{-2}$ per day cohort, 119 days; and 70 mg m$^{-2}$ per day cohort, 79 days). Treatment was discontinued in all patients because of progressive disease.

### Safety and efficacy

All patients were evaluable for safety. Adverse events were observed in all patients. Although four patients died within 90 days of the first administration, the causes of their deaths were not related to the treatment. There were four treatment-related serious adverse events, namely leucopenia, neutropenia, thrombocytopenia, and pneumonia, in a patient at the 30 mg m$^{-2}$ dose level in the first course. The major treatment-related adverse events are listed in Table 2. The main toxicities were leucopenia (81% of patients), neutropenia (71.4%), decreased red blood cell counts (66.7%), decreased haematocrit (66.7%), nausea (66.7%), decreased appetite (61.9%), lymphocytopenia (61.9%), and decreased haemoglobin levels (57.1%).

In the initial cohort at a dose level of 30 mg m$^{-2}$ per day, one of the three patients developed DLTs, involving grade 4 leucopenia, neutropenia, and thrombocytopenia. An additional three patients were enroled at the same dose level, and no DLTs were observed. At a dose of 40, 50, and 60 mg m$^{-2}$, no DLTs occurred in any of the three patients enroled. At a dose of 70 mg m$^{-2}$, one patient developed a DLT involving grade 4 neutropenia. However, no DLTs were observed in the additional three patients enroled at the same dose level. The second course was delayed in three of the six patients, and the third course was delayed in four of the four patients in the 70 mg m$^{-2}$ treatment cohort because of the development of grade 3 or 4 neutropenia.

All patients were evaluable for tumour response according to RECIST version 1.0. Although no complete or partial responses were observed, 11 patients achieved stable disease, resulting in a disease control rate (DCR) of 52.3%. The treatment could be continued for 12 weeks in 8 patients. The median progression-free survival (PFS) and overall survival (OS) were 2.6 months and 10.2 months, respectively (Figure 1).

In colorectal cancer patients ($n = 18$), the DCR was 50.0%, including 6 patients who were able to continue treatment over 12 weeks. The median PFS and OS in this cohort were 2.4 and 9.8 months, respectively.

### Pharmacokinetics

Pharmacokinetics were evaluated using plasma and urine samples (Table 3). Systemic concentrations of TFT and TPI increased linearly in correlation with increasing oral doses. Plasma TFT concentrations tended to increase with repeated administrations, and on day 12, $AUC_{0-10}$ of TFT was about 2.6 times higher than that measured on day 1. In contrast, there were no obvious changes in the PK parameters of TPI and FTY after repeated administrations. These tendencies were similar to the results after repeated, once daily administration. A significant inverse correlation was observed between the percent change in neutrophil count and TFT $C_{max}$ ($r = -0.678$, $P < 0.001$) or $AUC_{0-10}$ ($r = -0.753$, $P < 0.001$; Figure 2). The cumulative percentage of the TFT and TPI doses excreted in the urine from 0 to 10 h after drug administration was 1% to 8% and 19% to 23%, respectively.

## DISCUSSION

TAS-102, a thymidine analogue, is an orally administered antitumour drug. On the basis of this phase I trial, the RD for the subsequent phase II trial of TAS-102 in Japanese patients was determined to be 70 mg m$^{-2}$ per day twice a day for 5 days a week for 2 weeks, followed by 2-week rest. The treatment was well tolerated at the phase II doses.

The common grade 3 and 4 adverse events were haematological toxicities. Two patients experienced DLTs during cycle 1; one patient developed grade 4 neutropenia, leucopenia, and thrombocytopenia after treatment at a dose of 30 mg m$^{-2}$ per day, and the other patient experienced grade 4 neutropenia and leucopenia after treatment at a dose of 70 mg m$^{-2}$ per day. Thus, the safety profiles of TAS-102 in the Japanese patients were similar to those of the American patients reported in previous studies (Green *et al*, 2006; Hong *et al*, 2006; Overman *et al*, 2008a; 2008b). Of the 21 patients, 3 were treated with G-CSF 3 weeks after the first administration of each cycle. Two of these three patients received G-CSF at cycle 1 to treat neutropenia evaluated as DLTs and neutropenia resolved. Seven patients received antibiotics prophylactically to treat the adverse events. Blood tests performed 14 days or later after the first administration were useful for finding the nadir of toxicities, because the toxicities tended to be observed about 21 days after the first administration in each course. TAS-102 treatment could be continued in almost all patients with adequate management of toxicity without developing serious adverse events. In this study, we did not examine the effects of a dose of 80 mg m$^{-2}$ per day, because grade 3 neutropenia was observed in three of six patients

**Table 2** The most common treatment-related adverse events

| Dose (mg m$^{-2}$ per day) | 30 ($n=6$) | | | 40 ($n=3$) | | | 50 ($n=3$) | | | 60 ($n=3$) | | | 70 ($n=6$) | | | Total ($n=21$) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | All | ⩾G3 | ⩾G4 | All | ⩾G3 | ⩾G4 | All | ⩾G3 | ⩾G4 | All | ⩾G3 | ⩾G4 | All | ⩾G3 | ⩾G4 | All (%) | ⩾G3 (%) | ⩾G4 (%) |
| *Haematological toxicities* | | | | | | | | | | | | | | | | | | |
| Leucopenia | 4 | 1 | | 2 | | | 2 | 1 | | 3 | 1 | | 6 | 4 | | 17 (81.0) | 7 (33.3) | |
| Neutropenia | 2 | | 1 | 2 | | | 2 | 1 | | 3 | 3 | | 6 | 4 | | 15 (71.4) | 8 (38.1) | 1 (4.8) |
| Red blood cell count decreased | 2 | | | 2 | | | 3 | | | 2 | | | 5 | 1 | | 14 (66.7) | 2 (9.5) | |
| Haematocrit decreased | 3 | | | 2 | | | 3 | 1 | | 2 | | | 4 | 1 | | 14 (66.7) | 1 (4.8) | |
| Lymphocytopenia | 3 | 1 | | 1 | | | 3 | | | 2 | | | 4 | 3 | | 13 (61.9) | 4 (19.0) | |
| Anaemia | 4 | 1 | | | | | 2 | 2 | | 2 | | | 4 | 4 | | 12 (57.1) | 7 (33.3) | |
| Thrombocytopenia | 2 | 1 | 1 | | | | 1 | | | 1 | | | 5 | | | 9 (42.9) | 1 (4.8) | 1 (4.8) |
| Blood albumin level decreased | | | | 1 | | | 1 | | | 1 | | | 1 | | | 4 (19.0) | | |
| Blood bilirubin level increased | | | | 1 | | | | | | 1 | | | 2 | | | 4 (19.0) | | |
| Aspartate aminotransferase level increased | 1 | | | 1 | | | | | | 1 | | | 1 | | | 4 (19.0) | | |
| Blood alkaline phosphatase level increased | 1 | | | 1 | | | | | | 1 | | | 1 | | | 4 (19.0) | | |
| Protein total decreased | | | | 1 | | | | | | 1 | | | 1 | | | 3 (14.3) | 1 (4.8) | |
| Monocyte count increased | 1 | 1 | | | | | | | | | | | 2 | | | 3 (14.3) | | |
| Alanine aminotransferase increased | 1 | | | | | | | | | 1 | | | 1 | | | 3 (14.3) | | |
| *Nonhaematological toxicities* | | | | | | | | | | | | | | | | | | |
| Nausea | 4 | | | | | | 2 | | | 3 | | | 5 | | | 14 (66.7) | | |
| Appetite loss | 5 | 1 | | | | | | | | 3 | | | 5 | | | 13 (61.9) | 1 (4.8) | |
| Fatigue | 3 | 1 | | | | | 2 | | | 1 | | | 2 | | | 8 (38.1) | 1 (4.8) | |
| Vomiting | 2 | | | | | | 1 | | | 2 | | | 1 | | | 6 (28.6) | | |
| Diarrhoea | 1 | | | | | | 2 | 1 | | 1 | | | 1 | | | 5 (23.8) | 1 (4.8) | |
| Albuminuria | 1 | | | 1 | | | | | | 2 | | | 1 | | | 5 (23.8) | | |
| Stomatitis | | | | 1 | | | | | | 1 | | | 1 | | | 3 (14.3) | | |
| Weight loss | | | | | | | 1 | | | | | | 2 | | | 3 (14.3) | | |
| Bloating | 2 | | | | | | 1 | | | | | | | | | 3 (14.3) | | |

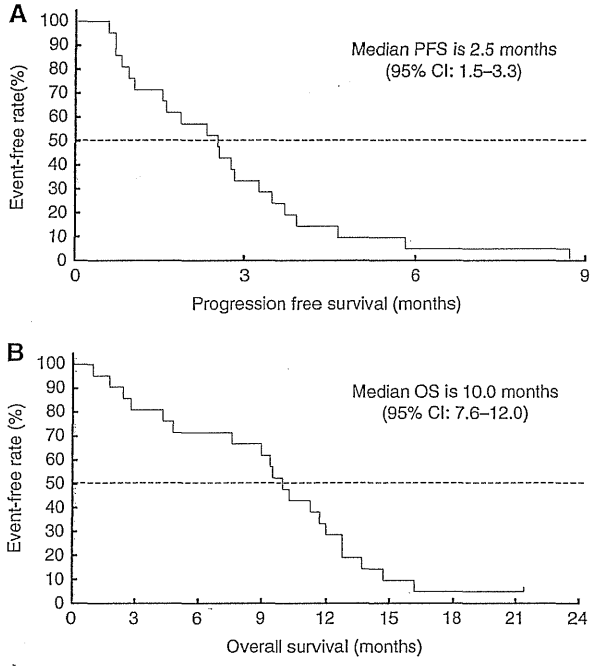


**Figure 1** Kaplan–Meier plots of the median progression-free survival (PFS) (**A**) and median overall survival (OS) (**B**) in all patients.

at a dose of 70 mg m$^{-2}$ per day, and the higher dose (80 mg m$^{-2}$ per day) was thought not to be tolerated on the basis of the similarity in the toxicities observed in this study and those in the US study (Green *et al*, 2006).

Dose-dependent increases in TFT and TPI exposures were observed for doses up to 70 mg m$^{-2}$ per day. Although the number of patients included in the PK evaluation was limited and the administration schedules differed, it seems that there are no large differences in the PK parameter of TFT, the active component of TAS-102, between the Japanese and American patients, because CL/F, $T_{max}$, and $t_{1/2}$ values of American patients at a dose of 50 mg m$^{-2}$ per administration are approximately comparable to those of Japanese patients at a dose range from 15 to 35 mg m$^{-2}$ per administration. However, the RD in this study was higher than that in the US study, which had the same schedule design as this study (Green *et al*, 2006). The data obtained from several phase I studies in the United States provided information about managing various adverse events and about when to perform blood tests, and when to skip drug administrations. Therefore, in this trial, patients were hospitalised during cycle 1 for evaluation of DLTs and blood tests, and observation of symptoms was performed more closely and frequently compared with that in the US study. Thus, our study suggested a different RD as compared with the US study. Therefore, we are going to perform the dose-matching study in the United States to determine the optimal dose for global study.

Only 1% to 8% and approximately 20% of the administered doses were excreted into the urine as TFT and TPI, respectively. TPI and FTY exposure on day 12 did not show obvious alterations with repeated administration of TAS-102, but TFT exposure on day 12 was 2.6 times that on day 1. Moreover, the TFT exposure on day 12 was significantly correlated with the percent change of neutrophil count from baseline. These results suggest that the triphosphate form of TFT is incorporated into DNA in the neutrophils in a concentration-dependent manner, which results in sequential increases in single-strand DNA breaks. Therefore, 70 mg m$^{-2}$ per day, the maximum dose in this study, is expected to be effective in a phase II study.

Although partial responses and complete responses were not confirmed, stable disease was observed in 11 patients. The DCR was 33.3% each at doses of 30, 40, and 50 mg m$^{-2}$ per day; 100.0% at a dose of 60 mg m$^{-2}$ per day; and 66.7% at a dose of 70 mg m$^{-2}$ per day. These findings indicate the tendency that the DCR correlated with the dose. The median PFS in all patients was 2.5 months. The median PFS was 1.7 months at doses $\leqslant$50 mg m$^{-2}$ per day and 3.3 months at doses $\geqslant$60 mg m$^{-2}$ per day. These result suggest that TAS-102 has a potent anti-tumour effect at a dose of $>$60 mg m$^{-2}$ per day. Although this agent did not reach the MTD, 70 mg m$^{-2}$ per day was determined as the RD for the subsequent studies.

**Table 3** Pharmacokinetic parameters of TFT, TPI, and an inactive form (FTY) after administration of TAS-102 on day 1 and day 12

| Dose ($mg\,m^{-2}$ per day) | 30 ($n=6$) | | 40 ($n=3$) | | 50 ($n=3$) | | 60 ($n=3$) | | 70 ($n=6$) | |
|---|---|---|---|---|---|---|---|---|---|---|
| Day | 1 | 12 | 1 | 12 | 1 | 12 | 1 | 12 | 1 | 12 |
| *TFT* | | | | | | | | | | |
| $C_{max}$ ($ng\,ml^{-1}$) | 1009 ± 491 | 1205 ± 421 | 1840 ± 737 | 2747 ± 610 | 2450 ± 1021 | 2757 ± 1173 | 3677 ± 1459 | 5437 ± 1685 | 3338 ± 767 | 4752 ± 1697 |
| $T_{max}$ (h) | 1.7 ± 1.3 | 1.6 ± 0.7 | 1.2 ± 0.8 | 1.7 ± 0.6 | 1.5 ± 0.9 | 1.3 ± 0.6 | 1.2 ± 0.8 | 1.3 ± 0.6 | 1.3 ± 0.5 | 1.9 ± 1.6 |
| $AUC_{0-10}$ ($ng\,h\,ml^{-1}$) | 2037 ± 773 | 5478 ± 2849 | 4347 ± 535 | 9994 ± 2109 | 4281 ± 1380 | 8656[a] ± NA | 8229 ± 1441 | 23672 ± 7844 | 8678 ± 1786[b] | 20950 ± 2237 |
| $AUC_{inf}$ ($ng\,h\,ml^{-1}$) | 2055 ± 793 | — | 4373 ± 568 | — | 4297 ± 1387 | — | 8435 ± 1645 | — | 8672 ± 1710 | — |
| $t_{1/2}$ (h) | 1.39 ± 0.38 | 2.44 ± 1.57 | 1.17 ± 0.15 | 1.52 ± 0.34 | 1.49 ± 0.59 | 1.96 ± 0.10 | 1.88 ± 0.73 | 2.33 ± 1.26 | 1.41 ± 0.38 | 1.97 ± 0.51 |
| CL/F ($l\,h^{-1}\,kg^{-1}$) | 0.168 ± 0.034 | — | 0.124 ± 0.035 | — | 0.178 ± 0.055 | — | 0.103 ± 0.014 | — | 0.118 ± 0.018 | — |
| Vd/F ($l\,kg^{-1}$) | 0.324 ± 0.095 | — | 0.204 ± 0.030 | — | 0.384 ± 0.175 | — | 0.273 ± 0.089 | — | 0.234 ± 0.054 | — |
| Ae (%) | 3.59 ± 3.82 | — | 4.85 ± 5.61 | — | 7.64 ± 4.49 | — | 0.96[a] ± NA | — | 3.69 ± 3.42[b] | — |
| *TPI* | | | | | | | | | | |
| $C_{max}$ ($ng\,ml^{-1}$) | 25.8 ± 14.7 | 44.1 ± 51.8 | 43.1 ± 6.5 | 41.8 ± 14.7 | 54.2 ± 28.5 | 50.2 ± 13.1 | 136.1 ± 77.5 | 99.6 ± 43.8 | 76.6 ± 32.1 | 70.0 ± 43.4 |
| $T_{max}$ (h) | 2.6 ± 1.6 | 2.8 ± 1.5 | 1.7 ± 0.6 | 2.7 ± 1.2 | 1.7 ± 0.6 | 2.7 ± 1.2 | 2.7 ± 1.2 | 2.7 ± 1.2 | 2.3 ± 0.8 | 2.3 ± 0.8 |
| $AUC_{0-10}$ ($ng\,h\,ml^{-1}$) | 117 ± 84 | 234 ± 283 | 166 ± 29 | 161 ± 41 | 214 ± 79 | 300[a] ± NA | 521 ± 338 | 447 ± 278 | 281 ± 99[b] | 317 ± 182 |
| $AUC_{inf}$ ($ng\,h\,ml^{-1}$) | 129 ± 96 | — | 170 ± 29 | — | 222 ± 79 | — | 542 ± 360 | — | 302 ± 96 | — |
| $t_{1/2}$ (h) | 2.27 ± 0.74 | 2.89 ± 0.83 | 1.53 ± 0.17 | 1.82 ± 0.18 | 1.78 ± 0.27 | 4.01 ± 3.57 | 1.66 ± 0.37 | 2.21 ± 0.62 | 1.67 ± 0.22 | 2.37 ± 0.93 |
| CL/F ($l\,h^{-1}\,kg^{-1}$) | 1.52 ± 0.67 | — | 1.52 ± 0.50 | — | 1.66 ± 0.56 | — | 0.91 ± 0.40 | — | 1.83 ± 1.06 | — |
| Vd/F ($l\,kg^{-1}$) | 4.90 ± 2.37 | — | 3.30 ± 0.93 | — | 4.31 ± 1.85 | — | 2.06 ± 0.62 | — | 4.42 ± 2.68 | — |
| Ae (%) | 19.4 ± 12.2 | — | 22.9 ± 5.1 | — | 20.0 ± 9.6 | — | 20.0[a] ± NA | — | 19.0 ± 7.5[b] | — |
| *FTY* | | | | | | | | | | |
| $C_{max}$ ($ng\,ml^{-1}$) | 248 ± 83 | 198 ± 49 | 453 ± 91 | 398 ± 4 | 645 ± 23 | 470 ± 174 | 753 ± 293 | 512 ± 41 | 878 ± 228 | 560 ± 92 |
| $T_{max}$ (h) | 2.1 ± 1.6 | 2.3 ± 0.8 | 1.7 ± 0.6 | 2.0 ± 0.0 | 1.5 ± 0.9 | 1.7 ± 0.6 | 1.5 ± 0.9 | 1.2 ± 0.8 | 2.0 ± 0.0 | 2.3 ± 1.4 |
| $AUC_{0-10}$ ($ng\,h\,ml^{-1}$) | 993 ± 392 | 1301 ± 524 | 1740 ± 172 | 2259 ± 411 | 1901 ± 316 | 2401[a] ± NA | 2653 ± 537 | 3095 ± 538 | 3165 ± 341[b] | 3622 ± 1094 |
| $AUC_{inf}$ ($ng\,h\,ml^{-1}$) | 1016 ± 407 | — | 1776 ± 216 | — | 1915 ± 327 | — | 2710 ± 559 | — | 3492 ± 693 | — |
| $t_{1/2}$ (h) | 1.34 ± 0.30 | 4.57 ± 2.74 | 1.32 ± 0.40 | 4.55 ± 2.90 | 1.18 ± 0.18 | 4.79 ± 2.50 | 1.62 ± 0.32 | 9.60 ± 5.31 | 1.57 ± 0.38 | 7.27 ± 2.95 |

Abbreviations: TFT = trifluorothymidine; TPI = thymidine phosphorylase inhibitor; AUC = area under the concentration-time curve; NA = not applicable. Cumulative urinary excretion rate was calculated within 10 h. [a]$n=2$. [b]$n=5$.

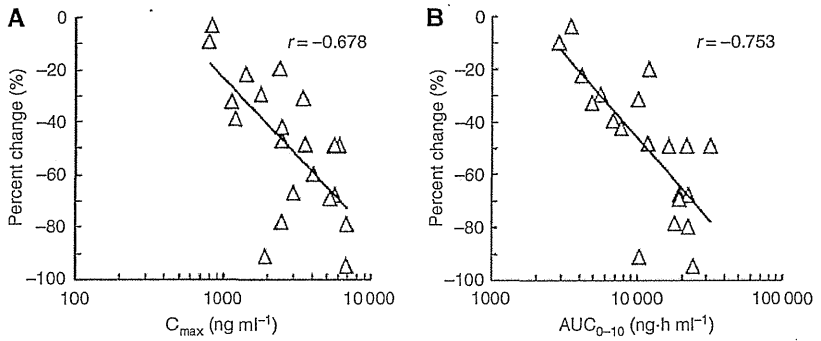


**Figure 2** Relationship between pharmacokinetic parameters of trifluorothymidine (TFT; $C_{max}$ and $AUC_{inf}$) and the percent change of the neutrophil count of patients treated with 30–70 $mg\,m^{-2}$ per day of TAS-102 on day 12 in 1 course. (**A**) Relationship between TFT $C_{max}$ and the percent change of the neutrophil count. (**B**) Relationship between TFT $AUC_{inf}$ and the percent change of the neutrophil count. Each symbol denotes an individual value.

In this study, 18 of the 21 patients had colorectal cancer. The DCR, median PFS, and OS in the colorectal cancer patients was 50.0%, 2.4 months, and 9.8 months, respectively; however, the median OS of panitumumab and cetuximab in colorectal cancer patients refractory to 5-FU, irinotecan, and oxaliplatin was 8.6 and 6.4 months, respectively (Saltz *et al*, 2004; Hecht, *et al*, 2007). These results suggest that TAS-102 showed anti-tumour effects in colorectal cancer patients refractory to standard treatment and thus may be a promising agent, and it is meaningful to conduct the phase II trials of TAS-102 on colorectal cancer patients.

Recently, chemotherapy based on 5-FU, such as FOLFOX and FOLFIRI, have been established as a first- and second-line therapy for colorectal cancer, but tertiary and subsequent therapies remain to established. Although cetuximab and panitumumab were effective in colorectal cancer patients refractory to standard therapy, these agents were not effective in patients with K-ras mutations. These results suggest that the efficacy of TAS-102 in patients refractory to 5-FU may be because of the differences in anti-tumour mechanisms of both agents, and the efficacy was presumed not to be affected by the K-ras status. The recent randomised phase II trial for colorectal cancer in Japan based on our phase I data has shown promising preliminary results (Kuboki *et al*, 2011). The PK and safety profiles reported in this phase I trial are important to determine the optimal dose and schedule used in a clinical setting. Studies on combination of TAS-102 with other molecular-target drugs should be performed in the future.

In conclusion, our Phase I study showed that TAS-102 was well tolerated up to doses of 70 $mg\,m^{-2}$ per day in Japanese patients with advanced solid tumours. The safety and efficacy of TAS-102 for colorectal cancer patients should be investigated in phase II and III trials.

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## Conflict of interest

The authors declare no conflict of interest.

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# *KRAS* mutations in primary tumours and post-FOLFOX metastatic lesions in cases of colorectal cancer

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BACKGROUND: *KRAS* mutations are predictive markers for the efficacy of anti-EGFR antibody therapies in patients with metastatic colorectal cancer. Although the mutational status of *KRAS* is reportedly highly concordant between primary and metastatic lesions, it is not yet clear whether genotoxic chemotherapies might induce additional mutations.

METHODS: A total of 63 lesions (23 baseline primary, 18 metastatic and 24 post-treatment metastatic) from 21 patients who were treated with FOLFOX as adjuvant therapy for stage III/IV colorectal cancer following curative resection were examined. The DNA samples were obtained from formalin-fixed paraffin-embedded specimens, and *KRAS*, *NRAS*, *BRAF* and *PIK3CA* mutations were evaluated.

RESULTS: The numbers of primary lesions with wild-type and mutant *KRAS* codons 12 and 13 were 8 and 13, respectively. The mutational status of *KRAS* remained concordant between the primary tumours and the post-FOLFOX metastatic lesions, irrespective of patient background, treatment duration and disease-free survival. Furthermore, the mutational statuses of the other genes evaluated were also concordant between the primary and metastatic lesions.

CONCLUSION: Because the mutational statuses of predictive biomarker genes were not altered by FOLFOX therapy, specimens from both primary tumours and post-FOLFOX tumour metastases might serve as valid sources of DNA for known genomic biomarker testing.

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**Keywords:** colorectal cancer; genomic biomarker; *KRAS*; anti-EGFR antibody; oxaliplatin

Molecular Diagnostics

*KRAS* mutations are predictive markers for the poor efficacy of anti-EGFR antibody therapies in patients with metastatic colorectal cancer (Lievre *et al*, 2006; Benvenuti *et al*, 2007; Di Fiore *et al*, 2007; Frattini *et al*, 2007; Khambata-Ford *et al*, 2007; Amado *et al*, 2008; De Roock *et al*, 2008; Freeman *et al*, 2008; Karapetis *et al*, 2008; Lievre *et al*, 2008). Point mutations in the *KRAS* gene occur early in the progression from colorectal adenoma to carcinoma and are detected in 35–40% of patients, regardless of their Dukes stage (Andreyev *et al*, 1998). More than 90% of the *KRAS* mutations in these patients have been detected in codons 12 (GGT) and 13 (GGC) (Oliveira *et al*, 2004). Activating mutations at codons 61 and 146 have also been reported in a small number of these tumours. In addition, mutations in the molecules involved in signalling pathways downstream of EGFR, such as *NRAS*, *BRAF* and *PIK3CA*, have also been reported in colorectal cancers. These mutations have been suggested to modify the efficacy of anti-EGFR antibody therapies, although their predictive value has not yet been established (De Roock *et al*, 2010).

Oxaliplatin [*trans*-R,R-1,2-diaminocyclohexaneoxalatoplatinum (II), L-OHP] is a third-generation platinum (Pt)-containing anti-tumour compound. It is frequently administered as a component of FOLFOX therapy in combination with 5-FU for patients with metastatic colorectal cancer. Oxaliplatin induces DNA damage associated with intra- and inter-strand cross-links (Pt-GG adducts) and can induce gene mutations (Woynarowski *et al*, 2000; Hah *et al*, 2007; Sharma *et al*, 2007). The mutagenic activity of oxaliplatin has been demonstrated in cultured cells (Silva *et al*, 2005).

The *KRAS* mutation status of primary and metastatic lesions is reportedly highly concordant (Oudejans *et al*, 1991; Losi *et al*, 1992; Suchy *et al*, 1992; Zauber *et al*, 2003; Weber *et al*, 2007; Etienne-Grimaldi *et al*, 2008; Santini *et al*, 2008; Garm Spindler *et al*, 2009; Loupakis *et al*, 2009; Perrone *et al*, 2009; Baldus *et al*, 2010; Italiano *et al*, 2010; Knijn *et al*, 2011). However, whether long-term treatment with genotoxic chemotherapies, such as oxaliplatin, can induce additional mutations in metachronous metastatic lesions has not yet been well examined.

Assuming that FOLFOX therapy has the potential to alter the biomarker mutation profile, it is important to determine whether

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the primary or relapsed tumour represents the more appropriate source of DNA for testing. We examined the mutation status of *KRAS* and other biomarker genes in primary and synchronous/metachronous metastatic lesions in patients with stage III/IV colorectal cancer treated with adjuvant FOLFOX therapy following curative resection.

## PATIENTS AND METHODS

### Patient selection

A total of 63 lesions from 21 patients who had received adjuvant FOLFOX therapy for stage III/IV colorectal cancer following curative resection at the National Cancer Center Hospital East, Japan, between January 2006 and December 2009 were examined.

All patients were treated with a modified FOLFOX6 regimen, with a reduced oxaliplatin dose of 85 mg m$^{-2}$ administered every 14 days, and 12 cycles were planned as the full therapy course (Andre *et al*, 2004; Allegra *et al*, 2009). FOLFOX therapy was discontinued when tumour relapse was demonstrated by imaging or when intolerable adverse events occurred.

### DNA samples and mutational analyses

The DNA samples were obtained from macroscopically dissected formalin-fixed paraffin-embedded specimens cut into 10-$\mu$m-thick sections. Genomic DNA was extracted using the EZ1 Advanced XL and EZ1 DNA Tissue Kits (Qiagen, Hilden, Germany) according to the manufacturer's instructions (Bando *et al*, 2011). Mutations in *KRAS* codons 12 and 13 were detected using the ARMS/Scorpions technology-based *KRAS* PCR Kit (Qiagen) according to the manufacturer's instructions. Mutations in *KRAS* codons 61 and 146, *NRAS* codons 12, 13 and 61, *BRAF* codon 600 and *PIK3CA* codons 542, 545, 546 and 1047 were detected using the multiplex PCR-Luminex method-based MEBGEN Mutation Kit (Medical & Biological Laboratories, Nagoya, Japan). Mutations detected with the MEBGEN Mutation Kit were confirmed by direct sequencing. Mutations in *PIK3CA* codons 542, 545 and 546 were further confirmed using the ARMS/Scorpions technology-based PI3K Mutation Test Kit (Qiagen). The study was approved by the Institutional Review Board of the National Cancer Center.

## RESULTS

### Patient and tumour site characteristics

We reviewed 151 consecutive cases of stage III/IV colorectal cancer treated with an adjuvant FOLFOX therapy after curative resection. Among these cases, 21 patients developed metastatic tumours that were diagnosed during or after the FOLFOX therapy and surgically resected. The patient and tumour site characteristics are shown in Table 1. The primary tumour sites were the colon and rectum in 8 and 13 patients, respectively. The most abundant primary tumour histopathological type was differentiated adenocarcinoma. Well- and moderately differentiated adenocarcinomas and mucinous adenocarcinomas were observed in 5, 14 and 2 patients, respectively. All metastatic tumours exhibited histology concordant with that of the associated primary colorectal adenocarcinoma.

In all, 12 patients had stage III disease, whereas the remaining 9 patients had synchronous metastatic lesions and were diagnosed as stage IV at the initial operation. There were 12 synchronous metastatic lesions in the patients with stage IV disease. In addition, six metastatic lesions were detected in five patients with stage III disease at operation that were resected prior to the start of FOLFOX therapy. These 18 lesions were regarded as 'pre-FOLFOX' metastatic lesions. The pre-FOLFOX metastases were found in the liver (11 lesions), lung (5 lesions), as a local recurrence (1 lesion) and as a subcutaneous recurrence (1 lesion). Meanwhile, 24 metastatic lesions in the 21 patients were detected during or after FOLFOX therapy. These lesions were regarded as 'post-FOLFOX' metastatic lesions. The post-FOLFOX metastases were found in the liver, lung, as a local recurrence and lymph node in 6, 14, 3 and 1 patients, respectively.

**Table 1** Characteristics

| Patient characteristics | Number |
|---|---|
| Sex (female/male) | 8/13 |
| Median age (range) | 64 (36–75) years |
| *Primary tumour site* | |
| Colon | 8 |
| Rectum | 13 |
| *Histopathological type of primary site* | |
| Well-differentiated adenocarcinoma | 5 |
| Moderately differentiated adenocarcinoma | 14 |
| Mucinous adenocarcinoma | 2 |
| *Stage before initial operation* | |
| III | 12 |
| IV (synchronous metastases) | 9 |
| **Tumour site characteristics** | |
| *Metastases* | |
| Pre-FOLFOX | 18 |
| Synchronous | 12 |
| Metachronous | 6 |
| Post-FOLFOX | 24 |
| *Sites of metastases* | |
| Pre-FOLFOX | |
| Liver | 11 |
| Lung | 5 |
| Local recurrence | 1 |
| Subcutaneous | 1 |
| *Post-FOLFOX* | |
| Liver | 6 |
| Lung | 14 |
| Local recurrence | 3 |
| Lymph node | 1 |

The median number of FOLFOX therapy cycles administered was 9 (3–12 cycles). Five patients experienced relapse during FOLFOX therapy (case 1, 2, 3, 7 and 12), whereas the remaining 16 patients experienced relapse after the end of FOLFOX therapy. The median disease-free survival, calculated from the time of the last operation until post-FOLFOX recurrence, was 409 days (97–1077). The median period from the start of FOLFOX therapy until recurrence was 373 days (35–1029). Relapses developed within 180 days after the end of FOLFOX therapy in 10 of the 21 patients (Table 2).

### Mutational status of *KRAS* and other genes

The mutational statuses of *KRAS* and other genes in primary and metastatic lesions are shown in Table 3. Mutations in *KRAS* codons 12 and 13 were detected in 13 of the 21 primary colorectal tumours. Among the remaining eight tumours with wild-type *KRAS* codons 12 and 13, two tumours exhibited *KRAS* codon 146 mutations (A146V and A146T) and one tumour exhibited *NRAS* codon 61 mutation (Q61H). Two tumours exhibited mutations in *PIK3CA* codon 542 (E542K), one tumour exhibited a *KRAS* G12S mutation and one tumour had no mutations in any of the genes examined. No apparent mutations of *KRAS* codon 61, *NRAS* codon

**Table 2** FOLFOX treatment, metastasis status and tumour recurrence sites

| Case | Primary site | Histopathological type | Pre-FOLFOX metastatic site | Synchronous/ metachronous | FOLFOX cycles | DFS (days) | Days from end of FOFLOX until recurrence | Post-FOLFOX recurrence site |
|---|---|---|---|---|---|---|---|---|
| 1 | Rectum | Mode | — | — | 3 | 124 | 6 | Liver |
| 2 | Colon | Mode | Liver | Synchronous | 4 | 97 | −16[a] | Liver |
| 3 | Colon | Mode | Liver | Synchronous | 4 | 116 | 26 | Liver |
| 4 | Rectum | Well | Local recurrence | Metachronous | 4 | 469 | 363 | Local recurrence |
| 5 | Rectum | Mode | — | — | 5 | 827 | 603 | Lung |
| 6 | Colon | Mode | — | — | 5 | 350 | 244 | Lymph node |
| 7 | Rectum | Mode | Liver | Synchronous | 8 | 214 | 1 | Lung |
| | | | Lung | Synchronous | | | | |
| 8 | Rectum | Muc | — | — | 8 | 538 | 318 | Lung |
| 9 | Colon | Well | — | — | 8 | 1077 | 903 | Liver |
| 10 | Colon | Mode | Liver | Synchronous | 8 | 344 | 120 | Lung |
| | | | Liver | Synchronous | | | | Lung |
| | | | Lung | Synchronous | | | | |
| 11 | Colon | Muc | Lung | Synchronous | 9 | 721 | 401 | Lung |
| 12 | Rectum | Well | Liver | Synchronous | 9 | 109 | −88[a] | Liver |
| 13 | Rectum | Mode | Liver | Metachronous | 11 | 328 | 120 | Liver |
| | | | Lung | Metachronous | | | | |
| 14 | Rectum | Mode | Subcutaneous | Metachronous | 12 | 519 | 156 | Lung |
| 15 | Colon | Mode | — | — | 12 | 388 | 176 | Local recurrence |
| 16 | Rectum | Mode | Liver | Synchronous | 12 | 466 | 210 | Lung |
| 17 | Rectum | Well | Lung | Synchronous | 12 | 556 | 264 | Lung |
| 18 | Colon | Mode | Liver | Metachronous | 12 | 531 | 231 | Lung |
| | | | | | | | | Lung |
| 19 | Rectum | Mode | Liver | Synchronous | 12 | 409 | 217 | Lung |
| 20 | Rectum | Mode | — | — | 12 | 455 | 243 | Local recurrence |
| 21 | Rectum | Well | Liver | Metachronous | 12 | 346 | 71 | Lung |
| | | | | | | | | Lung |

Abbreviations: DFS = disease-free survival; mode = moderately differentiated adenocarcinoma; muc = mucinous adenocarcinoma; well = well-differentiated adenocarcinoma.
[a]The cases that FOLFOX therapies were administered after recurrence.

**Table 3** Mutational status of *KRAS* and other genes

| Case | Primary site | Mutation status | Pre-FOLFOX metastatic site | Mutation status | Post-FOLFOX recurrence site | Mutation status |
|---|---|---|---|---|---|---|
| 1 | Rectum | KRAS G12D | — | — | Liver | KRAS G12D |
| 2 | Colon | KRAS G12D | Liver | KRAS G12D | Liver | KRAS G12D |
| 3 | Colon | KRAS G12D | Liver | KRAS G12D | Liver | KRAS G12D |
| 4 | Rectum | KRAS G12R | Local recurrence | KRAS G12R | Local recurrence | KRAS G12R |
| 5 | Rectum | KRAS G12D | — | — | Lung | KRAS G12D |
| 6 | Colon | WT | — | — | LN | WT |
| 7 | Rectum | KRAS G12S | Liver | KRAS G12S | Lung | KRAS G12S |
| | | | Lung | KRAS G12S | | |
| 8 | Rectum | WT | — | — | Lung | WT |
| 9 | Colon | WT | — | — | Liver | WT |
| 10 | Colon | KRAS G12A | Liver | KRAS G12A | Lung | KRAS G12A |
| | | | Liver | KRAS G12A | Lung | KRAS G12A |
| | | | Lung | WT | | |
| 11 | Colon | KRAS G13D | Lung | KRAS G13D | Lung | KRAS G13D |
| 12 | Rectum | KRAS A146V | Liver | KRAS A146V | Liver | KRAS A146V |
| 13 | Rectum | KRAS G12V | Liver | KRAS G12V | Liver | KRAS G12V |
| | | | Lung | KRAS G12V | | |
| 14 | Rectum | KRAS G12D | Subcutaneous | KRAS G12D | Lung | KRAS G12D |
| 15 | Colon | WT | — | — | Local recurrence | WT |
| 16 | Rectum | KRAS G12S, PIK3CA E542K | Liver | KRAS G12S, PIK3CA E542K | Lung | KRAS G12S, PIK3CA E542K |
| 17 | Rectum | KRAS G12D | Lung | KRAS G12D | Lung | KRAS G12D |
| 18 | Colon | KRAS G12D | Liver | KRAS G12D | Lung | KRAS G12D |
| | | | | | Lung | KRAS G12D |
| 19 | Rectum | NRAS Q61H | Liver | NRAS Q61H | Lung | NRAS Q61H |
| 20 | Rectum | PIK3CA E542K | — | — | Local recurrence | PIK3CA E542K |
| 21 | Rectum | KRAS A146V | Liver | KRAS A146V | Lung | KRAS A146V |
| | | | | | Lung | KRAS A146V |

Abbreviations: LN = lymph node; WT = wild-type.

12 or 13, *BRAF* codon 600, or *PIK3CA* codon 1047 were detected in any sample in this study.

The degree of concordance of the gene mutations in primary and pre-FOLFOX metastatic lesions was examined. In case 10, a *KRAS* G12A mutation was detected in the primary lesion, whereas the metastatic lesion in the lung had wild-type *KRAS*. Although the histological features of the lung lesion were consistent with metastatic adenocarcinoma of the colon, no mutations in the metastatic lesion were detected, even after repeated high-sensitivity examinations. The remaining 17 metastatic lesions in 14 patients, including 2 liver metastatic lesions in case 10, showed the same mutational statuses as the primary tumours for all of the genes examined.

Then, the mutational statuses of the post-FOLFOX metastatic lesions were examined. The mutational statuses of all genes examined were identical in the 21 primary tumours and the corresponding 24 post-FOLFOX metastatic lesions, regardless of the sites involved, duration of FOLFOX treatment or disease-free survival period.

## DISCUSSION

Previous studies have reported a high concordance rate of the *KRAS* mutations in primary and metastatic tumours (Oudejans *et al*, 1991; Losi *et al*, 1992; Suchy *et al*, 1992; Zauber *et al*, 2003; Weber *et al*, 2007; Etienne-Grimaldi *et al*, 2008; Santini *et al*, 2008; Garm Spindler *et al*, 2009; Loupakis *et al*, 2009; Perrone *et al*, 2009; Baldus *et al*, 2010; Italiano *et al*, 2010; Knijn *et al*, 2011). However, in patients receiving long-term chemotherapy, the effects of genotoxic chemotherapies, such as oxaliplatin, have not been well investigated.

In this study, we examined 21 patients with metastatic colorectal cancer who received adjuvant FOLFOX therapy. The recurrent tumours in three patients who showed relapse within 4 months after the primary surgery or during the first 3 or 4 cycles of adjuvant FOLFOX therapy (cases 1–3) were regarded as synchronous metastases arising from micrometastases that likely existed prior to the start of the adjuvant chemotherapy. The remaining 18 patients who developed relapses more than 8 months from the end of adjuvant FOLFOX therapy or after more than 6 cycles of adjuvant FOLFOX therapy were regarded as having metachronous metastatic tumours that had developed after exposure to oxaliplatin. Among these cases, tumour relapse occurred within 180 days after FOLFOX therapy in 7 patients and more than 180 days after FOLFOX therapy in the remaining 11 patients. Regardless of the treatment duration, 8 of the primary tumours with wild-type *KRAS* codons 12 and 13 did not acquire *KRAS* mutations. The remaining tumours with *KRAS* mutations also did not show additional mutations after FOLFOX therapy. Furthermore, none of the other genes that might potentially affect the efficacy of anti-EGFR antibody therapy were altered.

*KRAS*, *NRAS* and *BRAF* mutations are all regarded as strong driver mutations that induce cell proliferation. These mutations might be acquired in the early stages of carcinogenesis and have generally been reported as mutually exclusive (Andreyev *et al*, 1998). Consistent with this observation, the *KRAS* and *NRAS* mutations in this study were found to be mutually exclusive. In the rest of the tumours, other unidentified driver mutations or amplifications may have activated the signalling pathways promoting cell proliferation. Considering the exclusive nature of the tested mutations, the acquisition of additional driver mutations may not be advantageous to these tumour cells for clonal selection. This could be one explanation for why the mutational statuses of *KRAS* and other genes were not altered during the development of metastatic tumours.

Our findings suggest that both the primary tumours and metastatic tumours arising during or after FOLFOX therapy could be valid sources of DNA for *KRAS* testing prior to treatment with anti-EGFR antibodies, although the number of cases in this study was limited. This finding should be further confirmed in a larger number of cases. Though collecting surgically resected metastatic tumour tissues is often difficult, circulating tumour cells may be a useful alternative DNA source for highly reliable and sensitive mutation detection systems such as the ARMS/Scorpion method for further analyses.

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ORIGINAL RESEARCH

# Application of miRNA expression analysis on exfoliated colonocytes for diagnosis of colorectal cancer

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**Background:** Several methods for the early detection of colorectal cancer to reduce its mortality rate have been reported. Here, we investigated the potential of a fecal micro RNA test for the early detection of colorectal cancer.

**Methods:** Patients with colorectal cancer (n = 299) and healthy volunteers (n = 116) with no abnormalities detected by screening colonoscopy were enrolled in this case-control study. Micro RNA expression in the colonocytes of patients with colorectal cancer (n = 47) and in healthy volunteers (n = 35) were analyzed in the training set, and the micro RNA expression in the colonocytes of patients with colorectal cancer (n = 252) and healthy volunteers (n = 81) was validated in the validation set.

**Results:** In the training study, significant differences in the relative expression level of miR-17-92 cluster, -106a, -135, and -146a were observed between patients with colorectal cancer and healthy volunteers ($P < 0.01$). The area under the receiver operating characteristic curve using miR-17, -18a, -19a, -19b, -20a, -92a, -106a, -135b, and -146a was more than 0.7. The overall sensitivity and specificity in the training study using these micro RNAs was 70.2% (33/47) and 74.3% (26/35), respectively. The overall sensitivity and specificity in the validation study was 67.5% (170/252) and 75.3% (61/81), respectively.

**Conclusion:** We have developed a fecal micro RNA test for exfoliated colonocytes for colorectal cancer screening. Further comparative study of this test for colorectal cancer screening is needed.

**Keywords:** colorectal cancer, fecal micro RNA, colonocytes, cancer screening, fecal RNA test

## Introduction

The early stage of colorectal cancer is curable by surgical resection, thus a suitable colorectal cancer screening test is necessary to reduce its mortality rate. The fecal occult blood test has been used widely as a screening test for colorectal cancer.[1–3] However, large-scale studies have shown that the sensitivity of the fecal occult blood test is not very high using total colonoscopy as a reference standard in all subjects.[4–7] Therefore, several attempts for the early detection of colorectal cancer have been reported. In fecal DNA-based analysis, the stool DNA test[6] was recommended as a colorectal cancer screening method.[8] Further, we have reported several DNA-based methods for the detection of early-stage colorectal cancer using direct sequence analysis[9] and single-strand conformation polymorphism analysis[10] in exfoliated colonocytes. However, the sensitivity and specificity of the stool DNA test were insufficient compared with that of the fecal occult blood test.[11] Another technical issue was that several mutation sites of adenomatous polyposis coli (*APC*), *Kras*, and *p53* genes in colorectal cancer

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tissue were not always identical in those genes.[12] In addition, the DNA mutation analysis was complicated and expensive. This may make the use of fecal DNA analysis for colorectal cancer screening unrealistic.

Gene expression analysis based on real-time reverse transcription polymerase chain reaction (RT-PCR) has been shown to be relatively simple and cost-effective. Several attempts to detect colorectal cancer by RT-PCR in fecal samples have been reported.[13–15] In those reports, the expression analyses of *COX2* and *MMP7* in fecal RNA, and *COX2*, *MMP7*, *MYBL2*, and *TP53* in colonocyte RNA were conducted.[13,15,16]

MicroRNAs (miRNAs) are small (18–25 nucleotide) noncoding RNA molecules that regulate the activity of specific mRNA targets and play a major role in development of cancer. miRNA downregulates multiple target gene expressions by degrading mRNA or blocking its translation into protein through RNA interference.[17,18] Several miRNAs, such as miRNA-21 (miR-21), the miR-17-92 cluster and miR-135, were found to be highly expressed in colorectal cancer tissue.[19–22] Several recent studies have clarified that the circulating miRNA in plasma is a potential marker for detection of colorectal cancer,[23,24] and is remarkably stable in plasma and protected from endogenous RNase activity.[25]

We have developed a fecal miRNA test using colonocyte RNA.[26] In the present study, we analyzed several miRNAs using an optimal internal control to improve the accuracy of the fecal miRNA test. Following selection of a suitable target and threshold in the training study, the fecal miRNA test was evaluated in a validation study to determine its potential for early detection of colorectal cancer.

## Materials and methods

### Fecal samples and isolation of exfoliated cells

Naturally evacuated fecal samples were obtained from patients with colorectal cancer before surgical resection. Fecal samples were also obtained from healthy volunteers a few weeks after screening colonoscopy. All patients with colorectal cancer and healthy volunteers were instructed to evacuate at home into a disposable 5 × 10 cm polystyrene tray (AsOne, Osaka, Japan) and then bring the sample to the reception counter at the outpatient clinic or the Cancer Prevention and Screening Center of the National Cancer Center. The fecal samples were processed immediately after they were brought to our laboratory.

For the isolation of colonocytes from naturally evacuated feces, we used two kinds of immunomagnetic beads tagged with antihuman EpCAM monoclonal antibodies, ie, Dynabeads Epithelial Enrich (Dynal, Oslo, Norway) and JSR beads (JSR, Tsukuba, Japan).[27] The ability to isolate cells from feces using Dynal beads and JSR beads was almost same. The samples were processed as described previously.[9] Briefly, the fecal sample was homogenized with a buffer (40 mL) consisting of Hanks' solution, 10% fetal bovine serum, and 25 mM HEPES buffer (pH 7.35) at 200 rpm for one minute using a Stomacher system (Seward, Thetford, UK). The homogenate was filtered through a nylon filter (pore size, 512 μm), and following the addition of 80 μL of the immunomagnetic beads, the sample mixture was incubated for 30 minutes under gentle rolling conditions at room temperature. The mixture on the magnet was incubated on a shaking platform for 15 minutes at room temperature. The supernatant was then removed and the colonocytes in the pellet were stored at –80°C until RNA extraction.

### miRNA array for selection of internal control and target miRNA

To determine the internal control for miRNA analysis and the suitable target of miRNA, the colonocyte RNA of five patients with colorectal cancer and five healthy volunteers was analyzed using the TaqMan MicroRNA Array v3.0 (Applied Biosystems, Foster, CA), in accordance with the manufacturer's instructions. RT-PCR was performed using an Applied Biosystems 7900HT fast real-time PCR system. Next, the target miRNAs were validated using total RNA extracted from both the cancer tissue and the normal mucosa of 31 patients with colorectal cancer.

### Fecal miRNA analysis in patients with colorectal cancer and healthy volunteers

From August 2003 to November 2003 and from June 2004 to July 2004, 47 patients with colorectal cancer and 35 healthy volunteers were enrolled into the training study, respectively. From November 2003 to November 2009 and from July 2004 to March 2005, 252 patients with colorectal cancer and 81 healthy volunteers were enrolled in the validation study, respectively. The characteristics of these patients and volunteers are summarized in Table 1. All the patients with colorectal cancer had undergone surgical resection of their primary cancer at the National Cancer Center Hospital, Tokyo, Japan. No remarkable changes were observed except Dukes' stage classification between the training study and the validation study. All the healthy volunteers were confirmed to have no symptoms and evident abnormalities (eg, adenoma or carcinoma, including hyperplastic polyps) by screening

**Table 1** Characteristics of CRC patients and healthy volunteers

| Characteristics | Training set | | Validation set | |
|---|---|---|---|---|
| | CRC patients (n = 47) | Healthy volunteers (n = 35) | CRC patients (n = 252) | Healthy volunteers (n = 81) |
| Age, years | | | | |
| Median | 62 | 60 | 63 | 59 |
| Range | 35–83 | 40–69 | 32–86 | 41–70 |
| Sex, no (%) | | | | |
| Male | 33 (70.2) | 19 (54.3) | 162 (64.3) | 33 (40.7) |
| Female | 14 (29.8) | 16 (45.7) | 90 (35.7) | 48 (59.3) |
| Tumor location, no (%) | | | | |
| Cecum | 2 (4.3) | | 17 (6.7) | |
| Ascending colon | 7 (14.9) | | 39 (15.5) | |
| Transverse colon | 2 (4.3) | | 15 (6.0) | |
| Descending colon | 2 (4.3) | | 10 (4.0) | |
| Sigmoid colon | 9 (19.1) | | 51 (20.2) | |
| Rectum | 25 (53.2) | | 120 (47.6) | |
| Tumor size, mm | | | | |
| Median | 38 | | 37 | |
| Range | 7–76 | | 9–160 | |
| Histology, no (%) | | | | |
| W/D | 21 (44.7) | | 143 (56.7) | |
| M/D | 23 (48.9) | | 93 (36.9) | |
| P/D | 2 (4.3) | | 7 (2.8) | |
| Mucinous carcinoma | 1 (2.1) | | 8 (3.2) | |
| Carcinoid tumor | | | 1 (0.4) | |
| Tumor depth, no (%) | | | | |
| T1 | 5 (10.6) | | 34 (13.5) | |
| T2 | 8 (17.0) | | 60 (23.8) | |
| T3 | 33 (70.2) | | 154 (61.1) | |
| T4 | 1 (2.1) | | 4 (1.6) | |
| Dukes' stage, no (%) | | | | |
| A | 10 (21.3) | | 78 (31.0) | |
| B | 9 (19.1) | | 69 (27.4) | |
| C | 21 (44.7) | | 88 (34.9) | |
| D | 7 (14.9) | | 17 (6.7) | |

**Abbreviations:** CRC, colorectal cancer; W/D, well-differantiated adenocarcinoma; M/D, moderately differentiated adenocarcinoma; P/D, poorly differentiated adenocarcinoma.

colonoscopy performed at the Research Center for Cancer Prevention and Screening, National Cancer Center, Tokyo. The median age of the healthy volunteers was relatively younger than that of the patients with colorectal cancer. Regarding gender, the number of women was relatively higher among the healthy volunteers than among the patients with colorectal cancer. All participants were provided with detailed information about the study, and each gave written informed consent to participate in the study, which was approved by the institutional review board of National Cancer Center, Japan.

## miRNA expression analysis using real-time PCR

Total RNA was extracted from the colonocytes isolated from the fecal samples using an miRNeasy Mini Kit (QIAGEN, Valencia, CA), and cDNA was synthesized using a TaqMan MicroRNA RT Kit (Applied Biosystems), in accordance with the manufacturer's instructions. RT-PCR was performed with precycling heat activation at 95°C for 20 seconds, followed by 40 cycles of denaturation at 95°C for 3 seconds, and annealing/extension at 60°C for 30 seconds, using an Applied Biosystems 7500 fast RT-PCR system. For the analysis of all miRNAs, we used the TaqMan microRNA assay (Applied Biosystems). miRNA expression analysis was conducted using the comparative Ct (threshold cycle) method. In this analysis, the formulae for the relative quantification of each gene were as follows: (dCt of each miRNA) = (Ct of each miRNA) − (Ct of miR-24), and (relative quantification of each miRNA) = $2^{-(\text{dCt of each miRNA})}$.

## Statistical analysis

Differences in relative quantification of the miRNAs were analyzed using the two-sided Mann–Whitney *U*-test.

Statistical analyses were performed using SPSS Statistics version 19 for Windows (IBM, Tokyo, Japan). $P < 0.05$ was considered to indicate a statistically significant difference.

# Results

## Suitable internal control of miRNA analysis

Of 749 miRNAs, the average number of PCR-successful miRNA was 180 (range 90–295) in patients with colorectal cancer and 157 (53–242) in healthy volunteers, respectively. Forty miRNAs could be detected in all five patients with colorectal cancer and five healthy volunteers using the TaqMan MicroRNA Array, and these miRNAs served as candidates for internal control (Figure 1). Average Ct values of these miRNAs in the patients with colorectal cancer and healthy volunteers were 27.72 (23.81–31.16) and 28.78 (25.04–32.94), respectively. Mean differences in Ct values of miR-16, 24, -200c, and U6 from the average Ct values of these miRNAs were $-0.12 \pm 0.99$, $-1.48 \pm 0.48$, $-2.57 \pm 1.04$, and $1.18 \pm 3.19$, respectively. miR-24 expression was the most stable and constant from among all miRNAs.

## Selection of target miRNAs for colorectal cancer detection

According to the results of miRNA array, 20 miRNAs were selected as candidates for miRNA analysis (Table 2). Using tissue RNA, miR-17, -18a, -19a, -19b, -20a, -21, -92a, -106a, -135a, -135b, -146a, -183, -223, and -454* in cancer tissue were expressed at significantly higher levels than those in normal tissue ($P < 0.05$). On the other hand, there was no significant difference of expression for miR-34a, -155, -191, -206, -564, and -1208 between cancer tissue and normal tissue ($P > 0.1$). These 14 miRNAs were selected to target miRNAs for detection of colorectal cancer.

## Relative quantification of each miRNA in colonocytes

The relative expression level of each miRNA was calculated using that of miR-24 as an internal control for 47 patients with colorectal cancer and 35 healthy volunteers in the training set (Table 1). We observed significant differences in the relative expression level of miR-17, -18a, -19a, -19b, -20a, -92a, -106a, -135a, -135b, and -146a between the patients

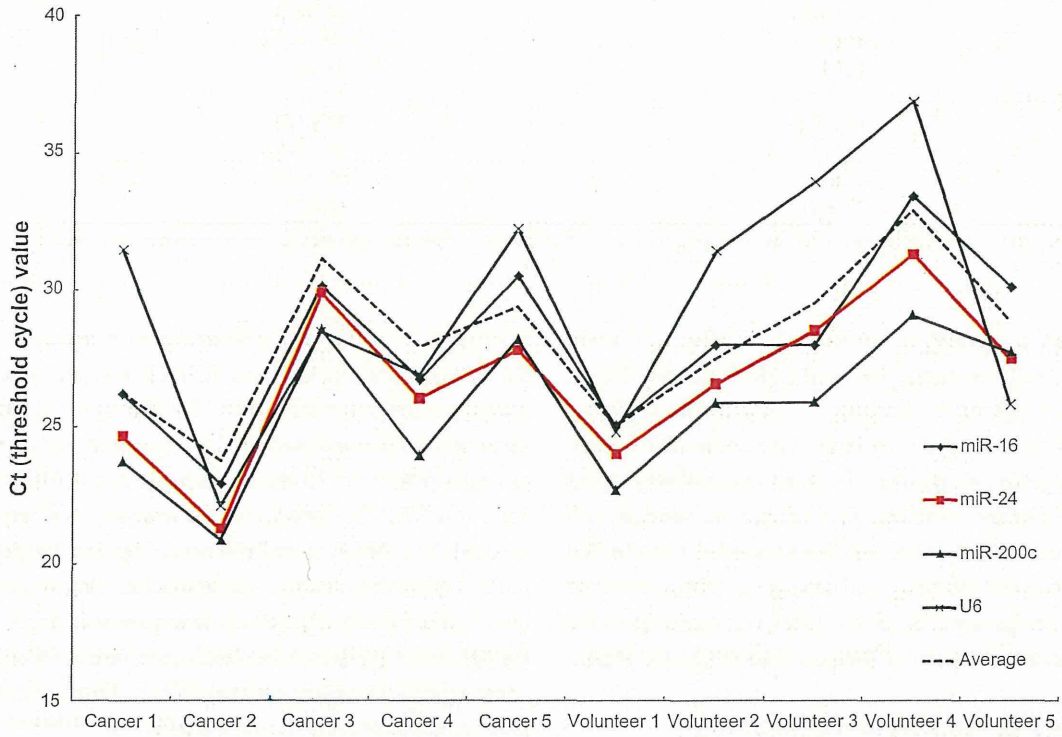


**Figure 1** Ct values of candidates for internal control. Of 749 miRNAs, 40 could be detected in all of five patients with colorectal cancer and five healthy volunteers using the TaqMan MicroRNA Array.

**Notes:** The average Ct values of these miRNAs in patients with colorectal cancer and healthy volunteers were 27.72 (23.81–31.16) and 28.78 (25.04–32.94). The differences in the Ct values of miR-16, 24, -200c, and U6 from the average Ct values of these miRNAs were $-0.12 \pm 0.99$ (average ± standard deviation), $-1.48 \pm 0.48$, $-2.57 \pm 1.04$, and $1.18 \pm 3.19$. The average Ct value of 40 miRNAs is indicated by the dotted line.

**Abbreviations:** Ct, threshold cycle; miRNA, micro RNA.

**Table 2** Mean values of relative quantifications of target miRNA in tissue samples

| | Colorectal cancer (n = 31) | Normal mucosa (n = 31) | *P* value |
|---|---|---|---|
| | Mean RQ (range) | Mean RQ (range) | |
| miR-17 | 1.50 (0–4.56) | 0.44 (0.20–0.95) | <0.001 |
| miR-18a | 0.037 (0.002–0.135) | 0.007 (0.001–0.020) | <0.001 |
| miR-19a | 0.007 (0.001–0.041) | 0.002 (0–0.005) | <0.001 |
| miR-19b | 0.040 (0.002–0.164) | 0.012 (0.002–0.040) | 0.001 |
| miR-20a | 0.472 (0.047–1.462) | 0.119 (0.026–0.284) | <0.001 |
| miR-21 | 0.850 (0.190–2.239) | 0.216 (0.065–0.757) | <0.001 |
| miR-34a | 0.024 (0.005–0.047) | 0.023 (0.010–0.039) | 0.8 |
| miR-92a | 5.117 (0.434–27.569) | 1.893 (0.728–3.779) | <0.001 |
| miR-106a | 0.311 (0.092–1.187) | 0.120 (0.054–0.286) | <0.001 |
| miR-135a | 0.008 (0.001–0.028) | 0.001 (0–0.002) | <0.001 |
| miR-135b | 0.092 (0.014–0.330) | 0.006 (0.001–0.024) | <0.001 |
| miR-146a | 0.216 (0.050–0.641) | 0.139 (0.033–0.387) | 0.001 |
| miR-155 | 0.144 (0.038–0.431) | 0.153 (0.059–0.437) | 0.4 |
| miR-183 | 0.012 (0.004–0.030) | 0.004 (0.001–0.009) | <0.001 |
| miR-191 | 0.515 (0.106–1.335) | 0.485 (0.117–1.250) | 0.5 |
| miR-206 | 0.002 (0–0.016) | 0.002 (0–0.010) | 0.6 |
| miR-223 | 0.416 (0.072–2.144) | 0.205 (0.044–0.754) | 0.006 |
| miR-454* | 0.0001 (0–0.0003) | 0.0001 (0–0.0002) | 0.03 |
| miR-564 | 0.0003 (0–0.0025) | 0.0003 (0–0.0022) | 0.2 |
| miR-1208 | 0.0001 (0–0.0008) | 0.0002 (0–0.0028) | 0.6 |

**Notes:** *P* value was analyzed by the Mann–Whitney *U*-test and $P < 0.05$ was considered to indicate a statistically significant difference.
**Abbreviation:** RQ, relative quantification.

with colorectal cancer and the healthy volunteers ($P < 0.01$). On the other hand, there was no significant difference in the relative expression level of miR-21, -183, -223, and -454* between the colorectal cancer patients and the healthy volunteers ($P > 0.1$, Table 3).

## Area under ROC curve

The data for sensitivity and specificity calculated using relative quantifications of miRNA in patients with colorectal cancer and healthy volunteers were blotted into a receiver operating characteristic (ROC) curve (Figure 2). Areas under the ROC curve using miR-21, -135a, -183, -223, and -454* were less than 0.6. On the other hand, areas under the ROC curve using miR-17, -18a, -19a, -19b, -20a, -92a, -106a, -135b, and -146a were more than 0.7.

## Sensitivity and specificity of miRNA expression analysis in training study

From the abovementioned results, we set miR-17, -18a, -19a, -19b, -20a, -92a, -106a, -135b, and -146a as a new miRNA set for detection of colorectal cancer. The thresholds of miR-17, -18a, -19a, -19b, -20a, -92a, -106a, -135b, and -146a were 2.1, 0.16, 0.57, 2.5, 1.4, 8.2, 3.2, 0.13, and 0.61, respectively (Table 4). The specificity of the healthy volunteers using each miRNA was set at 94.3% (33/35). The overall sensitivity of patients with colorectal cancer and the specificity of healthy volunteers were 70.2% (33/47, 95% confidence interval [CI] 55.1–82.7) and 74.3% (26/35, 95% CI 56.8–87.5), respectively.

**Table 3** Mean values of relative quantifications of target miRNA compared with an internal control, miR-24

| | CRC patients (n = 47) | Healthy volunteers (n = 35) | *P* value |
|---|---|---|---|
| | Mean RQ (range) | Mean RQ (range) | |
| miR-17 | 1.34 (0–3.76) | 0.94 (0–11.85) | <0.001 |
| miR-18a | 0.12 (0–0.96) | 0.04 (0–0.80) | <0.001 |
| miR-19a | 0.30 (0–1.55) | 0.12 (0–1.66) | <0.001 |
| miR-19b | 1.35 (0–7.89) | 0.71 (0–5.38) | <0.001 |
| miR-20a | 0.84 (0–3.56) | 0.33 (0–2.13) | <0.001 |
| miR-21 | 16.90 (0.28–66.49) | 12.02 (0–64.94) | 0.2 |
| miR-92a | 7.45 (0.38–35.02) | 2.74 (0–14.05) | <0.001 |
| miR-106a | 1.26 (0–4.08) | 0.78 (0–6.07) | <0.001 |
| miR-135a | 0.004 (0–0.043) | 0.00002 (0–0.0006) | 0.01 |
| miR-135b | 0.16 (0–2.21) | 0.02 (0–0.28) | <0.001 |
| miR-146a | 0.53 (0–3.05) | 0.13 (0–1.95) | <0.001 |
| miR-183 | 0.010 (0–0.202) | 0.009 (0–0.104) | 0.5 |
| miR-223 | 14.41 (1.59–49.90) | 16.33 (0.03–53.63) | 0.9 |
| miR-454* | 0.013 (0–0.560) | 0.003 (0–0.097) | 1 |

**Notes:** *P* value was analyzed by the Mann–Whitney *U*-test and $P < 0.05$ was considered to indicate a statistically significant difference.
**Abbreviations:** CRC, colorectal cancer; RQ, relative quantification.

## Sensitivity and specificity of miRNA expression analysis in validation study

After the training study, 252 patients with colorectal cancer and 81 healthy volunteers were validated in the validation study (Table 1). The thresholds of all miRNAs for the validation study were the same as those for the training study. The overall sensitivity of the patients with colorectal cancer and the specificity of the healthy volunteers were 67.5% (170/252, 95% CI 61.3–73.2) and 75.3% (61/81, 95% CI 64.5–84.2), respectively (Table 5). There was no remarkable difference between the training study and the validation study.

# Discussion

In our recent preliminary study, we analyzed the expression of miRNA in exfoliated colonocytes using oncogenic miRNAs, such as the miR-17-92 cluster, miR-21, and miR-135 normalized by U6.[26] We found that the expression analysis on the miRNA extracted from exfoliated colonocytes was feasible. In the present study, we adopted a more suitable internal control for miRNA expression and the optimal miRNA set for detecting colorectal cancer using TaqMan MicroRNA

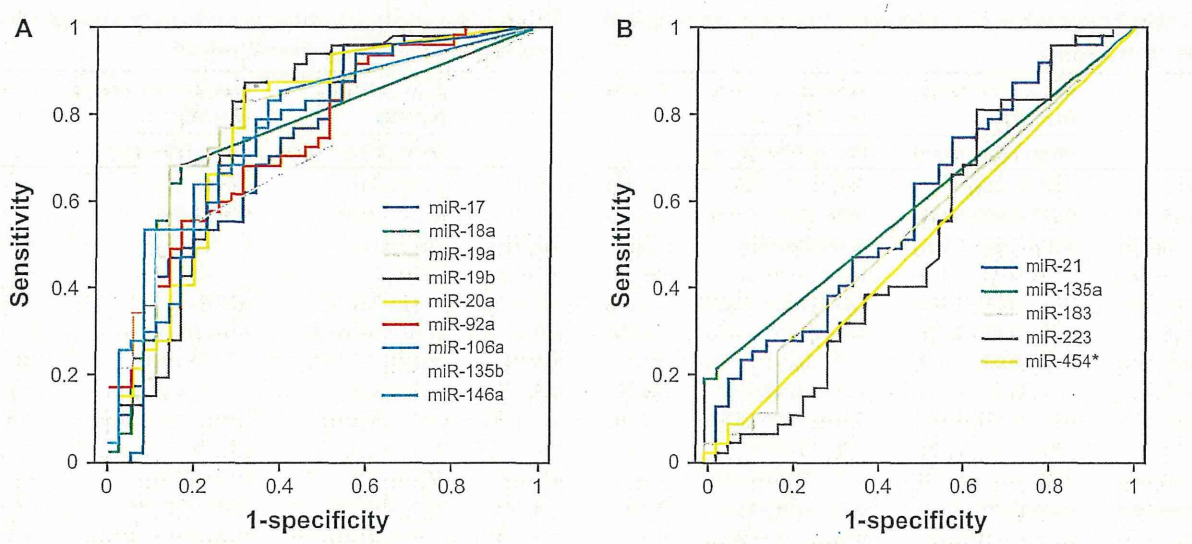


**Figure 2** Areas under the ROC curve. (**A**) ROC curve using miR-17, -18a, -19a, -19b, -20a, -92a, -106a, -135b, and -146a. Area under the ROC using these miRNAs were more than 0.7. (**B**) ROC curve using miR-21, -135a, -183, -223, and -454*.
**Note:** Areas under the ROC curve using these miRNAs were less than 0.6.
**Abbreviations:** miRNA, micro RNA; ROC, receiver operating characteristic.

Array. The highly stable expression in both patients with colorectal cancer and in healthy volunteers was necessary for the internal control. Because expression of miR-24 in the colonocytes of patients with colorectal cancer and healthy volunteers was more stable and constant than that of the miR-200 family or U6 that are sometimes used as a provisional internal control, miR-24 was adopted as an internal control in the present study. However, miR-24 was not used as an internal control in previous studies. Therefore, we believe that establishment of a universal internal control for miRNA analysis is urgently needed.

The miR-17-92 cluster, -21, -34a, -106a, -135, -146a, -155, -183, -191, -206, -223, -454*, -564, and -1208 were selected from 749 miRNAs as candidates for miRNA analysis using TaqMan MicroRNA Array. Among those, the miR-17-92 cluster, -21, -106a, -135, -146a, -183, -223, and -454* were highly expressed in colorectal cancer tissue compared with the normal mucosa in our preliminary results. To date, various reports have shown that the miR-17-92 cluster, -21, -106a, -135, and -223 were expressed more strongly in colorectal cancer tissue than in normal colorectal tissue.[19–22,28–31] Though it has been shown that miR-146a is highly expressed in several types of cancer tissue,[32,33] it has been reported that miR-146a is tumor suppressor miRNA.[34] These results are controversial; however, miR-146a was expressed to a significantly greater extent in colorectal cancer tissue than in normal mucosa in our study. Thus, we decided to use miR-17-92 cluster, -21, -106a, -135, -146a, -183, -223,

**Table 4** Sensitivity and specificity of each miRNA expression using optimal threshold in training set

| | Threshold | CRC patients (n = 47) | | Healthy volunteers (n = 35) | |
|---|---|---|---|---|---|
| | | No | Sensitivity (%) (95% CI) | No | Specificity (%) (95% CI) |
| Combined markers | | 33 | 70.2 (55.1–82.7) | 26 | 74.3 (56.8–87.5) |
| miR-17 | 2.1 | 8 | 17.0 (7.6–30.8) | 33 | 94.3 (80.9–99.3) |
| miR-18a | 0.16 | 11 | 23.4 (12.3–38.0) | 33 | 94.3 (80.9–99.3) |
| miR-19a | 0.57 | 7 | 14.9 (6.2–28.3) | 33 | 94.3 (80.9–99.3) |
| miR-19b | 2.5 | 6 | 12.8 (4.8–25.7) | 33 | 94.3 (80.9–99.3) |
| miR-20a | 1.4 | 10 | 21.3 (10.7–35.7) | 33 | 94.3 (80.9–99.3) |
| miR-92a | 8.2 | 15 | 31.9 (19.1–47.2) | 33 | 94.3 (80.9–99.3) |
| miR-106a | 3.2 | 1 | 2.1 (0.1–11.3) | 33 | 94.3 (80.9–99.3) |
| miR-135b | 0.13 | 13 | 27.7 (15.6–42.7) | 33 | 94.3 (80.9–99.3) |
| miR-146a | 0.61 | 13 | 27.7 (15.6–42.7) | 33 | 94.3 (80.9–99.3) |

**Abbreviations:** CRC, colorectal cancer; 95% CI, 95% confidence interval.

**Table 5** Sensitivity and specificity of miRNA expression (validation set)

| microRNA | CRC patients (n = 252) | | Healthy volunteers (n = 81) | |
|---|---|---|---|---|
| | No | Sensitivity (%) (95% CI) | No | Specificity (%) (95% CI) |
| Combined markers | 170 | 67.5 (61.3–73.2) | 61 | 75.3 (64.5–84.2) |
| miR-17 | 26 | 10.3 (6.8–14.8) | 77 | 95.1 (87.9–98.6) |
| miR-18a | 42 | 16.7 (12.3–21.8) | 76 | 93.8 (86.2–98.0) |
| miR-19a | 3 | 1.2 (0.2–3.4) | 81 | 100 (95.5–100) |
| miR-19b | 7 | 2.8 (1.1–5.6) | 80 | 98.8 (93.3–100) |
| miR-20a | 18 | 7.1 (4.3–11.0) | 79 | 97.5 (91.4–99.7) |
| miR-92a | 124 | 49.2 (42.9–55.5) | 78 | 96.3 (89.5–99.2) |
| miR-106a | 6 | 2.4 (0.9–5.1) | 80 | 98.8 (93.3–100) |
| miR-135b | 51 | 20.2 (15.5–25.7) | 77 | 95.1 (87.9–98.6) |
| miR-146a | 27 | 10.7 (7.2–15.2) | 77 | 95.1 (87.9–98.6) |

**Abbreviations:** CRC, colorectal cancer; 95% CI, 95% confidence interval.

and -454* for colorectal cancer detection using colonocytes in the present study.

In the training study, the expressions of miR-21, -183, -223, and -454* in exfoliated colonocytes of patients with colorectal cancer were not significantly different from those of healthy volunteers. Because relative expression of miR-135a was low in both patients with colorectal cancer and healthy volunteers, the area under the ROC curve using miR-135a was under 0.6. From the present results, we determined that miR-17, -18a, -19a, -19b, -20a, -92a, -106a, -135b, and -146a were useful for detection of colorectal cancer. The sensitivity and specificity of the miRNA assay in colonocytes was 70.2% and 74.3%, respectively. These results are almost the same as those of our previous studies,[9,10,16] and we have subsequently validated the miRNA set in the validation study.

Although the rate of patients with early-stage colorectal cancer was slightly high in the validation study compared with the training study, there were no remarkable changes between the characteristics of the training study and those of the validation study. The sensitivity and specificity of the miRNA assay in the validation study was 67.56% and 75.3%, respectively. The sensitivity and specificity of the fecal miRNA test were almost the same between the training study and the validation study. Furthermore, we could not find any specific difference between miRNA expression and the clinicopathological characteristics of colorectal cancer.

In summary, the fecal miRNA test using miR-17, -18a, -19a, -19b, -20a, -92a, -106a, -135b, and -146a was found to be useful for the detection of colorectal cancer in both the training study and the validation study. The present data may warrant further comparative study between fecal occult blood test and the fecal miRNA test for colorectal cancer screening in terms of sensitivity, specificity, and cost-effectiveness.

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## Disclosure

The authors report no conflicts of interest in this work.

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# Tailored immunoconjugate therapy depending on a quantity of tumor stroma

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**The purpose of this study was to clarify the appropriate combination of targeting antibody and conjugate-design of anti-tumor immunoconjugate depending on a quantity of tumor stroma. Most human solid tumors including pancreatic cancer (PC) forming hypovascular and stroma-rich tumor hinders the penetration of monoclonal antibodies (mAbs) into the cells, and that leads to failure of the conventional cell-targeting immunoconjugate strategy. To overcome this drawback, SN-38 as topoisomerase 1 inhibitor was conjugated to a mAb to collagen 4, a plentiful component of the tumor stroma via ester-bond. The immunoconjugate, which was able to release SN-38 in physiological condition outside the cells, was effective to stroma-rich PC-tumor. On the other hand, anti-CD 20 mAb-PEG-SN-38 via carbamate-bond as conventional immunoconjugate, enabled SN-38 to be released by a carboxylesterase inside of the tumor cell following the internalization, showed strong anti-tumor activity against malignant lymphoma as hypervascular and stroma-poor tumor. The conjugate-design, in parallel with the choice of targeting antibodies, should be selected to maximize the therapeutic effect in each individual tumor having a distinct stromal structure. (*Cancer Sci*, doi: 10.1111/cas.12062, 2012)**

Monoclonal antibody (mAb), which can target the tumor cell actively by the specific binding ability against corresponding antigen, easily extravasates from leaky tumor vessels but not from normal vessels, is long retained in the tumor by using active targeting and passive targeting based on the enhanced permeability and retention (EPR) effect.[1–4] Therefore, numerous mAbs have been developed and conjugated with anticancer agents (ACAs) or toxins to create an "immunoconjugate strategy".[5–8] Recent examples of the conjugates include anti-CD33 immunoconjugate-calicheamicin and anti-CD20 radiolabeled immunoconjugate, were effective to hematological malignancy such as malignant lymphoma and leukemia.[5] Heterogeneity of the tumor cells, however, prevents development of the immunoconjugate chemotherapy based on cell-specific antigen.[9–12] Moreover, conventional immunoconjugates depend on cleavage of conjugation site with intracellular biochemical (enzymatic) process after the cell-uptake of the conjugate.[13–16] In addition to such annoying characteristics of cancer cells themselves, most human solid tumors such as pancreatic cancer and gastric cancer, possess abundant stroma that hinders the distribution of mAbs (Fig. 1a).[17–20] To overcome these drawbacks, we developed a unique strategy whereby the cancer-stromal targeting (CAST) therapy by cytotoxic immunoconjugate bound to the collagen 4 or fibrin network in the tumor stroma, from which the payload released gradually and distributed throughout the tumor, resulting in the arrest of tumor growth due to induced damage to tumor cells and tumor vessels.[21,22] Besides, there have been a few reports describing tumor stromal targetingimmunoconjugates, a mAb against a cell surface antigen FAP as fibroblast targeting therapy, or a mAb against fibronectin for the targeting of tumor vascular endothelial cell in photodynamic therapy.[23,24] However, the merits and drawbacks of anti-stromal targeting immunoconjugate therapy in relation to the conjugate-design and the amount of tumor stroma have not yet been fully elucidated.

The purpose of this study was to clarify the appropriate combination of targeting antibody and conjugate-design of anti-tumor immunoconjugate depending on the quantity of tumor stroma. Hence, we selected two types of conjugate linker: ester-bond and carbamate-bond. We hypothesized that a combination of anti-stromal targeting mAb and a linker composed of ester-bond to release ACA outside the cells would be effective against the stroma-rich cancer. Conversely, anti-cancer cell targeting via carbamate-bond to release ACA inside the cells would be effective against stroma-poor cancer. It seemed that the outcome of immunoconjugate therapy against each individual tumor having distinct stromal structure was dependent on the selection of conjugation-design, as well as targeting mAb.

## Materials and Methods

**Antibodies and cells.** Anti-EpCAM (B8-4) and Anti-collagen 4 antibody (35-4) were prepared as previously reported.[21] Anti-human CD20 antibody (rituximab) was purchased from Daiichi-Sankyo (Tokyo, Japan). Human malignant lymphoma cell line RL was purchased from the American Type Culture Collection (Rockville, MD, USA). Human PC cell line SUIT2 was purchased from the Health Science Research Resources Bank (Osaka, Japan).

***In vivo* imaging and immunohistochemistry.** Immunohistochemistry was conducted using anti CD31 antibody (R&D Systems, Minneapolis, MN, USA), anti-collagen 4 antibody and anti-CD20 (rituximab), or anti-EpCAM antibody as first antibodies, Alexa 488-, 555- or 647-labeled anti-human, mouse, rat or goat IgG (Invitrogen, Carlsbad, CA, USA) as second antibodies.

For mouse-systemic *in vivo* imaging or tracking of antibody in the tissue, IRDye 800 (Li-Cor Biosciences, Lincoln, NE, USA) alexa-647 (Invitrogen) or Qdot 625 (Invitrogen) labeled antibodies were injected into the mice tail vein at 100 μg /body. Fluorescence images were obtained using OV110 (Olympus, Tokyo, Japan), BZ-9000 (Keyence, Osaka, Japan), LSM 710 (Carl Zeiss, LinkedIn Germany).

**Immunoconjugate.** The detailed process of chemical synthesis is shown in the Data S1. The final structure was composed of one maleimide for attachment of mAb, one $PEG_{12}$ (MW 865) spacer and one $PEG_{27}$ (MW 1422) ester-bond or

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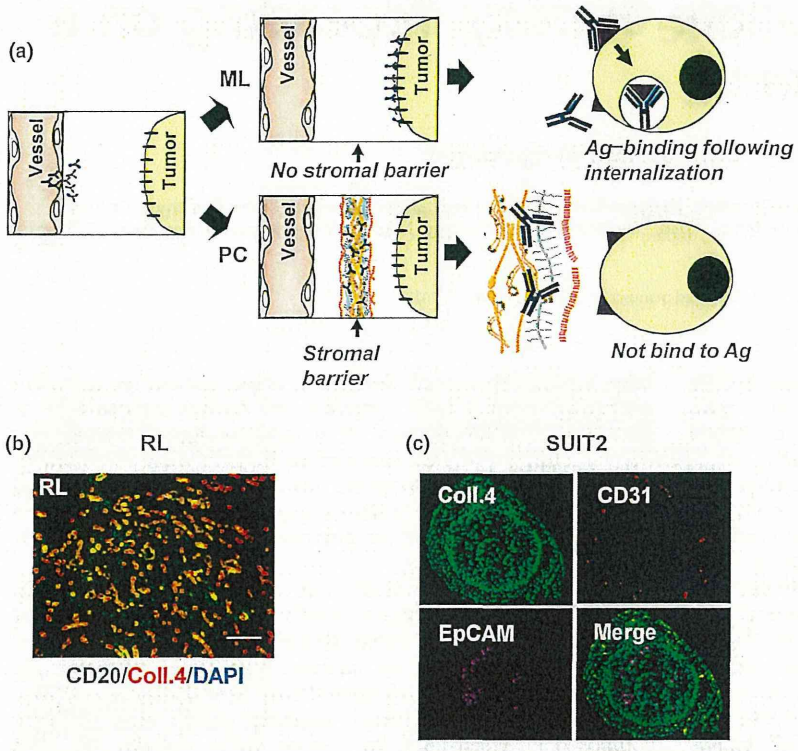


**Fig. 1.** The difference of tumor tissue stromal component as stromal barrier between malignant lymphoma and pancreatic cancer. (a) The schema of antibody delivery into the tumor cells. In the tumor having no stromal barrier like malignant lymphoma (ML), antibodies were delivered into the cancer cells, and can be internalized after antigen-binding. However, many human solid tumors including pancreatic cancer (PC) possess stromal barrier hindering the distribution of the immunoconjugates into cancer cells such that antigen-binding following antibody-intrernalization never occur. Ag, Antigen. (b) RL-tumor (ML) was stained with anti-CD20 (green), anti-collagen 4 (red) mAb and 4′6′-diamidino-2-phenylindole dihydrochloride (DAPI) (blue). Scale bar: 100 μm. (c) SUIT2-tumor (PC) was stained with anti-EpCAM (purple), anti-collagen 4 (green) and anti-CD31 (red) mAb. Co-existence of collagen 4 and CD 31 (yellow in Merge). Coll., collagen.

carbamate-bond for attachment of one SN-38 molecule. Interchain disulfides of the antibodies were reduced with 10 mM DTT (Sigma).[14] The numbers of free thiols were quantified with dinitrothiocyanobenzene (DNTB, Wako, Osaka, Japan). Reduced antibodies were reacted with maleimide-linker-SN-38 prodrugs in PBS containing 5 mM EDTA (pH 6) at room temperature for 1 h, then at 4°C overnight. The concentration of antibody-prodrug conjugates was determined using the Bradford method (Bio-Rad Protein Assay, 500-0006JA, Bio-Rad, Hercules, CA, USA). The numbers of residual thiols were quantified with dNTP. Each drug (SN-38)/antibody ratio was determined by comparing the numbers of free and residual thiols. In the characterization of the conjugates, statistical analysis was performed using Student's *t*-test.

**Animal model and anti-tumor effects.** Female BALB/c nude mice (5 weeks old) were purchased from SLC Japan (Shizuoka, Japan). Mice were inoculated subcutaneously in the flank with $5 \times 10^6$ cells of RL, or $2 \times 10^6$ of SUIT2. The length (L) and width (W) of tumor masses and body weight were measured every 4 days, and tumor volume was calculated using $(L \times W^2)/2$. All animal procedures were performed in compliance with the Guidelines for the Care and Use of Experimental Animals established by the Committee for Animal Experimental of the National Cancer Center. These guidelines meet the ethical standards required by law and also comply with the guidelines for the use of experimental animals in Japan. When the mean tumor volume reached approximately 140 $mm^3$ (RL) and approximately 70 $mm^3$ (SUIT2), mice were randomly divided into groups consisting of five mice. Immunoconjugates were administered on day 0 by the mice tail vein injection. The injection doses of antibody-SN-38 prodrug equal to an SN-38 dose of 3 mg/kg were determined by calculations based on drug (SN-38)/antibody ratio (range from 2629.08 to 3296.16 mg SN-38 per 1 mM antibody) for each drug. Statistical analysis was performed using ANOVA.

**Biochemistry and hematological examination.** Blood samples were taken from the healthy mice at 7 days after i.v. administration of immunoconjugates (at an equivalent SN-38 dose of 3 mg/kg). Hemograms were measured by using an auto-analyzer Celltacα MEK6358 (Nihon Kohden, Tokyo, Japan), and blood chemistry examinations were carried out by Nagahama LSL (Shiga, Japan).

**Anti-collagen antibody induced arthritis.** Female DBA/1J mice (5 weeks old) were purchased from SLC Japan. Anti-collagen 2 antibody (Chondrex, Redmond, WA, USA) or anti-collagen 4 antibody (clone 35-4, the same mAb in the immunoconjugate) were intraperitoneally administered on day 0 at 2 mg. Fifty micrograms of LPS (Chondrex) was intraperitoneally injected on day 3.

## Results

**Difference of tumor stromal component between malignant lymphoma and pancreatic cancer.** We first examined the difference of the stromal component influencing the drug delivery between malignant lymphoma RL and pancreatic cancer SUIT2. Anti-CD20- or anti-EpCAM-mAb, which is specific to lymphoma or epithelial carcinoma, respectively, was used as cancer cell-specific mAb.[5,25] Anti-collagen 4 mAb was prepared to evaluate the stromal component. RL tumor consisted of CD20-positive tumor cells and collagen-4-positive blood vessels, which was stained fine-linearly but not interspersed-fibrously like the intercellular-stroma (Fig. 1b). On the other hand, SUIT2-tumor reported as the histopathology relatively resembling original human pancreatic cancer,[21,26] consisted of EpCAM-positive cancer-cells and collagen-4-positive extracellular component, the latter was composed of both CD31-positive blood vessel wall (yellow in Merge, Fig. 1c) and high amount of CD31-negative stroma (green in Merge, Fig. 1c).

**Internalization and biodistribution of mAbs against malignant lymphoma or pancreatic cancer.** Cell-uptake of fluorescent anti-CD20- or anti-EpCAM-mAb against RL cells or SUIT2 cells was evaluated, respectively. Anti-CD20 mAb was internalized and colocalized with intracellular lysosome in RL cells at 12 h after the incubation. On the other hand, anti-EpCAM mAbs, the majority of which was still retained on cell-surface


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