Table 2. Response to Treatment in the Intention-to-Treat Population, According to Treatment Group.*

Response	Gefitinib (N=114)	Carboplatin–Paclitaxel (N=114)
	number of patients (percent)	
Complete response	5 (4.4)	0
Partial response	79 (69.3)	35 (30.7)
Complete or partial response†	84 (73.7)	35 (30.7)
Stable disease	18 (15.8)	56 (49.1)
Progressive disease	11 (9.6)	16 (14.0)
Response that could not be evaluated	1 (0.9)	7 (6.1)

* All responses differed significantly between the two groups ($P<0.001$ by Fisher's exact test).

† The percentage of patients in whom there was either a complete or a partial response was considered to be the rate of objective response.

progression-free survival from our phase 2 studies in patients with non–small-cell lung cancer and EGFR mutations. The data on overall survival first became available in 2008, when the combined analysis of Japanese phase 2 studies (Iressa — Combined Analysis of Mutation Positives [I-CAMP]) and the subgroup analyses of IPASS were reported.[7,22] We thus planned to have progression-free survival as the primary end point in the current study, because it allowed us to calculate the statistical power of the study.

Several studies have suggested that the EGFR copy number may be a better predictive biomarker for the efficacy of EGFR tyrosine kinase inhibitors than the presence of an EGFR mutation.[23] However, its predictive capacity has been reported only in placebo-controlled trials (Iressa Survival Evaluation in Lung Cancer [ISEL][24] and the BR.21 study[23]). Moreover, the subgroup analysis in IPASS showed that longer progression-free survival was significantly associated with sensitive EGFR mutations but not with a high EGFR copy number. We therefore believe that evaluation of the copy number is not necessary when an EGFR mutation test is available. In the current study, EGFR mutations were detected with the use of the PNA-LNA PCR clamp method, the usefulness of which has been validated.[15,16] With this method, EGFR mutations can be detected from small cytologic specimens, such as those from bronchial washings, pleural effusions, and sputum collection, which are frequently used for the diagnosis of advanced non–small-cell lung cancer. The results of the analyses are obtained within several days, so the treatment is usually not delayed. The PNA-LNA PCR clamp approach is readily available and is covered by health insurance in Japan.

The best timing of treatment with an EGFR tyrosine kinase inhibitor for patients with EGFR mutations remains undetermined. A recent study showed that overall survival did not differ significantly between first-line and second-line treatments with erlotinib.[25] Overall survival is considered to be influenced by the second-line or later treatment. In the current study, 95% of the patients in whom first-line carboplatin–paclitaxel failed crossed over to gefitinib therapy. Such a high crossover rate has not been reported in previous studies of EGFR tyrosine kinase inhibitors. For example, in IPASS, only 39% of patients in the first-line chemotherapy group later received an EGFR-tyrosine kinase inhibitor. Considering that in our study the median overall survival in the gefitinib group was 7 months longer than that in the chemotherapy group (30.5 months vs. 23.6 months), in which virtually all patients were given gefitinib as the second-line treatment, and that the rate of response to gefitinib was slightly worse in the second-line setting than in the first-line setting (58.5% vs. 73.7%), first-line gefitinib may be more effective than gefitinib as second-line or later therapy. This idea needs to be tested in studies with large samples or in a meta-analysis.

We believe that the prolonged progression-free survival provided by the use of first-line gefitinib is valuable for patients with advanced non–small-cell lung cancer, who have a poor prognosis. If gefitinib is administered as second-line or third-line treatment, patients may miss the opportunity to receive treatment with gefitinib because of rapidly progressive disease during or after first-line treatment. We believe that the current study, in combination with our previous study of patients with mutated-EGFR non–small-cell lung cancer and poor performance status,[26] establishes the clinical benefit of an EGFR tyrosine kinase inhibitor as first-line treatment in patients with non–small-cell lung cancer and sensitive EGFR mutations.

Predictable toxicity profiles were observed with gefitinib and with carboplatin–paclitaxel in the current study. Diarrhea and rash were seen more often in the gefitinib group, whereas hematologic and neurologic toxic effects were more common in the chemotherapy group. Gefitinib appears to

Table 3. Common Toxic Effects in the Safety Population, According to Treatment Group.*

Toxic Effect	Gefitinib (N=114)					Carboplatin–Paclitaxel (N=113)					P Value for Grade ≥3
	Grade 1	Grade 2	Grade 3	Grade 4	Grade ≥3	Grade 1	Grade 2	Grade 3	Grade 4	Grade ≥3	
	no. of patients				*no. (%)*	*no. of patients*				*no. (%)*	
Diarrhea	32	6	1	0	1 (0.9)	7	0	0	0	0	<0.001
Appetite loss	7	4	6	0	6 (5.3)	39	18	7	0	7 (6.2)	<0.001
Fatigue	8	1	3	0	3 (2.6)	19	11	1	0	1 (0.9)	0.002
Rash	38	37	6	0	6 (5.3)	8	14	3	0	3 (2.7)	<0.001
Neuropathy (sensory)	0	1	0	0	0	28	27	7	0	7 (6.2)	<0.001
Arthralgia	1	2	1	0	1 (0.9)	25	21	8	0	8 (7.1)	<0.001
Pneumonitis	3	0	2	1†	3 (2.6)	0	0	0	0	0	0.02
Aminotransferase elevation	20	13	29	1	30 (26.3)	31	5	0	1	1 (0.9)	<0.001
Neutropenia	5	1	0	1	1 (0.9)	4	9	37	37	74 (65.5)	<0.001
Anemia	19	2	0	0	0	35	32	6	0	6 (5.3)	<0.001
Thrombocytopenia	8	0	0	0	0	25	3	3	1	4 (3.5)	<0.001
Any	17	44	43	4†	47 (41.2)	4	25	41	40	81 (71.7)	<0.001

* Toxic-effect grades are based on the National Cancer Institute Common Terminology Criteria (version 3.0).
† One patient counted here had a grade 5 toxic effect.

be less toxic than carboplatin–paclitaxel. The only exception was interstitial lung disease; there were three cases of severe interstitial lung disease (≥grade 3) in the gefitinib group and none in the chemotherapy group; one of the cases was fatal. The patient who died was a woman who had no history of smoking and thus had a relatively low risk of interstitial lung disease. Gefitinib sometimes causes diffuse alveolar or interstitial damage, especially during the first 3 months of treatment.[27] The estimated incidence of interstitial lung disease is low in many countries (e.g., 0.3% in United States)[28] but is relatively high (4 to 6%) in Japan.[29,30] Every patient treated with an EGFR tyrosine kinase inhibitor should be carefully monitored for this toxic effect.

In conclusion, the efficacy of first-line gefitinib was superior to that of standard chemotherapy, with acceptable toxicity, in patients with advanced non–small-cell lung cancer harboring sensitive EGFR mutations. Selection of patients on the basis of EGFR-mutation status is strongly recommended.

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APPENDIX

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ORIGINAL ARTICLE

Phase II study of FOLFOX4 with "wait and go" strategy as first-line treatment for metastatic colorectal cancer

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Abstract
Purpose To evaluate the efficacy and safety of FOLFOX4 using "wait and go" strategy in treating metastatic colorectal cancer.
Methods The conventional FOLFOX4 was repeated every 2 weeks. We waited until the recovery of symptoms from persistent neurotoxicity within an added period of 2 weeks, before performing the next cycle ("wait and go" strategy).
Results We enrolled 58 patients, in whom a total of 481 cycles were administered (median 8 per patient; range 1–16). Toxicity was evaluated in 58 patients and response in 55. The major toxic effect was grade 3/4 neutropenia (33%). Painful paresthesia or persistent functional impairment was observed in 4 patients (7%). The response rate was 40% (95% confidence interval; 27.1–52.9%). The median progression-free survival time was 10.2 months, the 1-year survival rate was 89%, and the median overall survival time was 27.6 months.
Conclusions These findings indicate that this "wait and go" strategy reduces the frequency of persistent neuropathy while maintaining efficacy against metastatic colorectal cancer.

Keywords FOLFOX · Neuropathy · Metastatic colorectal cancer · Oxaliplatin · "Wait and go"

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Background

Oxaliplatin, a third-generation platinum anticancer drug, has been shown to be effective for the treatment of metastatic colorectal cancer (CRC) [1, 5, 9, 21]. Currently, the FOLFOX chemotherapy regimen, consisting of oxaliplatin, 5-fluorouracil (5-FU), and leucovorin (LV), has become the standard regimen as first-line treatment for metastatic colorectal cancer [5, 9, 21]. The European adjuvant trial for colon cancer (MOSAIC) demonstrated significant improvement in 3-year disease-free survival when oxaliplatin was added to infusional 5-FU and LV [1].

One of the well-known dose-limiting factors of oxaliplatin is a delayed-onset, cumulative, dose-related peripheral neuropathy, characterized by persistent paresthesias affecting the hands and feet, and which does not remit between cycles of treatment [5, 18]. Persistent peripheral neuropathy with pain or function impairment interfering with activities of daily living (grade 3) occurs in 10–20% of patients receiving total oxaliplatin doses >750–850 mg/m^2 [5, 9, 21]. Of great concern is the development of persistent peripheral neuropathy that requires complete discontinuation of oxaliplatin, regardless of its efficacy, to avoid a debilitating neuropathy, which may take 6–10 months to resolve [5, 7]. Although this neuropathy is largely reversible, safety data from the MOSAIC trial determined that at 4 years, a small minority of patients (<5%) have grade 3 persistent peripheral neuropathy after 6 months of adjuvant FOLFOX4 treatment [2]. Various schedules have been pursued to reduce neuropathy. A randomized trial of FOLFOX4 versus scheduled intermittent oxaliplatin (OPTIMOX 1) was associated with a slight reduction in grade 3 neuropathy (17.9% versus 13.3%, $P = 0.12$) without lack of efficacy in response or progression-free survival [22]. Despite equivalent efficacy, the OPTIMOX 1 "stop and go" strategy has not been widely adopted for all patients. This is probably as a result of variability in management of patients by different physicians, heterogeneity of the disease, and inability to reinstitute oxaliplatin at the time of progression, often because of persistent neuropathy [7].

For patients with unresectable metastatic disease, the duration of treatment is indefinite, extending until disease progression or until the treatment is no longer tolerated. Hence, it is imperative to manage appropriately the persistent peripheral neuropathy, which causes deteriorating in the quality of life during treatment. No single strategy, including calcium (Ca)–magnesium (Mg) supplementation [8, 11, 12] and various antineuropathic and antiepileptic medications [4, 10], has proven effective for preventing or reducing the cumulative neuropathy associated with oxaliplatin.

One possible approach to prevent grade 3 sensory neurotoxicity during treatment is to wait for the complete recovery of paresthesia or dysesthesia from persistent neurotoxicity until 29 days, followed by the subsequent course without dose modification. If paresthesia or dysesthesia continues over 29 days, the dose of oxaliplatin is reduced in the subsequent course, to maintain the antitumor effect of FOLFOX. We conducted the present phase II study to investigate this novel "wait and go" strategy.

Methods

The eligibility criteria for inclusion onto the study were as follows: adenocarcinoma of the colon or rectum; unresectable metastases; at least one measurable lesion of 1 cm or a residual nonmeasurable lesion; adequate bone marrow (hemoglobin >9.0 g/dl, leukocyte count lower limits of normal −12,000/mm^3, neutrophils <1,500/mm^3, platelet count 100,000/mm^3), liver (AST and ALT 2.5 upper limits of normal [UNL], total bilirubin 1.5 UNL, alkaline phosphatases 2.5 UNL), and renal function (creatinine less than UNL); Eastern Cooperative Oncology Group (ECOG) performance status (PS) of 0–2; and age 20–80 years. Previous adjuvant fluoropyrimidine chemotherapy, if given, must have been completed at least 2 weeks before inclusion. Patients with uncontrolled infection, massive ascites or pleural effusion, brain metastases, second malignancies, bowel obstruction, current watery diarrhea, a history of oxaliplatin-based adjuvant chemotherapy, or disease confined to previous radiation fields were excluded. Written informed consent was required and the Ethical Committee approved the study.

Chemotherapy

Eligible patients were treated with the FOLFOX4 regimen [1, 9, 21]. Each cycle comprised oxaliplatin 85 mg/m^2 and l-LV 100 mg/m^2 intravenously (IV) administered simultaneously for 2 h followed by 5-FU 400 mg/m^2 IV bolus followed by 5-FU 600 mg/m^2 infusion for 22 h on day 1, and the same therapy, without the oxaliplatin, administered on day 2 (total 46 h after the initial 2 h IV) of a 14-day treatment cycle. Pretreatment with a 5-hydroxytryptamine-3 antagonist and dexamethasone was strongly recommended, although the administration of intravenous calcium and magnesium was not permitted in order to prevent oxaliplatin-induced neuropathy. Treatment was continued until disease progression (PD), unacceptable toxicity, or patient choice.

Toxicity was assessed before starting each 2-week cycle using the National Cancer Institute-Common Toxicity Criteria (NCI-CTC) version 3.0. A specific scale was used for sensory neurotoxicity: grade 1 is brief paresthesia with complete regression before the next cycle, grade 2 is persistent paresthesia or dysesthesia without functional impairment over the next cycle, and grade 3 is painful paresthesia or persistent functional impairment (Table 1).

Table 1 Specific scale for sensory neurotoxicity

Grade	Sensory neurotoxicity
1	Brief paresthesia with complete regression before the next cycle (<15 days)
2	Persistent paresthesia or dysesthesia without functional impairment over the next cycle (≧15 days)
3	Painful paresthesia or persistent functional impairment

Chemotherapy was delayed until recovery if neutrophils <1,500/mm^3, platelets <75,000/mm^3, or for significant persistent non-hematological toxicity. If grade 4 neutropenia, grade 3/4 thrombocytopenia, or grade 3/4 gastrointestinal toxicities occurred, the FU dose was reduced to 300 mg/m^2 for the bolus component and 500 mg/m^2 for the infusion component and the oxaliplatin dose was reduced to 65 mg/m^2. In the case of grade 2 paresthesia at a new cycle of treatment, the next cycle of FOLFOX4 was delayed until the recovery of paresthesia from persistent neurotoxicity for up to 2 additional weeks (<29 days). If it persisted for 29 days, the oxaliplatin was reduced to 65 mg/m^2. If grade 3 paresthesia was present during treatment, oxaliplatin was omitted from the regimen.

Treatment was discontinued if subsequent reduction was indicated.

Evaluation

Pretreatment evaluation included complete patient histories, physical examinations, complete blood cell counts, biochemistry involving liver and renal functions, urinalysis, tumor markers including CEA and CA19-9, chest roentgenogram, electrocardiogram, and computed tomographic scans of the abdomen and chest. According to NCI-CTC version 3.0, toxicity and laboratory variables in complete blood cell counts, biochemistry, and urinalysis were assessed weekly during the first course, on days 1 and 15 from the second through to the sixth course and at least once during subsequent courses. CT scans were repeated to evaluate lesions every two courses and tumor markers were measured at the same time. Responses were evaluated according to the RECIST criteria [20]. To confirm partial response (PR) (30% or greater decrease in the sum of the longest dimensions of target lesions, referenced against the baseline sum of the longest dimensions of target lesions together with stabilization or decrease in size of nontarget lesions) or complete response (CR) (disappearance of all target and nontarget lesions together with normalization of tumor marker levels), tumor measurements were repeated no less than 4 weeks after objective response was firstly obtained. Responses were assessed by external review.

Overall survival (OS) was defined as the time from treatment initiation to death from any cause. Progression-free survival (PFS) was the time from treatment initiation to first documentation of disease progression detected by the external review or death from any cause (censored at second-line chemotherapy). Time-to-treatment failure (TTF) was the time from treatment initiation to discontinuation of treatment, first documentation of disease progression by the external review, or death from any cause.

Statistical evaluations

The phase II study was designed to test the null hypothesis that the true response probability is less than the clinically significant level of 25%. The response rate of first-line FOLFOX was reported to be from 45 to 50%. The alternative hypothesis of the response rate in this study was >45%, because the "wait and go" strategy to prevent grade 3 paresthesia might diminish the response. The probability of accepting treatment with a response probability (25%) was $P = 0.05$. The probability of rejecting treatment with a response rate of 45% was $P = 0.2$; therefore, the required number of patients was estimated to be 49. Allowing for a patient ineligibility rate of about 20%, we planned to enroll 60 patients. The 95% confidence interval (CI) was calculated for the RR, PFS, and TTF. OS, PFS, and TTF were calculated by the Kaplan–Meier method.

Results

Patients' characteristics

We enrolled 58 patients between March 2006 and April 2008, all of whom met all eligibility requirements and received at least one course of treatment. Patient characteristics are summarized in Table 2, and all patients were evaluated for toxicity and response. The median age of patients was 67.5 years (range, 37–80 years); 48 patients had an ECOG PS of 0 and 10 patients had an ECOG PS of 1. There were 13 patients with advanced disease with primary tumors and 45 patients in recurrent status. Primary sites were the colon in 35 patients and the rectum in 23 patients. Metastatic sites were in the liver in 39 patients, lungs in 17, lymph nodes in 21, and peritoneum in 11.

Safety

All 58 patients enrolled in the phase II study were assessable for safety and received 481 treatment courses (median, 8 courses; range, 1–16 courses). The median relative dose intensity was 76.9% for oxaliplatin, 76.7% for bolus FU, and 77.8% for infusion FU. The causes of treatment discontinua-

Table 2 Patients' profile ($n = 58$)

Characteristic	No. of patients %
Median age, years (range)	67.5 (37–80)
Sex	
Male	36
Female	22
ECOG PS	
0	48
1	10
2	0
Disease status	
Advanced	3
Recurrent	45
Primary tumor	
Colon	35
Rectum	23
Differentiation	
Well	11
Moderate	42
Poor	5
Metastatic sites	
Liver	39
Lymph node	21
Lung	17
Peritoneum	11
Others	4
No. of metastatic sites	
0	0
1	25
>1	33

tion were disease progression in 20 patients (34.5%), delayed recovery from toxicity such as neutropenia, thrombocytopenia, and liver dysfunction in 6 patients, withdrawal of consent, mainly due to economic issues, in eight cases, surgery for metastases in five patients, allergic reaction in five patients, subsequent reduction in four patients, and grade 3 paresthesia in four patients (6.9%). There were no serious unexpected adverse events and no treatment-related deaths.

The overall incidences (%) of hematological and non-hematological toxicities in the phase II study are listed in Table 3. Grade 3/4 neutropenia was the most common adverse event and occurred in 32.8% of all 58 patients. No patient had febrile neutropenia. With the exception of paresthesia, major non-hematological toxicities were liver dysfunction, anorexia, stomatitis, and diarrhea. Grade 3 non-hematological toxicities were diarrhea (1.7%) and nausea (1.7%). We observed grade 1 paresthesia in 24 patients (41.4%), grade 2 in 13 patients (22.4%), and grade 3 in four patients (6.9%). Cumulative incidence of paresthesia is shown in Fig. 1. The median times to onset of

Table 3 Observed adverse events according to number of patients

Event	Number of patients ($n = 58$)				
	NCI-CTC grade, version 3				
	1	2	3	4	3/4, %
Leucopenia	10	28	6	0	10.3
Neutropenia	0	9	9	10	32.8
Anemia	12	14	1	0	1.7
Thrombocytopenia	28	6	2	0	3.4
Anorexia	12	9	0	0	0
Nausea	15	6	0	0	0
Vomiting	6	2	0	0	0
Fatigue	12	6	0	0	0
Diarrhea	4	2	1	0	1.7
Constipation	1	0	0	0	0
Stomatitis	4	0	0	0	0
Abnormal AST	27	5	1	0	1.7
Abnormal ALT	17	4	0	0	0
Hyperbilirubinemia	7	1	0	0	0
Neuropathy[a]	24	13	4	–	6.9

[a] A specific scale was used for neuropathy (Table 1)

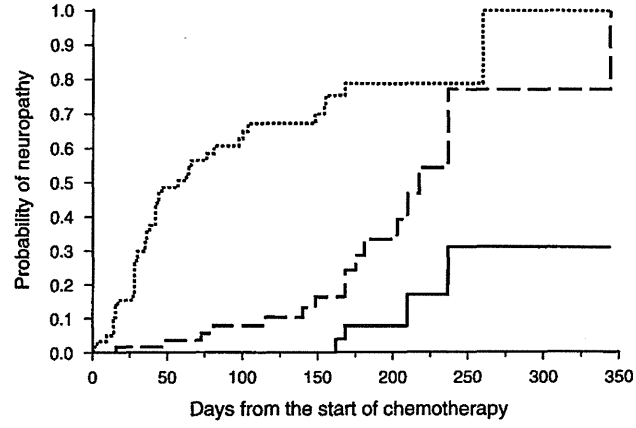

Fig. 1 Cumulative incidence of neuropathy. *Solid line*, grade 3 neuropathy ($n = 4$); *broken line*, grade 2 neuropathy ($n = 13$); *dotted line*, grade 1 neuropathy ($n = 24$)

paresthesias were 54.5 days for grade 1 and 213.5 days for grade 2, respectively. Grade 3 paresthesia was observed from 162 to 237 days from the start of chemotherapy. The median cumulative doses of oxaliplatin associated with paresthesia were 255 mg/m^2 for grade 1,764 mg/m^2 for grade 2, and 973 mg/m^2 for grade 3.

The dose reductions were required in 16 of all 58 patients (27.6%). Among these 16 patients, the reasons for dose reduction were grade 4 neutropenia in eight patients, grade 3/4 gastrointestinal toxicities in one patient, grade 3/4 thrombocytopenia in three patients, and grade 2 paresthesia in only one patient. The treatment delay within 2 weeks was observed in 50 of all 58 patients (86.2%) among 171 of

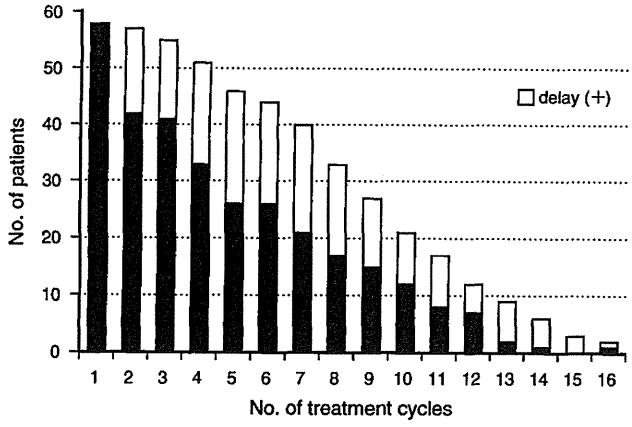

Fig. 2 The frequency of treatment delays in terms of treatment cycle. *Black bar*, numbers of patients who started the treatment within 29 days from the initial day of the previous chemotherapy cycle; *White bar*, numbers of patients who started the treatment over 29 days from the initial day of the previous chemotherapy cycle

all 481 treatment courses (35.6%). The frequency of treatment delay over 2 weeks was from 40.9 to 100% after the fourth treatment course (Fig. 2).

Efficacy

The response was assessed as CR, PR, stable disease (SD) (less than a 30% reduction and less than a 20% increase in the sum of the longest dimensions of target lesions, referenced against the baseline sum of the longest dimensions of target lesions together with stabilization or decrease in size of nontarget lesions), and progressive disease (PD) in 2, 20, 25, and 8, respectively, of the 55 patients in the efficacy analysis set (three were not assessable). The RR was 40.0% (95% CI 28.1–53.2%) and the disease control rate (CR + PR + SD) was 85.5% (95% CI 73.8–92.4%).

The median follow-up period was 15.5 months as of the data cut-off date, October 15, 2009. The median PFS was 10.2 months (95% CI 6.4–14.0 months) (Fig. 3), median overall survival time (MST) was 27.6 months (95% CI 20.6–35.6 months) (Fig. 4), and median TTF was 5.0 months (95% CI 3.6–5.1 months). The patients who received the second-line chemotherapy or the surgery for metastases without PD were censored at the date of image examination immediately before the second-line chemotherapy or the surgery for metastases in PFS analysis. The 1- and 2-year survival rate of MST was 89.0% (95% CI 80.7–97.3%) and 57.8% (95% CI 42.3–73.4%), respectively. Of the 58 patients, 46 (79.3%) discontinued treatment and received second-line chemotherapy.

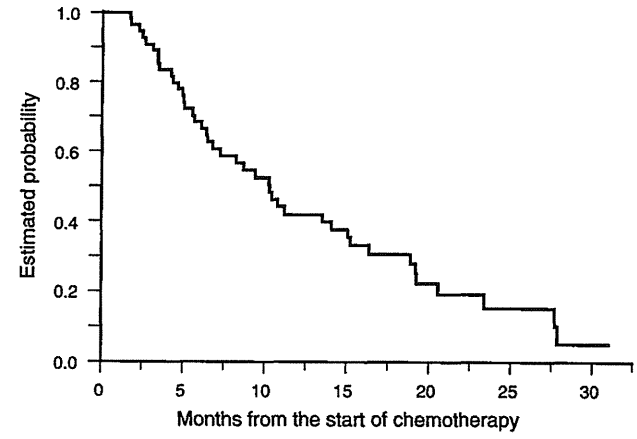

Fig. 3 Kaplan–Meier estimates of progression-free survival ($n = 58$)

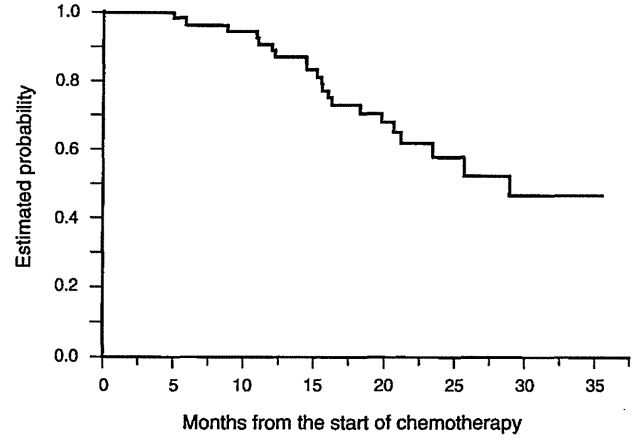

Fig. 4 Kaplan–Meyer estimates of overall survival ($n = 58$)

Discussion

We set out to determine whether the "wait and go" strategy for FOLFOX4 in the treatment of metastatic colorectal cancer would be effective. This is the first study of FOLFOX4 with the novel "wait and go" strategy, which minimizes painful paresthesia or persistent functional impairment during treatment by a 2-week wait for the recovery of paresthesia or dysesthesia from persistent neurotoxicity at the new cycle of treatment. Using this strategy, a very promising efficacy, low incidence of painful paresthesia or persistent functional impairment of 6.9% was obtained in our phase II study: an RR of 40.0%, a median PFS of 10.2 months, and an MST of 27.6 months with a 1-year survival rate of 89.0%. Our efficacy results are comparable to those of other recently reported FOLFOX4 regimens for metastatic colorectal cancer, although the RR of 40.0% is slightly lower than previously reported rates of 45% [9] to 49.5% [5]. One possible explanation might be that the frequency of treatment delay of up to 2 weeks in almost 40% of cases in the fourth and fifth treatment course might diminish the confirmation rate of response (Fig. 2). However, it is true that the RR of 40.0% with 95% CI from 28.1 to 53.2% met the primary endpoint of this study.

In this study, the allowance for a patient ineligibility rate was set at 20%, which is twice the ordinary rate of 10%, because the aim of this study was to evaluate the new "wait and go" strategy concept. Fortunately, all 58 accrued patients were treated with this strategy. During this study, the new molecular targeting drug, bevacizumab, was approved at April 2007 by the Japanese regulatory authorities, and the combination of bevacizumab and chemotherapy including the FOLFOX4 regimen became one of the standard therapies for metastatic colorectal cancer in Japan. The introduction of bevacizumab to clinical practice slowed patient accrual in this trail. At 2 years from the start of this study, the number of enrolled patients reached 58 patients, which was more than the required 49 patients initially estimated as necessary for statistical evaluation of this trial. We halted accrual of patients in April 2008 in accordance with the recommendation of the safety monitoring committee.

The grading system, originally developed by Levi and co-workers [16], takes into account both intensity and duration of symptom-related oxaliplatin-induced neurological toxicity. At present, the most commonly used neurological toxicity scale is the NCI-CTC, which considers only the intensity of neuropathy. Our grading system used in this study was consistent with that by Levi et al. [16, 17], in terms of the consideration of both intensity and duration of symptom-related oxaliplatin-induced neurological toxicity. The duration reported by Levi et al. was within 1 week or 2 weeks [16, 17]. Because the new cycle of FOLFOX4 is begun every 2 weeks, we decided on 2 weeks as an appropriate period to evaluate grade 1 or 2 paresthesia. However, the criteria for grade 3 neurological toxicity (painful paresthesia or persistent functional impairment) used in our study are similar to that of the NCI-CTC. Thus, our criteria are appropriate to indirectly compare the frequency of grade 3 neurological toxicity between other clinical trials and this trial.

The frequency of grade 3 neurological toxicity was 6.9% in this trial. In a European trial in advanced colorectal cancer, 18% of patients assigned to the FOLFOX4 regimen had grade 3 neurosensory toxicity during treatment [5]. The same rate was observed among patients assigned to the FOLFOX4 regimen in a North Central Cancer Treatment Group study in metastatic colorectal cancer [9]. In the Multicenter International Study of Oxaliplatin/5-Fluorouracil, Leucovorin in the Adjuvant Treatment of Colon Cancer (MOSAIC), 12.4% of patients treated with FOLFOX4 developed grade 3 paresthesia during therapy [1]. The rates of grade 3 neurotoxicity in those studies are higher than the 6.9% observed in this study. In the National Surgical Adjuvant Breast and Bowel Project (NSABP) C-07 study, the incidence of grade 3 neurotoxicity was reported to be 8.4% among patients treated with the FLOX regimen (500 mg/m^2 FU intravenous (IV) bolus weekly for 6 weeks plus 500 mg/m^2 LV IV weekly for 6 weeks with 85 mg/m^2 oxaliplatin IV administered on weeks 1, 3, and 5 of each 8-week cycle for three cycles [13, 14]). This lower incidence of grade 3 neurological toxicity was speculated to be partly due to the scheduled rest in the FLOX regimen. The 2-week wait in the FOLFOX4 regimen depending on the persistency of neurological toxicity might prevent grade 3 neurological toxicity, even in metastatic disease.

The dose reduction and discontinuation of oxaliplatin due to neurological toxicity has varied in different trials. Rothenberg et al. reported the 85 mg/m^2 oxaliplatin in FOLFOX4 was reduced to 65 mg/m^2 in cases of persistent paresthesia or dysesthesia with preserved function, but not activities of daily living (grade 2), or temporary (7–14 days) paresthesia or dysesthesia with pain or function impairment that interferes with activities of daily living (grade 3) [18]. Oxaliplatin was omitted from the regimen until recovery in the case of grade 2 persistent paresthesia or dysesthesia, or grade 3 temporary (1–14 days) paresthesia or dysesthesia. The incidence of grade 3 cumulative neuropathy is reported to be 3%. This lower incidence might be explained by the 6 cycles as the median number of treatment cycles, due to the second-line setting for progressive colorectal cancer after the irinotecan-containing regimen. In the study on first-line FOLFOX reported by de Gramount et al. [5], oxaliplatin was reduced in cases of persistent ($\geq$14 days) paresthesia or temporary (7–14 days) painful paresthesia or temporary functional impairment. In cases of persistent ($\geq$14 days) painful paresthesia or persistent functional impairment, oxaliplatin was omitted from the regimen until recovery. Paresthesia with pain and cumulative paresthesia interfering with function occurred in 10.5 and 16.3% of patients, respectively. The dose intensity was 76% for FU and 73% for oxaliplatin during all cycles, which is similar to the 76.7% for bolus FU and 77.8% for infusion FU and 76.9% for oxaliplatin in our study. Considering the similar dose intensity of oxaliplatin, the "wait and go" strategy might effectively prevent painful paresthesia or persistent functional impairment compared with previously reported conventional methods to reduce the dose and to discontinue oxaliplatin.

Our data have some limitations. First, our results were obtained in a single-armed phase II study including small number of patients. Additionally, FOLFOX4 was used without molecular targeting drugs such as bevacizumab [19] or anti-human epidermal growth factor receptor monoclonal antibodies [3, 6]. The independent studies are warranted to extrapolate this "wait and go" strategy to molecular targeting drug-containing regimens. Second, the primary endpoint in this trial was the RR, not the reduction in neurotoxicity. Prospective phase III trials, including larger numbers of patients, are needed to corroborate our

results. However, we believe that our results suggest that this "wait and go" strategy could be a treatment of choice for patients who are reluctant to encounter persistent neurological toxicity, especially in the palliative setting, with or without molecular targeting drugs. Third, we evaluated the neurological toxicity based on clinicians' reports. In 2006, the FDA recommended that patient-reported outcomes should be considered the gold standard in addition to physician observation. Written in layman language, patient-reported outcomes have been advocated by the NCI since 2006 alongside NCI-CTC. Patients' assessment tools should be used for greater accuracy of interpretation of patient-reported outcomes [15, 23].

In conclusion, the "wait and go" strategy may be effective to prevent painful paresthesia or persistent functional impairment during treatment while maintaining the efficacy of the FOLFOX4 regimen for metastatic colorectal cancer. Further evaluation is needed to examine whether this strategy can be compared with the "stop and go" strategy [22].

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Conflict of interest No authors have any conflict of interest.

Appendix

The following investigators participated in the study: Mitsugu Kochi, Ken Hagiwara (Nihon University School of Medicine, Tokyo, Japan); Yuki Tanabe (Asahikawa Medical University, Hokkaido, Japan); Eiji Meguro, Akinori Takagane, Makoto Kobayashi (Hakodate Goryokaku Hospital, Hokkaido, Japan); Hiroyuki Shibata, Kou Miura (Tohoku University, Miyagi, Japan); Masayuki Sato (Miyagi Cancer Center, Miyagi, Japan);, Yutaka Hoshino, Fumihiko Osuka (Fukushima Medical University, Fukushima, Japan); Michitaka Nagase (Jichi Medical University, Tochigi, Japan); Miki Adachi (IUHW Mita Hospital, Tokyo, Japan); Kenji Katsumata (Tokyo Medical University, Tokyo, Japan); Masanori Yoshino (Nippon Medical School Musashi Kosugi Hospital, Kanagawa, Japan); Reiji Aotake, Koji Doi (Fukui Red Cross Hospital, Fukui, Japan); and Takuji Fukui (Midori Municipal Hospital, Aichi, Japan).

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特 集 分子標的治療薬におけるバイオマーカーの役割

大腸癌における抗EGFR抗体薬のバイオマーカー

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Predictive Biomarkers of Anti-EGFR Monoclonal Anti-Body in Colorectal Cancer: Hiroshi Soeda, Hideki Shimodaira and Chikashi Ishioka (*[1]*Institute of Development, Aging and Cancer, Tohoku University*, *[2]*Dept. of Clinical Oncology, Tohoku University Hospital*)

Summary

The epidermal growth factor receptor (EGFR), a receptor tyrosine kinase, triggers a downstream signaling cascade through areas such as the RAS-RAF-MAPK and PI3K-AKT pathways, which are involved in cell proliferation, survival and motility. Inhibiting EGFR activation has demonstrated significant promise as a molecular targeting therapy for various solid tumors. Two monoclonal antibodies (mAbs) targeting EGFR, cetuximab and panitumumab, are established to be new treatment options for metastatic colorectal cancer (mCRC). Among activating mutations in downstream of EGFR, the *KRAS* mutation, which is present in 40% of mCRC patients, has shown to be a predictive biomarker for resistance to anti-EGFR antibody therapy based on Caucasian studies. However, only a small proportion of patients achieved an objective response and benefit from anti-EGFR antibody, even among those with wild-type *KRAS* tumors. Other downstream factors in EGFR signaling are now being explored, such as the *BRAF*, *PIK3CA*, *PTEN* genes. Cetuximab, a chimeric immunoglobulin 1 (IgG_1) monoclonal antibody, may also exert antitumor effects through antibody-dependent cell-mediated cytotoxicity (ADCC). ADCC is influenced by *FCγR IIa*-H131R and *FCγR IIIa*-V158F polymorphisms. Additional analysis of *BRAF*, *PIK3CA*, *PTEN* and *FcγR* genes in *KRAS* wild-type patients could narrow down the selection of patients who are most likely to benefit from anti-EGFR antibody therapy. **Key words**: Anti-EGFR antibody, Predictive biomarker, Colorectal cancer, *KRAS*, **Corresponding author**: Hiroshi Soeda, Department of Clinical Oncology, Institute of Development, Aging and Cancer, Tohoku University, 4-1 Seiryo-machi, Aoba-ku, Sendai 980-8575, Japan

要旨 治癒切除不能な進行・再発大腸癌を対象にする分子標的治療薬に，抗EGFR抗体薬であるcetuximabとpanitumumabがある。抗EGFR抗体薬はリガンドのEGFRへの結合を阻害し，リガンドによる受容体チロシンキナーゼの活性化およびその下流のRASを介する細胞増殖シグナルを抑制する。抗EGFR抗体薬は海外の臨床試験で，*KRAS*遺伝子に点突然変異のある大腸癌で治療成績が期待できないことが示されているが，*KRAS*遺伝子に加えて*BRAF*遺伝子，*PIK3CA*遺伝子などでもバイオマーカーとなり得るかの検討が行われている。また，cetuximabはIgG_1抗体であり，その治療成績の一部は抗体依存性細胞介在性傷害反応（ADCC）に依存し，Fcγ受容体の遺伝子多型に影響を受けるとされている。今後，*KRAS*遺伝子とともにEGFR下流遺伝子やFcγ受容体の遺伝子多型を検討することは，大腸癌の個別化治療をさらに前進させるものと期待される。

はじめに

進行・再発大腸癌に対するがん薬物療法はこの10年で著しく進歩し，分子標的治療薬の導入により生存期間中央値が2年を超えるまでに治療成績が向上してきた[1]。進行・再発大腸癌の一次・二次治療の標準治療はFOLFIRI, FOLFOX療法に抗VEGF抗体であるbevacizumabの併用が主流であったが，本邦においてもcetuximabが2008年7月にEGFR陽性の治癒切除不能な進行・再発結腸・直腸癌に対して承認を受け，抗EGFR抗体薬が使用可能となり，さらに治療の選択が広がった。NCCN (National Comprehensive Cancer Network) のガイドラ

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インでは，現在のところ一次治療としてFOLFOX，XELOX，FOLFIRI療法に分子標的治療薬を併用する治療法が推奨されている。

抗EGFR抗体薬のcetuximabはマウス骨髄細胞株由来のIgG_1サブクラスのキメラ化モノクローナル抗体である。また，panitumumabはIgG_2サブクラスの完全ヒト化モノクローナル抗体である。

Ⅰ．抗EGFR抗体とEGFRシグナル伝達経路

上皮細胞増殖因子受容体（以下EGFR）は，細胞膜に存在する受容体ですべての上皮細胞に発現する。EGFRの主要な細胞内シグナル伝達経路としては図1に示すように，Ras/Raf/MAPK経路，PI3K/Akt経路，JAK/STAT経路がある[2-4]。正常組織におけるEGFRは，細胞の分化，増殖，発達，維持の調節に重要な役割を果たしている。一方，癌細胞ではEGFRの機能が亢進し，癌の増殖，浸潤，転移，生存，血管新生などに関与する。抗EGFR抗体薬は，EGFRのリガンドであるEGFおよびTGFαのEGFRへの結合を阻害し，リガンドによる受容体チロシンキナーゼの活性化を阻害する[5]。

抗EGFR抗体薬は海外の臨床試験で，*KRAS*遺伝子に点突然変異のある大腸癌で抗腫瘍効果がないことが示されている。なお，大腸癌においてはcetuximab，panitumumabともにEGFRの発現強度と治療成績には関連性が認められていない[6]。

Ⅱ．KRAS

*KRAS*遺伝子の恒常的活性化型点突然変異は膵癌，胆管癌，大腸癌，肺癌など多くのヒトがんの発がん過程において重要な役割を担っている[7]。*KRAS*遺伝子は大腸癌において40%程度の頻度で活性型の変異が検出される。そのうち90%以上がコドン12および13に認められ，低い頻度でコドン61および146などにも認められる。コドン12および13はKRASの活性化部位の周辺に位置している[8]。*KRAS*遺伝子の点突然変異はRASの変異蛋白質のGTPase活性を低下し，細胞内シグナル伝達経路において活性型にとどまらせ，下流へのシグナルが恒常的に持続すると考えられている[9]。したがって，*KRAS*遺伝子変異がある場合，EGFRを標的分子としても下流のシグナル伝達がブロックされず，抗腫瘍効果が得られない可能性が示唆されている。

実際，一次治療については，CRYSTAL試験[10]では，*KRAS*遺伝子野生型の患者集団においてFOLFIRI＋cetuximab群がFOLFIRI群に比し奏効率，無増悪生存期間（以下PFS）中央値，全生存期間（以下OS）で有意に良好であった。OPUS試験[11]では，*KRAS*遺伝子野生型の患者集団においてFOLFOX4＋cetuximab群がFOLFOX4群に比し奏効率，PFS中央値で有意に良好であった。

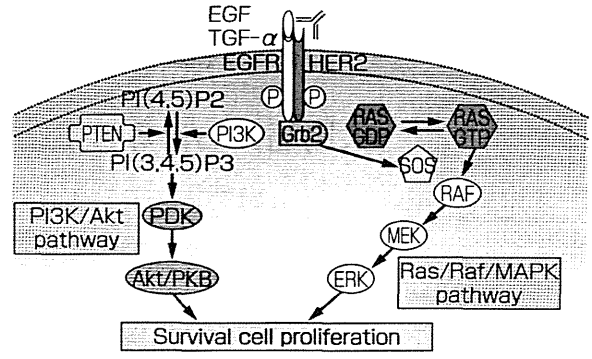

図1 EGFRシグナル伝達係路

このような結果を受けて，わが国の大腸癌治療ガイドラインが改訂され，2010年4月に本邦においても*KRAS*遺伝子検査が保険適応となった。すなわち，抗EGFR抗体薬投与前の検査が必須となり，国内の大腸癌診療は欧米と同レベルに到達したと考えられる。

Ⅲ．BRAF

*KRAS*遺伝子に変異がなくとも抗EGFR抗体薬が必ずしも有効とは限らず，*KRAS*遺伝子以外にも感受性に関連する因子が存在することが考えられる。その候補として，RASを介するシグナル伝達の下流因子のなかで細胞増殖にかかわるBRAFが第一にあげられる。BRAFはセリン・スレオニンキナーゼであり，N-末端側にRAS結合部位，C-末端側にキナーゼ部位が存在する。活性化したKRAS蛋白質が直接結合し，キナーゼ活性によりMAPK経路の一員としてシグナルを下流に伝達する。*BRAF*遺伝子変異は甲状腺癌，悪性黒色腫，大腸癌，卵巣癌，前立腺癌などで報告されている[12]。大腸癌における*BRAF*遺伝子の変異頻度は10%程度で[13]，その80%以上はキナーゼ部位の1アミノ酸置換V600Eである[14]。過去に*BRAF*遺伝子変異については，後ろ向き研究の結果であるものの，三次治療以降のcetuximabあるいはpanitumumab治療に対して奏効例を認めないという報告がなされている[15,16]。また，一次治療でのcetuximab治療において，*KRAS*遺伝子野生型患者における*BRAF*遺伝子変異型は治療効果予測因子ではなく予後不良因子になるという報告もある[17]。

Ⅳ．PI3K

*KRAS*遺伝子および*BRAF*遺伝子以外のバイオマーカーとして，腫瘍の進行および転移能の亢進をもたらす

フォスファチジルイノシトール3リン酸（以下PI3K）も候補にあげられる。PI3Kはその上流の受容体型チロシンキナーゼやRASにより活性化されるが，自身の触媒または調節ドメインに対応する遺伝子変異によっても活性化され，PI3K/Akt経路の活性化を介して細胞死を抑制する機能を担う。

PI3Kの触媒サブユニットのp110αをコードする*PIK3CA*遺伝子には，乳癌，尿路系癌，子宮癌，大腸癌，頭頸部癌などで変異がみつかり注目されている[18,19]。大腸癌では*PIK3CA*遺伝子の変異は15%程度の頻度で，その変異は1塩基置換を生じるものが多く，exon 9（E542K，E545Kなど）および20（H1047Rなど）を解析することで80%以上を網羅できるとされている[13]。ただ，*PIK3CA*遺伝子の変異については，抗EGFR抗体薬の治療成績に関連性があるという報告[20]と関連性がない[21]という相反する報告があり，治療成績との関連は今のところ不確定である。また，exon 20の変異のみが治療効果予測因子になり得るという報告もある[22]。

また，PI3Kの調節サブユニットのp85αをコードする*PIK3R1*遺伝子に関しても大腸癌で約8%の頻度で変異があったと報告されている[23]。*PIK3CA*遺伝子と同様，治療成績との関連は今のところ不確定である。

V．PTEN

*PTEN*はPI3K/Akt経路を阻害するがん抑制遺伝子であり，PI3Kを脱リン酸化する。*PTEN*遺伝子の変異は様々な癌で報告されている[24]。また，PTENの発現消失は20～30%前後の大腸癌に認められている。臨床研究においては，原発巣ではPTENの発現の有無でcetuximab投与の治療成績は変わらないものの，転移巣ではPTEN発現患者で治療成績が良好であったとの報告がある[25,26]。

また免疫組織化学染色法でのPTEN非発現患者は，後ろ向き研究の結果ではあるが，*KRAS*・*BRAF*遺伝子ともに野生型の患者におけるcetuximab治療で，治療効果予測因子にはならないものの，不良な予後予測因子であったと報告されている[27]。

Ⅵ．Amphiregulin，epiregulin

amphiregulin（以下AREG）やepiregulin（以下EREG）はEGFRのリガンドであり，EGF，TGFαよりも結合能が弱いことが知られている。ただし，進行・再発大腸癌における予後予測因子あるいは抗EGFR抗体薬の治療効果予測因子である可能性が報告されており，がん化の過程で中心的な役割を果たしていると考えられる。

*KRAS*遺伝子変異のように治療効果が期待できない患者を選別する役割を果たすことは難しいが，*KRAS*野生型においてAREGやEREGが高発現している場合，cetuximab＋irinotecan併用療法を行うことで良好な治療成績が得られると報告された[28]。*KRAS*変異型では，AREGやEREGの発現と治療成績に相関は認めなかった。

Ⅶ．抗EGFR抗体とADCC活性

ADCCとは，標的細胞に結合した抗体がナチュラルキラー細胞やT細胞，好中球，マクロファージなどのエフェクター細胞上のFc受容体と結合することで，抗体依存的に誘導される標的細胞介在性傷害活性である。抗CD20抗体（rituximab），抗HER2抗体（trastuzumab）をはじめとするIgG_1サブクラスの抗体薬治療に特異的な利点として注目されており，Fcγ受容体の遺伝子多型により治療成績に差が生じることが報告されている[29,30]。IgG_1抗体であるcetuximabにおいても，EGFR依存性シグナル伝達経路の阻害の他にADCCの誘導が寄与していることが知られており[31,32]，*FcγRⅡa*と*FcγRⅢa*の遺伝子多型（FcγⅡa-H/R[131]，FcγRⅢa-V/F[158]）に影響を受けるとされている。そのため，Fcγ受容体の遺伝子多型はIgG_1サブクラスであるcetuximabの治療効果予測のための分子マーカーとして期待されている。

FcγRⅡa，*Ⅲa*遺伝子については，欧米からの報告[33]によりcetuximabに対してより高い治療成績が期待されている遺伝子型は131H，158Vであり，これはrituximab，trastuzumabで示された傾向と一致している。HapMapプロジェクトによると日本人におけるH/Hの遺伝子型は約70%を占めるとされており，この頻度は欧米人に比し高頻度である。反対にV/Vの遺伝子型は約2%であり，この頻度は欧米人に比しやや低頻度である。*FcγRⅡa*，*Ⅲa*遺伝子を総合で考えると，cetuximabの治療成績が期待される遺伝子多型をもつ頻度は，日本人において欧米人よりもやや多く，ADCC活性が治療成績に影響すると仮定するならば，欧米人に比し日本人は良好な治療成績が期待できると考えられる。

Ⅷ．抗EGFR抗体薬投与における皮膚症状

抗EGFR抗体薬において皮膚障害は最も頻度の高い毒性であり，grade 2以上の皮膚障害はその治療成績に関連すると報告されている[34]。ただし，抗EGFR抗体薬による治療経過中の最高gradeと治療成績を比較している報告が大部分であり，治療が長期間に及び総投与量が多くなる患者が，より高度のgradeに分類されやすくなっている可能性がある。そのため，皮膚障害が治療効

果予測因子になり得ると厳密には結論付けられないと考えられる。皮膚障害と治療成績の関連性を新たに示すためには，治療開始後早期に期間を限定した皮膚障害のgradeの解析が今後必要になると考えられる。

おわりに

現在，患者の個別化医療分野において，薬剤の効果を予測するバイオマーカーを開発することは最も重要な課題の一つである。抗EGFR抗体薬の治療成績は，*KRAS*遺伝子変異の有無により大きく異なることが明らかになり，大腸癌の薬物療法に初めて個別化医療が導入された。今後，*KRAS*遺伝子同様に*BRAF*遺伝子や*PIK3CA*遺伝子などについてもバイオマーカーとなり得るか検討することは，大腸癌の個別化治療をさらに前進させるものと期待される。バイオマーカーを用いたがん分子標的治療薬による治療を行うためには様々な臨床試験による検証が必要であるが，治療効果を最大限に引きだし副作用を軽減することで，患者のケアに劇的な改善をもたらし，さらに医療費削減にも貢献できるものと考えられる。

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Induction of apoptosis by cytoplasmically localized wild-type p53 and the S121F mutant super p53

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Abstract. After DNA damage, p53 is accumulated in the nucleus and transactivates downstream genes and induces apoptosis. There are two pathways in p53-dependent apoptosis, the transactivation-dependent and -independent pathway. In this study, we constructed p53-inducible glioblastoma cell lines and analyzed them for the induction of apoptosis and transactivation of p53-downstream genes after the nuclear or cytoplasmic expression of p53. To sequester p53 in the cytoplasm, we used p53 mutant with arginine to glycine substitution at residue 306 (R306G). Wild-type p53 retained the ability to arrest the cell cycle, and a p53 mutant with serine to phenylalanine substitution at residue 121 (S121F), which has a strong ability to induce apoptosis, retained this ability even when both the wild-type and p53 and S121F mutant were exclusively sequestered from the nucleus into the cytoplasm. Notably, cytoplasmically sequestered wild-type p53 and S121F mutant transactivated the downstream genes with distinct expression profiles, and the strong apoptotic ability of S121F was not associated with its transactivation activity. These results underscore the existence of transactivation-independent apoptosis and cytoplasmic function of p53.

Introduction

TP53 tumor suppressor gene is one of the most commonly mutated genes in human neoplasia, and approximately 80% of these mutations are missense mutations (1,2). The gene product, p53 protein, is a nuclear transcriptional activator that is activated by post-translational modification, including phosphorylation and acetylation, in response to DNA-damaging stresses. Activated p53 is stabilized, accumulates in the nucleus and binds to p53-responsive elements (p53REs) in the promoter region of p53-downstream genes (3). Transactivation of these genes, including *p21WAF1*, *MDM2*, *p53AIP1*, *BAX*, *NOXA* and *PUMA*, results in cell cycle arrest and apoptosis.

Most p53 mutants with a single amino acid substitution found in human neoplasm lose the ability to bind to p53REs, and this functional defect is thought to be one of the most important oncogenic events caused by *TP53* mutation (4). Therefore, the translocation of p53 into the nucleus is crucial for normal p53 function. Cytoplasmic sequestration of wild-type p53 was observed in undifferentiated neuroblastoma, breast cancer, retinoblastoma, colorectal carcinoma and glioblastoma cells (5-7). In all these cells, wild-type p53 is inactivated since it is retained in the cytoplasm. Although the precise mechanism underlying the cytoplasmic sequestration remains unclear, several molecular mechanisms have been proposed: i) a mutation in the bipartite sequence of p53 (residues 305 and 306) (8) or a truncated mutation of the nuclear localization motif receptor protein importin-α (9); ii) hyperactive nuclear export by an MDM2-dependent pathway (10); and iii) overexpression of cytoplasmic tethering proteins, such as mortalin (11), cullin 7 (12) and PARC (13). The mutations in the bipartite sequence have been analyzed comprehensively, and these mutants were shown to lose transactivation activity in a yeast functional assay (14).

In contrast to tumor-derived loss-of-function p53 mutants, other types of p53 mutants (super p53s) have a stronger ability to induce apoptosis than wild-type p53. Among these, a p53 mutant with a serine to phenylalanine substitution at residue 121 (S121F) has a distinct affinity to bind p53REs from wild-type p53 (15). S121F induces a more potent apoptosis than wild-type p53 in mammalian cell lines. The transcriptional activity of S121F for downstream genes, however, is less efficient than that of wild-type p53 (16). In addition, different expression profiles among super p53s have been reported (17). These results suggest that transactivation-independent cytoplasmic activity occurs in p53-dependent apoptosis and that S121F may be a diverged mutant with enhanced cytoplasmic activity.

To test this hypothesis, we expressed wild-type and S121F p53 in the nucleus or cytoplasm of p53-null SF126 glioblastoma cells using a p53 mutant with an arginine to glycine substitution at residue 306 (R306G), and analyzed them for induction of apoptosis and transactivation of p53-downstream genes following the p53 induction.

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Key words: p53, tumor suppressor, apoptosis, subcellular localization

Materials and methods

Construction of stable SF126 glioblastoma cell lines. The plasmids pCR259-WTp53, pCR259-S121F and pCR259-R306G were previously constructed (14). pCR259-S121F-R306G was constructed by inserting a small fragment of pCR259-R306G into the *Bsu*36I/*Eag*I site of pCR259-S121F. The small *Nhe*I/*Eag*I fragments of the four pCR259-based plasmids were inserted into the *Nhe*I/*Not*I site of pcDNA5/TON (Invitrogen, Carlsbad, CA, USA). The resulting plasmids were designated pcDNA5/TON-WTp53, pcDNA5/TON-S121F, pcDNA5/TON-R306G and pcDNA5/TON-S121F-R306G, respectively. The stable SF126 cell lines expressing tetracycline-inducible p53 were constructed according to the protocol described in the T-Rex™ System (Invitrogen) using the four pcDNA5/TON-based plasmids. For each category, several stable clones were selected by hygromycin B (100 μg/ml) and two independent stable clones were used.

Western blot analysis. The cell lines were harvested and the cells were resuspended in lysis buffer containing 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA and 1% protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). Cell lysates were centrifuged for 10 min at 4°C. The supernatants were resolved by SDS-PAGE and transferred to PVDF membranes. The membranes were incubated with anti-p53 (FL-393; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and mouse anti-actin (Sigma-Aldrich), followed by incubation with goat anti-rabbit Alexa Fluor 680 IgG (Invitrogen) and goat anti-mouse IR Dye 800 CW IgG (Rockland, Gilbertsville, PA, USA). Expression of both p53 and β-actin was visualized using an Odyssey Infrated Imaging System (LI-COR, Lincoln, NE, USA).

Immunofluorescent analysis. Each cell line was cultured on poly-D lysine-coated Lab-Tek Chamber Slides™ (Nalge Nunc, Rochester, NY, USA) until 70% confluence was achieved. At 24 h after the addition of 10 ng/ml doxycycline or phosphate-buffered solution (PBS), the cells were fixed with acetone-methanol (1:1) and incubated for 20 min at -20°C. After washing with PBS and blocking with 5% non-fat milk in PBS containing 0.05% Tween-20 for 1 h, cells were incubated with FITC-conjugated mouse anti-p53 (DO-1 FITC; Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted at 1:500, stained with propidium iodide and then visualized on an LSM5 PASCAL (Carl Zeiss, Jena, Germany).

Cell proliferation assay. Cells ($4x10^3$) were seeded and incubated in a 96-well plate for 24 h. Doxycycline (10 ng/ml) or PBS was added to the medium and the cells were then cultured until 72 h at 37°C. At 0, 24, 48 and 72 h after the addition of doxycycline, 10 μl of the Cell Counting Kit-8 (Dojin Laboratories, Kumamoto, Japan) was added to each well and the cells were incubated for 2 h at 37°C. Absorbance at 490 nm was read with a microplate reader. Each data point is derived from triplicate experiments. The absorbance values at 24, 48 and 72 h were normalized by the value at 0 h.

Cell cycle analysis by fluorescence-activated cell sorting. Cells ($1x10^6$) were seeded and incubated in a 10-cm culture plate for 24 h, and then incubated in the presence of doxycycline (10 ng/ml). After 24 h, the cells were collected and stained with propidium iodide (50 μg/ml). The stained cells were filtered through 50-μm nylon mesh and analyzed using a Cytomics FC500 (Beckman Coulter, Miami, FL, USA). The subfraction of cells in each phase of the cell cycle was calculated using Multicycle software (Phoenix Flow Systems, San Diego, CA, USA). The average subfraction value of two independent cell lines was calculated.

Quantitative real-time PCR analysis. Total RNA was extracted from cells in the presence or absence of doxycycline using an RNeasy Mini kit (Qiagen, Gaithersburg, MD, USA). RNA (1 μg) was converted to cDNA using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) with random hexamers. TaqMan Gene Expression Assay was performed on the ABI 7500 real-time PCR System (Applied Biosystems) according to the manufacturer's protocol. Assay ID was as follows; GAPDH, Hs99999905_m1; ACTB (β-actin), Hs99999903_m1; MDM2, Hs01066938_m1; p21 (CDKN1A), Hs00355782_m1; BAX, Hs00180269_m1; NOXA (PMAIP1), Hs00560402_m1; PUMA (BBC3), Hs00248075_m1; and P53AIP1, Hs00223141_m1. The expression level of each p53 target gene was collected by either β-actin or GAPDH (data not shown). A relatively induced expression was measured as a ratio of the collected value of doxycycline presence against that of doxycycline absence. Two independent clones were analyzed, and the data were shown as a mean of four replicates with an error bar of standard deviation.

Results

Cytoplasmic sequestration of p53 by R306G mutation. To examine the cytoplasmic activity of wild-type p53 and the S121F mutant, we constructed a series of stable SF126 glioblastoma cell lines. These cells expressed wild-type p53, S121F, R306G or S121F-R306G double mutants in the presence of doxycycline (Fig. 1). To examine the cellular localization of doxycycline-induced p53, an immunofluorescent analysis was performed (Figs. 1 and 2). Both wild-type p53 and S121F localized mostly to the nucleus. In the presence of the R306G mutation in the same p53 molecule, wild-type p53 and S121F were exclusively sequestered from the nucleus to the cytoplasm. To quantify the degree of sequestration of p53, 100 cells of each cell clone were scored as having nuclear, cytoplasmic or both nuclear and cytoplasmic patterns (Fig. 2). Both wild-type and S121F thoroughly localized in the nucleus (>95% of cells), and no cell showed a cytoplasmic pattern. By contrast, both wild-type and S121F with R306G localized in the cytoplasm (>95%) or exhibited cytoplasmic and nuclear patterns. None of the cells expressing p53 exhibited any nuclear pattern. These results indicate that R306G sequestered p53 from the nucleus to the cytoplasm. This result is reasonable since R306G is a mutation in the bipartite sequence of p53 (residues 305 and 306) as described above.

Inhibition of cell proliferation by cytoplasmically localized wild-type p53 and S121F. To examine the effect of cytoplasmically localized wild-type p53 and the S121F mutant on cell proliferation, we cultured two independent cell lines for each

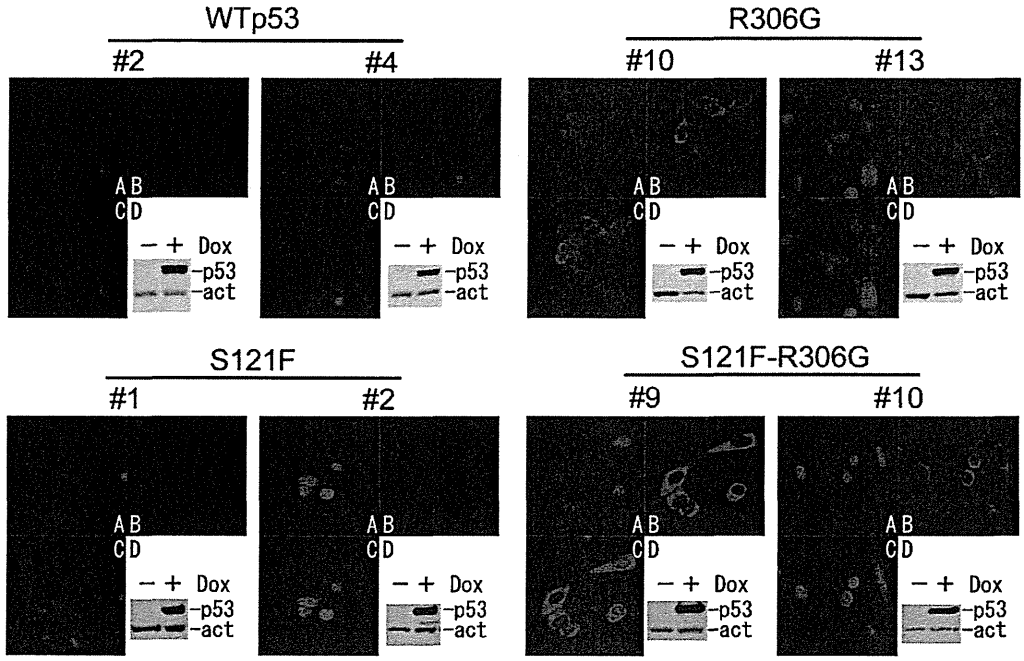

Figure 1. Inducible expression and subcellular localization of p53. Two independent clones in each category were analyzed by immunofluorescent analysis. (A) Propidum iodide; (B) p53; (C) merge of (A) and (B). (D) Western blotting of doxycycline-dependent p53 expression; β-actin was the internal control. WT, wild-type.

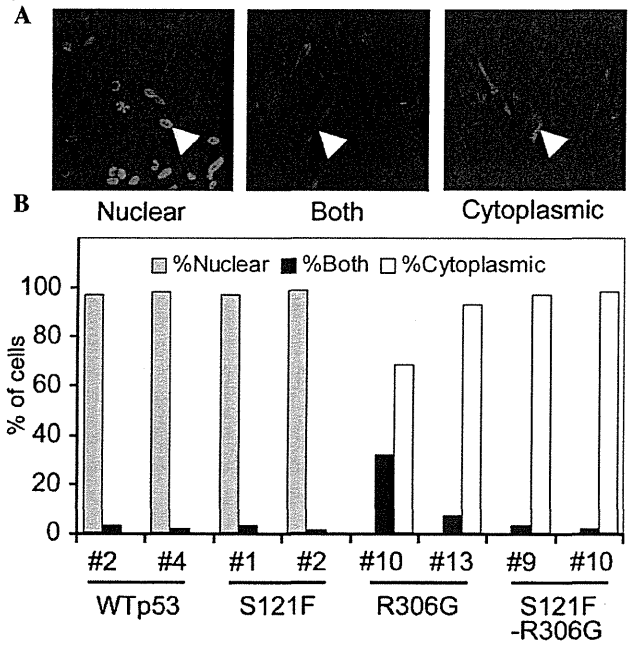

Figure 2. Quantitative analysis of the subcellular localization of wild-type p53 and S121F. (A) Subcellular localization of p53 was visualized as described in Fig. 1, and was classified as nuclear, cytoplasmic or both nuclear and cytoplasmic. (B) One hundred cells were analyzed and the percentage of each category is shown. WT, wild-type

category (wild-type, S121F, R306G, S121F-R306G and null p53) and estimated the viable cells at 24, 48 and 72 h after p53 induction by doxycycline (data not shown). The results at 48 h are shown in Fig. 3A. Although the cytoplasmic sequestration of wild-type p53 considerably disturbed the inhibitory effect against cell proliferation by wild-type p53, some inhibitory effects remained. The cytoplasmic sequestration of S121F did not disrupt the strong inhibitory effect on cell proliferation. These results indicate a cytoplasmic function of p53 on cell proliferation in both wild-type p53 and S121F.

Induction of apoptosis by cytoplasmically localized wild-type p53 and S121F. To evaluate the ability of wild-type p53 and the S121F mutant to induce apoptosis, each cell line was cultured and analyzed for a percentage of cell-cycle phase by fluorescence-activated cell sorting 24 h following the p53 induction by doxycycline (Fig. 3B and C). As shown in Fig. 3B, compared to the null p53 control, wild-type p53 clearly arrested cells at the G1 (49.9-59.4%) and G2 + M (12.3-32.1%) phases of the cell cycle and subsequently reduced the S-phase fraction (33.1-2.3%). The sub-G1 fraction (apoptosis fraction) was only slightly increased (4.7-6.2%) at 24 h, whereas at 48 h a substantial increase was observed (1.7-16.1%; data not shown). The cytoplasmic sequestration of p53 did not affect the cell cycle (G1, 60.5%; G2 + M, 31.6%), but slightly affected both the S phase (5.9%) and the sub-G1 phase (2%). S121F markedly increased the sub-G1 fraction (44.5%) without an increase in the G1 (33.1%) and G2 + M (8.3%) fractions, indicating strong apoptotic induction without cell cycle arrest. The cytoplasmic sequestration of S121F affected the sub-G1 (26.7%), G1 (41.2%) and G2 + M (19.6%) fractions only partially, indicating that a strong induction of apoptosis of S121F was retained despite the cytoplasmic sequestration. These results were consistent with the results of the cell proliferation analysis and again indicated a cytoplasmic function of p53 on both cell cycle arrest and apoptosis.

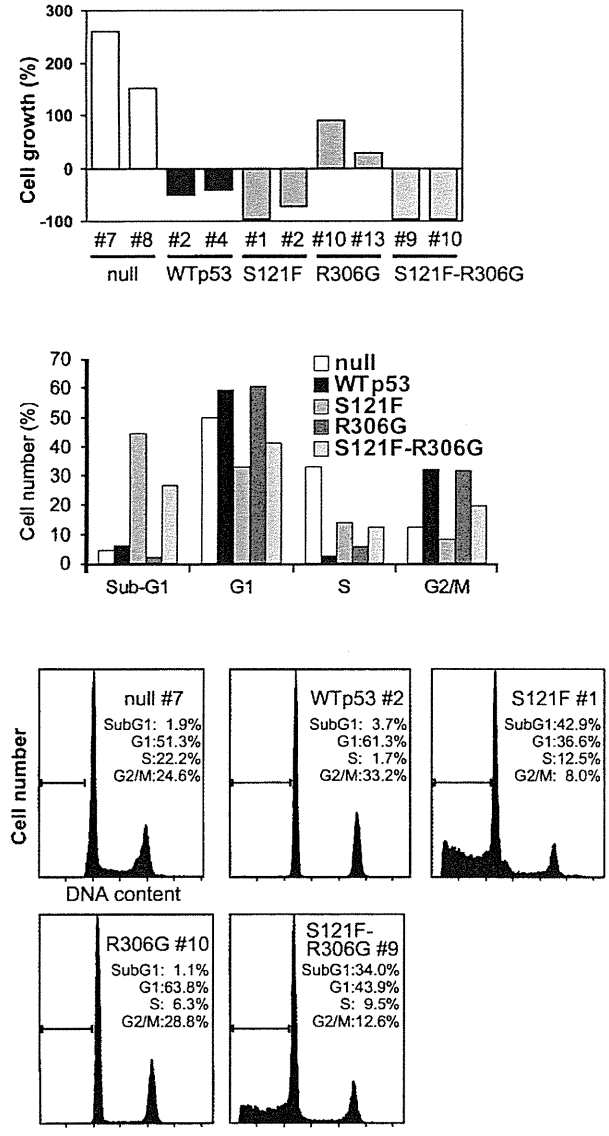

Figure 3. Inhibition of cell proliferation and induction of apoptosis by wild-type p53 and S121F. (A) Cell proliferation assays were performed at 24, 48 and 72 h after the addition of doxycycline. The average of triplicate data at 48 h is shown. (B) Cell cycle analysis by fluorescence-activated cell sorting. The average subfraction value of two independent cell lines at 24 h after the addition of doxycycline is shown. (C) Representative DNA histogram and subfractions at 24 h.

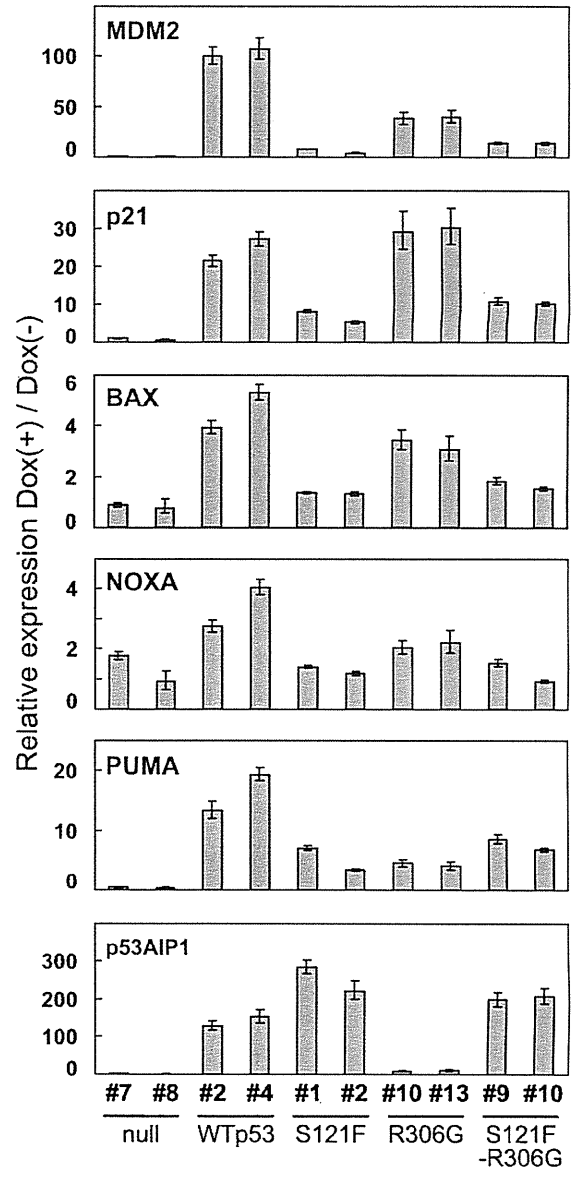

Figure 4. Transactivation of six p53 target genes by quantitative real-time PCR analysis. The expression level of each p53 target gene was collected by β-actin. Relative induced expression is shown as a ratio of the collected value of doxycycline presence against that of doxycycline absence. Two independent clones were analyzed, and the data are shown as a mean of four replicates with an error bar of standard deviation.

Transactivation of p53 target genes by cytoplasmically localized wild-type p53 and S121F. To examine the effect of cytoplasmic sequestration on transcriptional activation by wild-type p53 and S121F, transcripts of the p53-downstream genes, *MDM2*, *p21WAF1*, *BAX*, *NOXA*, *PUMA* and *p53AIP1*, were quantitated by real-time quantitative PCR analysis at 24 h after p53 induction (Fig. 4). Of the six genes, all except *p53AIP1* were less efficiently transactivated by S121F than by wild-type p53. The results showing a lower ability of S121F than wild-type p53 on transactivation were mostly consistent with our previous findings, with the exception of the result of *p53AIP1* (17), which is consistent with the previous hypothesis that S121F may cause a transactivation-independent apoptotic pathway. Notably, the cytoplasmic sequestration of wild-type p53 did not completely inactivate transactivation, but it reduced the level of transactivation in 5 of the 6 target genes (wiht the exception of *p21WAF1*). Of note, the cytoplasmic sequestration of S121F did not change the expression profile of the target genes. The ability of cytoplasmically sequestered S121F on transactivation was also confirmed when the expression level of each p53 target gene was collected by GAPDH (data not shown).

Discussion

The cytoplasmic sequestration of p53 did not completely inactivate p53 function, suggesting the cytoplasmic function of p53. This finding may be the reason that mutations on the