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ORIGINAL PAPER

Prediction of response and prognostic factors for Ewing family of tumors in a low incidence population

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Abstract
Purpose There is some unknown reason Ewing family of tumors (EFTs) is much less common on Asia and Africa than in the Western Caucasian population. This study analyzed the prediction of response and prognostic factors for Ewing family of tumors (EFTs) in an Asian population with a low incidence.
Methods We retrospectively reviewed 94 patients with EFTs between 1978 and 2006. Fifteen patients received local therapy only. Statistical analyses were performed for 79 patients, including those who received systemic chemotherapy, to identify factors related to chemotherapy responsiveness, event-free survival, and overall survival.
Results Of the 79 patients whose records were analyzed, the 5-year event-free rate and overall survival (OS) rate were 41 and 54%, respectively. The response rate to first-line chemotherapy was 61% in 70 patients with assessable lesions. A significant predictor of response was existence of a non-pelvic primary tumor ($P = 0.04$). Significant prognostic factors for OS were age, performance status, and metastases at the time of diagnosis ($P < 0.01$, respectively). Fifty-four patients had disease progression or recurrence after first-line treatment. The time to progression was 3.4 months after salvage treatment. Progression during first-line treatment was significantly associated with time to progression after salvage treatment ($P = 0.01$). All patients treated without chemotherapy in first-line treatment were recurred with poor prognosis.
Conclusion A non-pelvic primary tumor was a favorable predictor of responsiveness to chemotherapy. Chemoresistant patients might less benefit from second line chemotherapy. Chemotherapy in first-line treatment should not be omitted, even if primary tumor was extirpated completely.

Keywords Ewing family of tumors · Predictive factor · Prognostic factor · Response · Chemotherapy · Asian population

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Introduction

The Ewing family of tumors (EFTs) is a group of rare malignant tumors that mostly arise in bone, although a significant proportion of patients have soft tissue primaries. EFTs share histological, immunohistochemical and

cytogenetic characteristics; in the past, they have also been identified as Ewing sarcomas of bone or soft tissue, malignant peripheral primitive neuroectodermal tumors, primitive neuroepitheliomas or Askin tumors. (Miser et al. 1987) The vast majority of these tumors arise in children and young adults. The treatment of EFTs consists of a multidisciplinary approach including surgery, radiotherapy, and combination chemotherapy. During the past three decades, the prognosis of patients with EFTs has improved considerably, as shown in several clinical trials, mainly because of improved chemotherapy regimens (Burgert et al. 1990; Grier et al. 2003; Jurgens et al. 1988; Nesbit et al. 1990; Paulussen et al. 2001; Sluga et al. 2001).

For reasons that remain unknown, EFTs rarely occurs in Asian and African-American populations. The incidence of EFTs in Asian populations is lower than in Western populations (Guo et al. 1999) According to the Japanese Musculoskeletal Tumor Committee, 473 patients with EFTs of bone were registered during 1972–2003, the population of Japan is 120 million (The JOA Musculo-Skeletal Tumor Committee 2003a) Registration of malignant soft tissue tumor had starting from 2003, and 11 patients with EFTs of extra-osseous primary were registered in 2003. (The JOA Musculo-Skeletal Committee 2003b) Only three reports on the clinical outcome of Japanese patients with EFTs have been made (Obata et al. 2007; Ozaki et al. 2002; Yamada et al. 2006). It is controversy that the prognosis of patients with EFTs were relatively poorer compared with the major Euro-American studies. However, the recent report described the clinical outcome of patients with localized EFTs of bone were virtually equivalent (Obata et al. 2007).

Several clinical and biologic characteristics can assist in determining the prognosis and directing the intensity of therapy. These characteristics include age, primary tumor location and size, the presence or absence of metastases, the serum lactate dehydrogenase level, and the response to therapy (Bacci et al. 2000; Catterill et al. 2000; Obata et al. 2007; Rodriguez-Galindo et al. 2003; Sluga et al. 2001). Although chemotherapeutic regimens and treatment strategies based on prognostic factors have been advanced, previous reports from developing countries indicate that similar results were not obtained in non-western population (Cardenas-Cardos et al. 1999; Jenkin et al. 2002; Villarroel et al. 1997) Thus, previously reported prognostic factors may not have the same influence on clinical outcome in patients belonging to populations with a low incidence, even if developed countries.

The aim of this study was to analyze the clinical characteristics and prognostic factors of EFTs in an Asian population with a low incidence.

Methods

Patients

We retrospectively reviewed the records of 94 patients with EFTs; all records were retrieved from a database of patients treated at the National Cancer Center Hospital (Tokyo, Japan) between September 1978 and April 2006. Two experienced musculo-skeletal pathologists (T.H. or K.S.) had diagnosed or reviewed all biopsy or surgical specimens after performing histological or immunohistochemical examinations. Molecular genetic studies such as PCR or FISH had been performed in cases with available specimens (Yamaguchi et al. 2005).

Treatment

In the present study, all the patients had received single modality therapy or various combinations of multi-modality therapy. Therapy for local control was individualized: surgery alone, radiation therapy alone or a combination of surgery and radiotherapy was performed, as suitable. Various systemic chemotherapy regimens were used. The 94 patients were classified into four groups according to their first-treatment systemic therapy regimen: group I consisted of patients treated with systemic chemotherapy, including vincristine, doxorubicin, and cyclophosphamide with or without actinomycin D; group II consisted of patients treated with multi-drug chemotherapy regimens, including vincristine, doxorubicin, actinomycin D and ifosphamide (VAIAdr) or vincristine, doxorubicin, and cyclophosphamide, alternating with ifosfamide and etoposide (VAdrC/IE); group III consisted of patients treated with systemic treatment including various chemotherapy regimens (Meyers et al. 1995, 1998) (T9 protocol, $n = 1$; T11 protocol, $n = 3$; T12 protocol, $n = 1$; vincristine plus etoposide plus cyclophosphamide plus cisplatin, $n = 2$; vincristine plus ifosfamide plus cisplatin, $n = 1$; doxorubicin plus cisplatin, $n = 2$; and etoposide plus cisplatin, $n = 1$); group IV consisted of patients receiving local therapy, including surgery or radiotherapy, without systemic chemotherapy. Some patients received high-dose consolidation therapy and peripheral blood stem cell transplantations or autologous bone marrow transplantations. Salvage treatment after recurrence was classified in the same manner as the first-line treatments.

Response assessment

Objective responses were evaluated according to the WHO criteria (World Health Organization 1979) Patients with no bidimensionally measurable lesions were considered ineligible for the objective response evaluation and were classified as not evaluable (NE). Systemic chemotherapy was

discontinued if clinical or radiological evidence of progression was present.

Statistical analysis

Event-free survival was measured from the first day of treatment until the observation of evidence of the first local, regional, or distant recurrence or progression of the tumor or the final day of follow-up without recurrence. Time to progression was measured from the first day of salvage treatment until disease progression or the final day of follow-up without disease progression, and the overall survival was measured from the first day of treatment until death or the final day of follow-up.

After excluding patients treated with local therapy only, pretreatment and treatment variables were investigated for their relation to event-free survival, and overall survival using both univariate and multivariate Cox regression analyses. The variables were selected after considering the possible effects on prognosis indicated by our experience and previous investigations (Bacci et al. 2000; Catterill et al. 2000; Obata et al. 2007; Rodriguez-Galindo et al. 2003; Sluga et al. 2001). The variables were followed as: gender (male versus female), age (<15 years vs. 15≤), Eastern Cooperative Oncology Group performance status (0 vs. 1≤), primary tumor type (bone versus soft-tissue), primary tumor site (non-pelvic as extremities or axial sites versus intra-thoracic or abdominal), primary tumor size (≤8 cm vs. 8<), disease type (localized versus metastatic), serum lactate dyhydrogenase level (elevated vs. normal or unknown), serum neuron-specific enolase level (elevated vs. normal or unknown). The median event-free survival, time to progression, and overall survival were estimated using the Kaplan–Meier method. We used univariate and multivariate logistic regression analysis to assess the relationship between pretreatment and treatment variables and the response to chemotherapy. A statistical analysis was also performed to identify factors associated with the time to progression in patients treated with salvage therapy. The statistical analyses were performed using SAS, version 9.1.3 (SAS Institute, Cary, NC, USA), and the significance level was set at $P = 0.05$ (two-sided).

Results

Patient characteristics

Fifty-five men and 39 women with a median age at the time of diagnosis of 22 years (range 2–70 years) were enrolled in this study. The median Eastern Cooperative Oncology Group performance status was 0 (range 0–2). Forty-nine patients (52%) had primary tumors in bone and the others had primary tumors in soft tissue. The primary tumor sites are listed in Table 1. Sixty-four primary tumors (68%) were located in the trunk, and the remaining 30 tumors were located in extremities. The median largest dimension of the primary tumor was 7 cm (range 1.5–29 cm). Twenty-two patients had metastasis at the time of diagnosis. The median number of sites involved in each of the 22 patients with metastases was 2 (range 1–4).

Treatment

Of the 94 patients, 79 had received chemotherapy as their first-line treatment and the remaining 15 patients had been treated without chemotherapy (2 patients had undergone a combination of surgery and radiation therapy, 11 patients had undergone surgery, and 2 patients had undergone radiation therapy). When grouped according to their chemotherapy regimen, 4 patients received group I treatments, 62 patients received group II treatments, and 13 patients received group III treatments. Twenty-two patients received high-dose chemotherapy as their first-line chemotherapy treatment (1 patient in group I, 20 patients in group II, and 1 patient in group III). Among the patients that received chemotherapy, 9 patients received chemotherapy in an adjuvant setting (7 patients in group II, including 2 patients who received high-dose chemotherapy; and 2 patients in group III). Among the 79 patients who received chemotherapy, 23 patients also underwent a combination of surgery and radiation therapy, 26 patients underwent surgery, 17 patients underwent radiation therapy, and 13 patients did not undergo local therapy.

Response to chemotherapy

The response rate of 70 patients whose response to chemotherapy was assessable was 61% [95% confidence interval

Table 1 Sites of primary tumors in 94 patients with EFT

Tumor location	N	%
Osseous	49	
Skull	3	3.2
Trunk	13	13.8
Pelvic	14	14.9
Upper extremities	8	8.5
Lower extremities	11	11.7
Extra-osseous	45	
Head and neck	4	4.2
Trunk	8	8.5
Intra-thoracic	5	5.3
Intra-abdominal	17	18
Upper extremities	3	3.2
Lower extremities	8	8.5

(CI): 50 to 73%; 6 complete responses (CR), 37 partial responses (PR), 17 no changes (NC) or NE, and 10 progressive diseases (PD)]. Performance status, primary tumor size, and primary tumor site were significantly associated with response in univariate analysis. A multivariate logistic regression analysis indicated that the only significant predictor of response was a non-pelvic primary tumor [hazard ratio (HR), 3.01; 95% CI, 1.02–8.91; $P = 0.04$).

Outcome

Of the 79 patients, the 5-year event-free rate and overall survival rate were 41 and 54%. The median event-free survival and overall survival were 2.0 and 6.1 years, respectively (Fig. 1). Among the 57 patients without metastasis, the 5-year event-free rate and overall survival rate were 47 and 68%, respectively. And the 22 patients with metastasis, the 5-year event-free rate and overall survival rate were 30 and 37%, respectively. Age, primary tumor size, primary tumor site, and disease type were significantly associated with event-free survival in univaliate analysis. And a multivariate Cox regression analysis disclosed that metastasis at the time of diagnosis was a significant adverse prognostic factor of event-free survival ($P = 0.02$, Table 2). Age, primary tumor size, performance status, and disease type were significantly associated with OS in univariate analysis. And Cox regression analysis disclosed significant adverse prognostic factors for OS were an age ≥15 years, a performance status ≥1, and metastasis at the time of diagnosis, respectively ($P < 0.01$, Table 2).

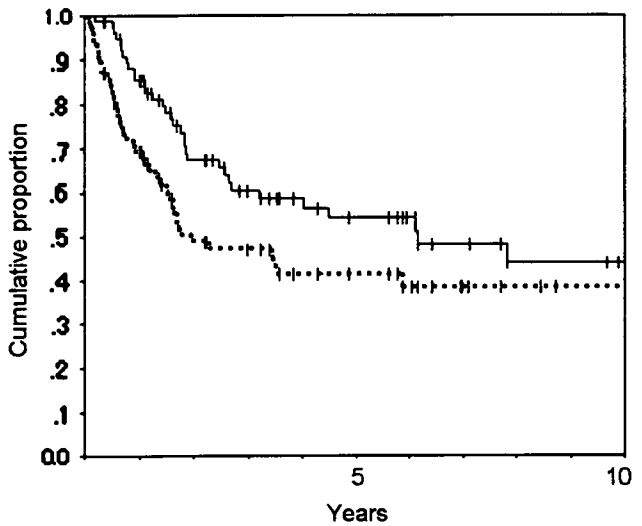

Fig. 1 Kaplan–Meier analysis of event-free survival (*dotted line*) and overall survival (*solid line*) in 79 patients who received chemotherapy. The *vertical bars* indicate censored cases

Salvage treatment

Of the 94 patients, 54 had received second line treatment for disease progression or recurrence. The median number of disease sites was 1 (range 1–3). The most common disease sites were the lung ($n = 24$), bone ($n = 15$), primary tumor site ($n = 11$), lymph node ($n = 7$), liver ($n = 5$), peritoneum ($n = 4$), soft-tissue ($n = 4$), and brain ($n = 2$). The majority of patients (85%) had received chemotherapy, and eight patients had received local therapy—including one patient who had undergone surgery. When grouped according to their chemotherapy regimen, 4 patients received group I treatments, 21 patients received group II treatments, and 21 patients received group III treatments, including 5 patients who received high-dose chemotherapy as a second line treatment (2 patients in group II, 3 patients in group III). The time to progression was 3.4 months after second line treatment. Progression during first-line treatment was significantly associated with time to progression after second line treatment (HR, 2.56; 95% CI, 1.21–.39; $P = 0.01$). The duration of the event-free survival was not significantly associated with the time to progression.

After second line treatment, 53 patients had developed progression. In these patients, 37 patients had received third line treatment including 20 patients had been treated by chemotherapy, 1 patients received group I treatments, 3 patients received group II treatments, and 16 patients received group III treatments. The other 17 patients had been treated by local therapy, 13 patients received radiation therapy, 4 patients received surgery. After third line treatment, 12 patients had been treated with chemotherapy and 7 patients had received local therapy in forth line treatment. Although 5 patients had received further treatment, all heavily treated patients had died.

Table 2 Multivariate analyses in 79 patients treated with chemotherapy

Variables	Event-free survival			Overall survival		
	HR	95% CI	*P* value	HR	95% CI	*P* value
Metastasis at the time of diagnosis	2.34	1.16–4.72	0.02	2.71	1.31–5.65	<0.01
Age ≥ 15	–	–	–	4.76	1.60–14.2	<0.01
PS ≥ 1	–	–	–	2.91	1.55–5.43	<0.01

HR Hazard ratio, 95% CI, 95% confidence interval, *PS* ECOG performance status

Patients treated without chemotherapy in first-line treatment

In this study, 15 patients did not receive chemotherapy as part of their first-line treatments. The median age of these patients was 39 years (range 20–53 years). None of these patients had metastasis, and most of the patients (87%) had extra-osseous primary tumors. Two-thirds of the patients had a primary tumor size ≤80 mm. Nine patients had pelvic primary tumors, and only two patients had primary tumors in their extremities. A univariate analysis indicated that age, percentage of extra-osseous primary tumor sites, and percentage of pelvic primary tumor sites were significantly different among these 15 patients, compared with the other 79 patients. Two-thirds of these cases were admitted during the last 5 years of the study period. Regrettably, all patients had recurred, 13 patients had developed systemic recurrence and the other had local recurrence. The median time to recurrence was 9.4 months. The most common systemic disease sites was lung ($n = 6$), liver ($n = 5$), bone ($n = 4$), lymph nodes ($n = 3$), and miscellaneous ($n = 2$). In 13 patients with systemic recurrence, all patients had received group II treatment, including 5 patients had received additional local therapy (3 patients in radiation therapy and 2 patients in surgery). Despite chemotherapy in group II was performed for systemic recurrence, 12 of 13 patients had died and only 1 patient survived over 2 years after systemic recurrence. In patients with local recurrence (intra-thoracic and lower extremities in each patient), they had received group II treatment. Although patient with intra-thoracic tumor had progression again, the patient treated with group III treatment and survives over 2 years after local recurrence. The other patient survives over 1 year without recurrence. The median overall survival of these patients was 2.9 years, significantly shorter than that of the other 79 patients ($P = 0.03$, log-rank test).

Discussion

This retrospective study revealed that predictive factor of response in first-line chemotherapy and the progressive disease in first line chemotherapy was associated with outcome of second line treatment. The prognostic factors and prognosis in EFTs patients with low incidence may be similar to that of patients in previous reports. This study suggested that appropriate timing of systemic chemotherapy was important to achieve good prognosis, even if local therapy as surgery had successful in patients with localized disease of EFTs.

The incidence of EFTs in Asian countries is generally lower than that of Caucasian populations (Guo et al. 1999). Previous studies have described the background characteristics and treatment results in Japanese populations, which have a low incidence of EFTs (Obata et al. 2007; Ozaki et al. 2002; Yamada et al. 2006). Two studies of EFTs were small sample size less than 20 patients, and the largest study of EFTs of bone included 243 patients. Recent study suggested the there was no considerable differences in clinical background in patients with EFTs of bone. (Obata et al. 2007) When comparing the present study with reports from Western countries, the present study showed a higher frequency of soft-tissue primary tumors. Our hospital is a specialist orthopedic cancer referral center, and the present study describes no small sample to be reported in an Asian population. Differences among populations are difficult to judge because of selection biases. Previous studies reporting different frequencies of genetic aberrations may explain the different incidences and prognoses among populations (de Alava et al. 1998; Ozaki et al. 2002). We had insufficient material for statistical analyses of any possible relation between genetic alteration in our patients' tumors and their prognosis.

Limitations of this study were retrospective nature and the considerable heterogeneity of the treatment regimens. However, the majority of the patients had received multidrug-chemotherapy regimens consisting of VAIAdr or VAdrC/IE. Thus, the treatment outcome among the patients who received chemotherapy, adjusted for the presence of metastasis, was probably representative.

Advances in systemic chemotherapy have generally contributed to the improvement of treatment results (Sluga et al. 2001). In the present study, some of the patients were treated without chemotherapy, within the last 5 years. These patients were relatively older and had higher incidences of soft-tissue and pelvic primary tumors. Although none of these patients had metastatic disease at the time of their diagnosis, the prognosis of the patients without chemotherapy was clearly poorer. Present study demonstrates that even in patients with small primary tumors had been completely extirpated with sufficient margin, appropriate timing of systemic chemotherapy has an important role for cure. Population with low incidence leads to less experienced physicians. However, EFTs arouses from various site and adult patients with atypical primary tumors have a particularly poor prognosis. Therefore, promoting of multimodality treatment strategy and education for physicians will improve clinical outcome.

Although it is not a true prognostic factor that can be assessed at the time of diagnosis, the radiologic response to initial chemotherapy appeared to be a strong predictor of overall survival. (Sluga et al. 2001) The present study indicated that patients with non-pelvic primary tumors responded well to chemotherapy. Thus, this favorable subset of patients with EFTs may have a better prognosis. Previous report attributed the poor prognosis in pelvic primary

tumors to difficulties in local therapy, especially surgical resection (Catterill et al. 2000). This problem associated with primary site will not be solved easily. Biological and molecular characteristics may be explored among patients with pelvic primary tumors, and new molecular targeted therapy should be developed to prolong survival in this group.

The prognosis of patients with relapsed disease is poor. Many reports have investigated chemotherapy regimens that can be effective in producing temporary disease control in second line settings (El Weshi et al. 2004; Shankar et al. 2003) In addition, high-dose chemotherapy and peripheral blood stem cell transplantation have been studied as second line treatments for patients with relapsed EFTs (Burdach et al. 1993; Stewart et al. 1996). Although a subgroup of patients benefit from these treatments, both the role and indications for second line treatment remain uncertain. A previous study reported that patients with a short first remission derived little benefit from second line therapy. (Shankar et al. 2003) Although the duration of remission was not associated with the time to progression in the present study, the patients who had progressive diseases during their first-line treatments were significantly associated with a poor outcome after second line treatment. Therefore, patients with these poor prognostic factors should receive palliative therapy, rather than aggressive second line treatment.

Previous reports mostly including EFTs of bone have analyzed prognostic factors such as metastatic disease, patient age, tumor size, and pelvic primary tumor location (Bacci et al. 2000; Catterill et al. 2000; Obata et al. 2007; Rodriguez-Galindo et al. 2003; Sluga et al. 2001). Although the outcome of treatment in the present study was slightly worse than that reported in Western populations, the prognostic factors for event-free and overall survival identified in the present report were similar to those mentioned in previous reports. The current staging system (localized or metastatic) is clearly important for categorizing patients. In addition, risk-adapted strategies using prognostic factors should help to clarify the best treatment strategy in each risk group.

Much remains to be done to improve the outcome of patients with EFTs, especially in countries with low incidences. Because of the difficulty of conducting clinical trials in populations with low incidences, joining nationwide treatment study or international study group would be important to develop a common staging system and common treatment guideline in worldwide.

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Clinical Efficacy of S-1 in Pretreated Metastatic Breast Cancer Patients

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Background: S-1, an oral fluoropyrimidine carbamate, is an active and well-tolerated agent against solid cancer. However, the clinical efficacy of S-1 in patients with metastatic breast cancer has not been determined.
Methods: We retrospectively evaluated the efficacy of S-1 and identified its adverse effects in patients with metastatic breast cancer who had failed to respond to prior chemotherapy regimens. All the patients were treated at the National Cancer Center Hospital and received S-1 twice daily at a dose of 80 mg/m^2 for 4 weeks, followed by a 2-week rest interval.
Results: Between 2003 and 2007, 37 women with metastatic breast cancer received S-1 as a third line or greater chemotherapy regimen. All the patients had been previously treated with both anthracyclines and taxanes prior to S-1 chemotherapy. The median order of S-1 administration was as a fifth-line treatment, and 23 patients (62%) received S-1 as their final anticancer drug. One (3%) partial response and two (5%) stable diseases were observed. The median time to progression (TTP) was 84 days. Grade 2 adverse events, such as diarrhea, stomatitis and neutropenia occurred in 5 (16%), 1 (3%) and 1 (3%) patients, respectively.
Conclusions: S-1 was safety administered to heavily treated metastatic breast cancer patients with limited efficacy. Further evaluation of S-1 is necessary to elucidate its clinical role in breast cancer treatment.

Key words: S-1 – metastatic breast – cancer – chemotherapy

INTRODUCTION

Treatment of patients with metastatic breast cancer (MBC) aims to prolong survival while relieving symptoms and maintaining a good quality of life (QOL).

Capecitabine is an orally administered fluoropyrimidine that has been reported to be effective in both monotherapy and combination therapy regimens. Capecitabine as a single agent produced an overall response rate (RR) of 29% and a median time to disease progression of 4.6 months in large phase II trials in taxane-pretreated MBC patients (1–3). Since capecitabine can sustain the QOL of MBC patients, it has been widely used as a third-line or subsequent chemotherapy regimen for heavily treated patients.

On the other hand, S-1 is another orally administered fluorinated pyrimidine that has been reported to be a well-tolerated and active agent against solid cancers. In a phase II study of S-1, the RR was 41.7% and the median survival time was 872 days among taxane-pretreated patients with MBC; S-1 has been approved in Japan as a salvage chemotherapy for patients who have received anthracycline and taxane (4,5). In addition, S-1 has been used mainly for the treatment of cancers of the digestive tract (6–8), and its efficacy is well known. However, the clinical usefulness of S-1 in patients with MBC is uncertain. Here, we describe the efficacy and tolerability of S-1 in a clinical setting.

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PATIENTS AND METHODS

PATIENTS

A retrospective analysis was performed on patients with MBC who received S-1 monotherapy between January 2003 and December 2006 at the National Cancer Center Hospital (NCCH). The patient population was identified from a

database at the NCCH. All the patients had received chemotherapy previously. They were followed up until death or, if they were still alive, to their last visit prior to March 2007.

The best response for each patient was assessed according to the WHO criteria (8). A complete response (CR) was defined as the disappearance of all clinical and radiographic evidence during two observations performed at least 4 weeks apart. A partial response (PR) was defined as a decrease of 30% or more in the sum of the products of the biperpendicular diameters of measurable lesions. Stable disease (SD) was defined as a <30% decrease and a <25% increase in the sum of the products of the biperpendicular diameters of measurable lesions and no appearance of new lesions; these conditions had to be maintained for at least 12 weeks. Progressive disease was defined as a greater than 25% increase in the sum of the products of the biperpendicular diameters of measurable lesions or the appearance of new lesions. The clinical benefit rate was defined as the proportion of patients who achieved either a CR, PR or SD. The National Cancer Institute common toxicity criteria (9) were adopted to determine toxicity.

Treatment

S-1 was administered orally twice daily (80 mg/m^2) for 28 days followed by 14 days of rest. Treatment was continued until disease progression, unacceptable adverse effects or withdrawal of the patient's consent. In the case of Grade 2 or worse toxicity, S-1 administration was interrupted and not resumed until the toxicity had resolved or improved to Grade 1.

The time to progression (TTP) was calculated from the day of commencement of S-1 administration until the day of documented progression. Overall survival (OS) was calculated from the start date of S-1 to the date of death from any cause. TTP and OS were analysed according to the Kaplan–Meier estimates.

RESULTS

Thirty-seven patients received S-1 as a greater than second-line chemotherapy for MBC between January 2003 and December 2006 at NCCH. Table 1 shows the patient's characteristics. The median age was 49 (28–70) years. The Eastern Cooperative Oncology Group (ECOG) performance statuses of the patients were all <2. The sites of metastatic disease were the bone and/or soft tissue in only six patients (16%) and involved visceral sites in 31 patients (84%). Table 2 shows the chemotherapy regimens that were administered prior to S-1. The median number of chemotherapy regimens used before the administration of S-1 including adjuvant and neoadjuvant treatments, was 4, and 23 patients (62%) received S-1 as their final chemotherapy regimen. All the patients had previously received both anthracyclines and taxanes, 13 patients (35%) had received vinorelbine and 11 patients (30%) had received oral 5FU-derivatives prior to the administration of S-1. All the patients who had responded to treatment had exhibited adequate progression-free intervals from the prior taxane administration until the subsequent taxane administration. Three patients received the same taxane regimen twice, once as adjuvant chemotherapy and the second time in combination with Trastuzumab after recurrence. Prior oral 5FU-derivatives included in other regimens were CMF (five patients), UFT (five patients), 5'DFUR (five patients) and CPT-11 (one patient). Sixteen patients (43%) with ER-positive diseases had received hormone therapy, and 13 patients (35%) with HER2-positive diseases had received Trastuzumab as a monotherapy or in combination with taxane or vinorelbine.

Table 1. Patient characteristics

	No. of patients (n = 37)	% of patients
Median age (years; range)	49 (28–70)	
Metastatic sites involved		
Bone/Soft tissue	6	16
Visceral	31	84
Oestrogen receptor		
Positive	16	43
Negative	21	57
Progesteron receptor		
Positive	17	46
Negative	20	54
HER2/neu status		
Positive	13	35
Negative	24	65

Table 2. Prior chemotherapy

Prior chemotherapy	No. of patients (n = 37)	% of patients
No. of regimens used		
2/3/4/5/6/7/8	4/10/10/4/2/0/3	
Median (range)	4 (2–8)	
Neoadjuvant chemotherapy	6	16
Adjuvant chemotherapy	17	46
S-1 was the last regimen	23	62
Prior chemotherapy		
Anthracycline	37	100
Taxane	37	100
Vinorelbine	13	35
Capecitabine	1	3

The median number of administration days was 70 (6–415 days). The RR was 3%, with no cCR and 3% (1/37) PR. The overall clinical benefit rate (CR, PR and SD for more than 6 months) was 8% (3/37). The median TTP was 84 days (range, 6–415) (Fig. 1; note that a colour version of this figure is available as supplementary data at http://www.jjco.oxfordjournals.org). The median OS from the start of S-1 treatment was 284 days (range, 14–1511), and six patients (16%) were still alive at the last follow-up. Nine patients (24%) received S-1 for more than 100 days. Six out of these nine patients had visceral involvement. Two out of seven patients had oestrogen receptor-positive diseases and four of them were HER2-positive.

Overall, S-1 was well tolerated. Table 3 shows the adverse events in response to S-1 chemotherapy. Toxicities of Grade 3 or more were not reported. The most common toxicities arising from S-1 administration were diarrhea (33%) and nausea (30%). Most of the adverse events were Grade 1, and none of the S-1-related adverse events were fatal. The most frequent reasons for treatment discontinuation were disease progression (30 patients, 81%) and adverse event (seven patients, 19%). The adverse events that were encountered were Grade 2 diarrhea (five cases), Grade 2 stomatitis (one case) and Grade 2 neutropenic fever (one case).

DISCUSSION

The number of patients with MBC who have been pretreated with anthracyclines and/or taxanes are increasing. However, the optimal chemotherapy for patients with MBC who have been pretreated with both anthracyclines and taxanes has not been determined. These patients require palliative therapy that offers a chance of prolonging life with minimal toxicity according to the antitumor response and the alleviation of tumor-related symptoms.

In this study, S-1 chemotherapy produced a 3% RR and an 8% rate of clinical benefit in previously treated patients

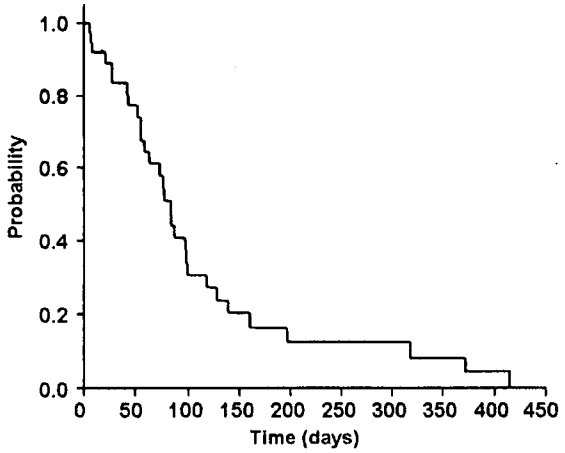

Figure 1. Kaplan–Meier curve for time to progression (TTP). Median TTP was 84 (range 6–415) days.

Table 3. Treatment-related adverse event of TS-1

	Gradre 1 (%)	Grade 2 (%)
Diarrhea	7 (19)	5 (14)
Stomatitis	5 (14)	1 (3)
Nausea/vomitting	11 (30)	0 (0)
Neutropenia	1 (3)	1 (3)
Disorder of liver function	2 (6)	0 (0)

with MBC who were refractory to both anthracyclines and taxanes. The median TTP was 84 days, and 24% of the patients received S-1 for more than 100 days. These results were worse than those reported in clinical trials. This discrepancy is probably because 11 patients had received other 5FU-derivatives prior to S1, the median order of S-1 administration was fifth line (most of the patients received S-1 chemotherapy as their final treatment), and most of the patients had multiple metastatic sites (84% had visceral metastases). The toxicity of S-1, however, was mild in these heavily treated patients, and S-1 is considered to be a feasible palliative chemotherapy in heavily treated MBC patients.

Several oral 5FU-derivatives have been used to treat MBC, but only S-1 and capecitabine have been tested in taxane-refractory MBC patients (10). The treatments were administered based upon physicians' decisions, but the reason why S-1, and not capecitabine, was selected in this study population is unclear. S-1 is a fluoropyrimidine that consists of 1-(2-tetrahydrofuryl)-5-fluorouracil (FTO), a pro-drug of 5-FU, and two other compounds, 5-chloro-2, 4-dihydroxypyrimidine (CDHP; gimestat) and potassium oxonate (OXO; otastat), in molar proportions of 1:0.4:1. CDHP is an inhibitor of dihydropirimidine dehydrogenase (DPD), which degenerates 80% of 5-FU in the liver and maintains the 5-FU level above a minimal effective concentration level. On the other hand, capecitabine is converted to 5'-DFUR either by human carboxyesterase (CE) or cytidine deaminase (CD), which is mainly localized in the human liver. 5'-DFUR is converted to the active form of 5-FU by thymidine phosphorylase (dThdPase) in human tumors. Low CE and CD activity levels are thought to protect the digestive wall and bone marrow from capecitabine toxicity.

Clinically, the reported RRs of capecitabine and S-1 in taxane-pretreated MBC patients are similar, but the toxicity profile seems to be different. Relatively severe diarrhea (14%, Grade 3) and hand-foot syndrome (10%, Grade 3) were observed in a phase II study for capecitabine (2,3), whereas the incidence of Grade 3 or severe diarrhea was relatively low (0.9%) and no hand-foot syndrome was observed in a phase II study of S-1 for MBC (4). A direct comparison of capecitabine and S-1 monotherapy is surely necessary, and since the antitumor activity of capecitabine might be relatively low in tumor cells with high DPD levels, an evaluation of the efficacy of S-1 after progression with

capecitabine or in tumors with high DPD expression levels is warranted.

Moreover, while the efficacy of capecitabine in combination therapy with other cytotoxics (11–16) or as first-line chemotherapy (17) has already been reported, few evidence of the efficacy of S-1 in combination therapy or first-line chemotherapy is available (18,19). The efficacy and safety of S-1 in combination with molecular-targeted drugs, such as antibodies and small molecule tyrosine kinase inhibitors, are also unknown. Further studies are thus required to elucidate the clinical role of S-1 in the management of breast cancer patients.

Conflict of interest statement

None declared.

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Synergistic antitumor activity of the novel SN-38-incorporating polymeric micelles, NK012, combined with 5-fluorouracil in a mouse model of colorectal cancer, as compared with that of irinotecan plus 5-fluorouracil

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The authors reported in a previous study that NK012, a 7-ethyl-10-hydroxy-camptothecin (SN-38)-releasing nano-system, exhibited high antitumor activity against human colorectal cancer xenografts. This study was conducted to investigate the advantages of NK012 over irinotecan hydrochloride (CPT-11) administered in combination with 5-fluorouracil (5FU). The cytotoxic effects of NK012 or SN-38 (an active metabolite of CPT-11) administered in combination with 5FU was evaluated *in vitro* in the human colorectal cancer cell line HT-29 by the combination index method. The effects of the same drug combinations was also evaluated *in vivo* using mice bearing HT-29 and HCT-116 cells. All the drugs were administered i.v. 3 times a week; NK012 (10 mg/kg) or CPT11 (50 mg/kg) was given 24 hr before 5FU (50 mg/kg). Cell cycle analysis in the HT-29 tumors administered NK012 or CPT-11 *in vivo* was performed by flow cytometry. NK012 exerted more synergistic activity with 5FU compared to SN-38. The therapeutic effect of NK012/5FU was significantly superior to that of CPT-11/5FU against HT-29 tumors ($p = 0.0004$), whereas no significant difference in the antitumor effect against HCT-116 tumors was observed between the 2-drug combinations ($p = 0.2230$). Cell-cycle analysis showed that both NK012 and CPT-11 tend to cause accumulation of cells in the S phase, although this effect was more pronounced and maintained for a more prolonged period with NK012 than with CPT-11. Optimal therapeutic synergy was observed between NK012 and 5FU, therefore, this regimen is considered to hold promise of clinical benefit, especially for patients with colorectal cancer.

Key words: NK012; SN-38; 5-fluorouracil; drug delivery system; colorectal cancer

The 5-year survival rates of colorectal cancer (CRC) have improved remarkably over the last 10 years, accounted for in large part by the extensively investigated agents after 5-fluorouracil (5FU). Irinoetcan hydrochloride (CPT-11), a water-soluble, semi-synthetic derivative of camptothecin, is one such agent that has been shown to be highly effective, and currently represents a key-drug in first- and second-line treatment regimens for CRC. CPT-11 monotherapy, however, has not been shown to yield superior efficacy, including in terms of the median survival time, to bolus 5FU/leucovorin (LV) alone.[1] In 2 Phase III trials, the addition of CPT-11 to bolus or infusional 5FU/LV regimens clearly yielded greater efficacy than administration of 5FU/LV alone, with a doubling of the tumor response rate and prolongation of the median survival time by 2–3 months.[1,2]

CPT-11 is converted to 7-ethyl-10-hydroxy-camptothecin (SN-38), a biologically active and water-insoluble metabolite of CPT-11, by carboxylesterases in the liver and the tumor. SN-38 has been demonstrated to exhibit up to a 1,000-fold more potent cytotoxic activity than CPT-11 against various cancer cells *in vitro*.[3] The metabolic conversion rate is, however, very low, with only <10% of the original volume of CPT-11 being metabolized to SN-38[4,5]; conversion of CPT-11 to SN-38 also depends on genetic interindividual variability of the activity of carboxylesterases.[6] Direct use of SN-38 itself for clinical cancer treatment must be shown to be identical in terms of both efficacy and toxicity.

Some drugs incorporated in drug delivery systems (DDS), such as Abraxane and Doxil, are already in clinical use.[7,8] The clinical benefits of DDS are based on their EPR effect.[9] The EPR effect is based on the pathophysiological characteristics of solid tumor tissues: hypervascularity, incomplete vascular architecture, secretion of vascular permeability factors stimulating extravasation within cancer tissue, and absence of effective lymphatic drainage from the tumors that impedes the efficient clearance of macromolecules accumulated in solid tumor tissues. Several types of DDS can be used for incorporation of a drug. A liposome-based formulation of SN-38 (LE-SN38) has been developed, and a clinical trial to assess its efficacy is now under way.[10,11]

Recently, we demonstrated that NK012, novel SN-38-incorporating polymeric micelles, exerted superior antitumor activity and less toxicity than CPT-11.[12] NK012 is characterized by a smaller size of the particles than LE-SN38; the mean particle diameter of NK012 is 20 nm. NK012 can release SN-38 under neutral conditions even in the absence of a hydrolytic enzyme, because the bond between SN-38 and the block copolymer is a phenol ester bond, which is stable under acidic conditions and labile under mild alkaline conditions. The release rate of SN-38 from NK012 under physiological conditions is quite high; more than 70% of SN-38 is released within 48 hr. We speculated that the use of NK012, in place of CPT-11, in combination with 5FU may yield superior results in the treatment of CRC. In the present study, we evaluated the antitumor activity of NK012 administered in combination with 5FU as compared to that of CPT-11 administered in combination with 5FU against CRC in an experimental model.

Material and methods

Cells and animals

The human colorectal cancer cell lines used, namely, HT-29 and HCT-116, were purchased from the American Type Culture Collection (Rockville, MD). The HT-29 cells and HCT-116 cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (Cell Culture Technologies, Gaggenau-Hoerden, Germany), penicillin, streptomycin, and amphotericin B (100 units/mL, 100 μg/mL, and 25 μg/mL, respectively; Sigma, St. Louis, MO) in a humidified atmosphere containing 5% CO_2 at 37°C.

BALB/c *nu/nu* mice were purchased from SLC Japan (Shizuoka, Japan). Six-week-old mice were subcutaneously (s.c.)

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inoculated with 1×10^6 cells of HT-29 or HCT-116 cell line in the flank region. The length (a) and width (b) of the tumor masses were measured twice a week, and the tumor volume (TV) was calculated as follows: $TV = (a \times b^2)/2$. All animal procedures were performed in compliance with the Guidelines for the Care and Use of Experimental Animals established by the Committee for Animal Experimentation 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.

Drugs

The SN-38-incorporating polymeric micelles, NK012, and SN-38 were prepared by Nippon Kayaku (Tokyo, Japan).[12] CPT-11 was purchased from Yakult Honsha (Tokyo, Japan). 5FU was purchased from Kyowa Hakko (Tokyo, Japan).

Cell growth inhibition assay

HT-29 cells were seeded in 96-well plates at a density of 2,000 cells/well in a final volume of 90 μL. Twenty-four hours after seeding, a graded concentration of NK012 or SN-38 was added concurrently with 5FU to the culture medium of the HT-29 cells in a final volume of 100 μL for drug interaction studies. The culture was maintained in the CO_2 incubator for an additional 72 hr. Then, cell growth inhibition was measured by the tetrazolium salt-based proliferation assay (WST assay; Wako Chemicals, Osaka, Japan). WST-1 labeling solution (10 μL) was added to each well and the plates were incubated at 37°C for 3 hr. The absorbance of the formazan product formed was detected at 450 nm in a 96-well spectrophotometric plate reader. Cell viability was measured and compared to that of the control cells. Each experiment was carried out in triplicate and was repeated at least 3 times. Data were averaged and normalized against the nontreated controls to generate dose-response curves.

Drug interaction analysis

The nature of interaction between NK012 or SN-38 and 5FU against HT-29 cells was evaluated by median-effect plot analyses and the combination index (CI) method of Chou and Talalay.[13] Data analysis was performed using the Calcusyn software (Biosoft, NY, USA). NK012 or SN-38 was combined with 5FU at a fixed ratio that spanned the individual IC_{50} values of each drug. The IC_{50} values were determined on the basis of the dose-response curves using the WST assay. For any given drug combination, the CI is known to represent the degree of synergy, additivity or antagonism. It is expressed in terms of fraction-affected (Fa) values, which represents the percentage of cells killed or inhibited by the drug. Isobologram equations and Fa/CI plots were constructed by computer analysis of the data generated from the median effect analysis. Each experiment was performed in triplicate with 6 gradations and was repeated at least 3 times. The resultant dose-response curves were averaged, to create a single composite dose-response curve for each combination.

In vivo analysis of the effects of NK012 combined with 5FU as compared to those of CPT-11 combined with 5FU

When the mean tumor volumes reached ~93 mm^3, the mice were randomly divided into test groups consisting of 5 mice per group (Day 0). The drugs were administered i.v. *via* the tail vein of the mice. In the groups administered NK012 or 5FU as single agents, the drug was administered on Days 0, 7 and 14. In the combined treatment groups, NK012 or CPT-11 was administered 24 hr before 5FU on Days 0, 7 and 14, according to the previously reported combination schedule for CPT-11 and 5FU.[14] Complete response (CR) was defined as tumor not detectable by palpation at 90 days after the start of treatment, at which time-point the mice were sacrificed. Tumor volume and body weight were measured twice a week. As a general rule, animals in which the tumor volume exceeded 2,000 mm^3 were also sacrificed.

Experiment 1. Evaluation of the effects of NK012 combined with 5FU and determination of the maximum tolerated dose (MTD) of NK012/5FU. By comparing the data between NK012 administered as a single agent and NK012/5FU, we evaluated the effects of the combined regimen against the s.c HT-29 tumors. A preliminary experiment showed that combined administration of NK012 15 mg/kg + 5FU 50 mg/kg every 6 days caused drug-related lethality (data not shown). To determine the MTD, therefore, we set the dosing schedule of the combined regimen at 5 or 10 mg/kg of NK012 + 50 mg/kg of 5FU three times a week.

Experiment 2. Comparison of the antitumor effect of NK012/5FU and CPT-11/5FU. Based on a comparison of the data between NK012/5FU and CPT-11/5FU against the s.c. HT-29 and HCT-116 tumors, we investigated the feasibility of the clinical application of NK012/5FU for the treatment of CRC. CPT-11/5FU was administered three times a week at the respective MTDs of the 2 drugs as previously reported, that is, CPT11 at 50 mg/kg and 5FU at 50 mg/kg, respectively.[14] NK012/5FU was administered once three times a week at the respective MTDs of the 2 drugs determined from Experiment 1.

Cell cycle analysis

Samples from the HT-29 tumors that had grown to 80–100 mm^3 were removed from the mice at 6, 24, 48, 72 and 96 hr after the administration of NK012 alone at 10 mg/kg or CPT-11 alone at 50 mg/kg. The samples were excised, minced in PBS and fixed in 70% ethanol at −20°C for 48 hr. They were then digested with 0.04% pepsin (Sigma chemical Co., St Lous, MO) in 0.1 N HCL for 60 min at 37°C in a shaking bath to prepare single-nuclei suspensions. The nuclei were then centrifuged, washed twice with PBS and stained with 40 μg/mL of propidium iodide (Molecular Probes, OR) in the presence of 100 μg/mL RNase in 1 mL PBS for 30 min at 37°C. The stained nuclei were analyzed with B-D FACSCalibur (BD Biosciences, San Jose, CA), and the cell cycle distribution was analyzed using the Modfit program (Verity Software House Topsham, ME).

Statistical analyses

Data were expressed as mean ± SD. Data were analysed with Student's t test when the groups showed equal variances (F test), or Welch's test when they showed unequal variances (F test). $p < 0.05$ was regarded as statistically significant. All statistical tests were 2-sided.

Results

Antiproliferative effects of NK012 or SN-38 administered in combination with 5FU

Figure 1*a* shows the dose-response curves for NK012 alone, 5FU alone and a combination of the two. The IC_{50} levels of NK012 and 5FU against the HT-29 cells were 39 nM and 1 μM, respectively, and the IC_{50} level of SN-38 was 14 nM (data not shown). Based on these data, the molar ratio of NK012 or SN-38:5FU of 1:1,000 was used for the drug combination studies.

Figures 1*b* and 1*c* show the median-effect and the combination index plots. Combination indices (CIs) of <1.0 are indicative of synergistic interactions between 2 agents; additive interactions are indicated by CIs of 1.0, and antagonism by CIs of >1.0. Figure 1*c* shows the combination index for NK012 and 5FU, when 2 drugs are supposed to be mutually exclusive. Marked synergism was observed between Fa 0.2 and 0.6. Theoretically, the CI method is the most reliable around an Fa of 0.5, suggesting synergistic effects of the combination of NK012 and 5FU. This synergistic effect was more evident than that of SN-38/5FU (Fig. 1*d*).

In vivo *effect of combined NK012 and 5FU*

Experiment 1. Dose optimization and effect of combined NK012 and 5FU against HT-29 tumors. Comparison of the relative tumor volumes on Day 40 revealed significant differences between

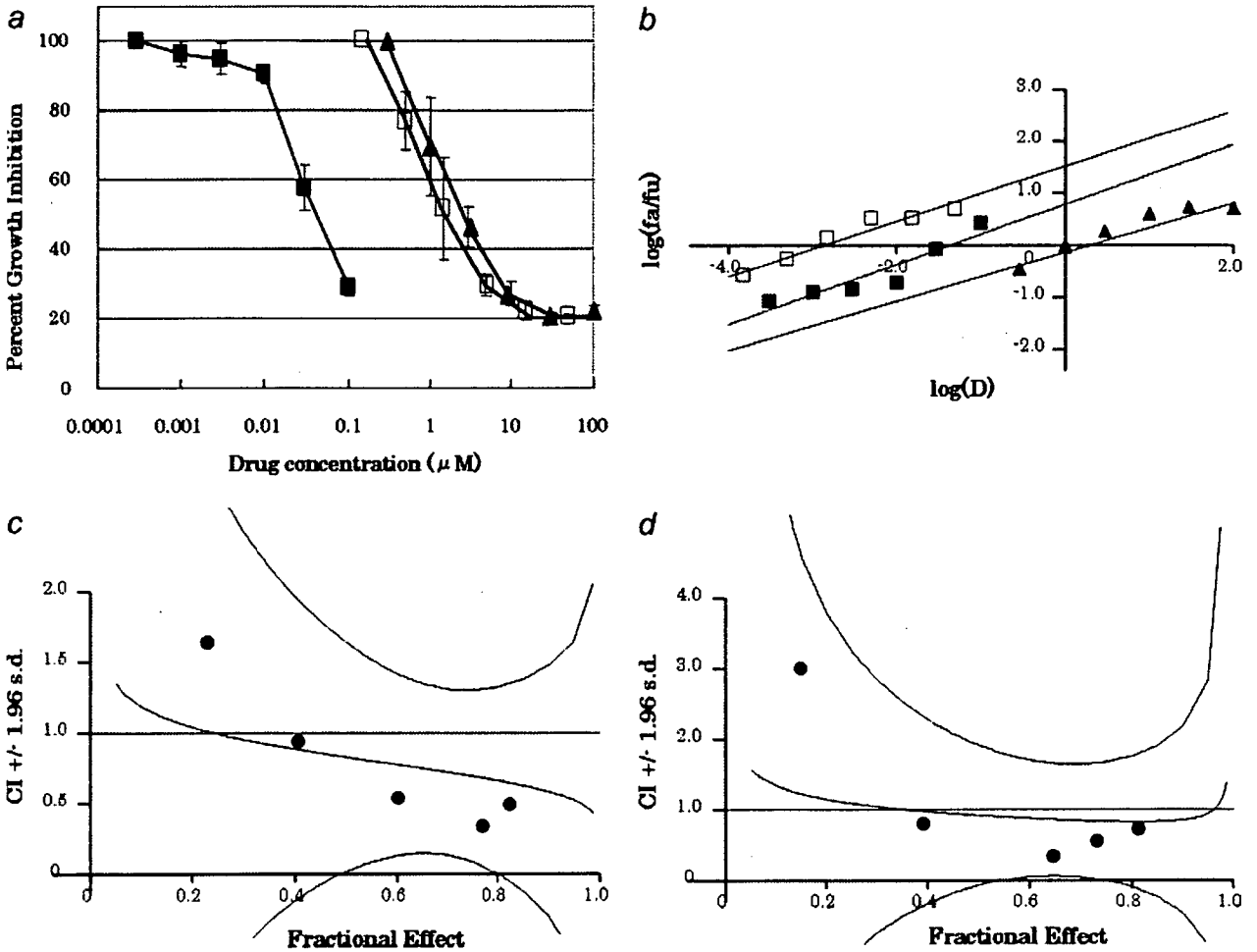

FIGURE 1 – Interaction of NK012 and 5FU *in vitro*. (*a*) Dose-response curves for NK012 alone (■), 5FU alone (▲) and their combination (□) against HT-29 cells. HT-29 cells were seeded at 2,000 cells/well. Twenty-four hours after seeding, a graded concentration of NK012 or 5FU was added to the culture medium of the HT-29 cells. Cell growth inhibition was measured by WST assay after 72 hr of treatment. Cell viability was measured and compared with that of the control cells. Each experiment was carried out independently and repeated at least 3 times. Points, mean of tripricates; bars, SD. (*b*) Median effect plot for the interaction of NK012 and 5FU. (*c*, *d*) Combination index for the interaction as a function of the level of effect (fraction effect = 0.5 is the IC_{50}). The straight line across the CI value of 1.0 indicates additive effect and CIs above and below indicate antagonism and synergism, respectively. The molar ratio of NK012/5FU (*c*) or SN-38/5FU (*d*) at 1:1,000 was tested by CI analysis. Black circles represent the CIs of the actual data points, solid lines represent the computer-derived CIs at effect levels ranging from 10 to 100% inhibition of cell growth, and the dotted lines represent the 95% confidence intervals.

those in the mice administered NK012 alone and those administered NK012/5FU at 5 mg/kg of NK012 ($p = 0.018$) (Fig. 2*a*). Although there was no statistically significant difference in the relative tumor volume measured on Day 54 between the mice administered NK012 alone and NK012/5FU at 10 mg/kg of NK012 ($p = 0.3050$), a trend of superior antitumor effect was demonstrated in the group treated with NK012/5FU at 10 mg/kg of NK012 (Fig. 2*a*). The CR rates were 20, 40 and 60% for 5 mg/kg NK012 + 50 mg/kg 5FU, 10 mg/kg NK012 alone and 10 mg/kg NK012 + 50 mg/kg 5FU, respectively. The schedule of 10 mg/kg NK012 + 50 mg/kg 5FU resulted in no remarkable toxicity in terms of body weight changes, and these doses were determined as representing the MTDs (Fig. 2*b*).

Experiment 2. Comparison of the antitumor effect of combined NK012/5FU and CPT-11/5FU against HT-29 and HCT-116 tumors. The therapeutic effect of NK012/5FU on Day 60 was significantly superior to that of CPT-11/5FU against the HT-29 tumors ($p = 0.0004$) (Fig. 3*a*). A more potent antitumor effect, namely, a 100% CR rate, was obtained in the NK012/5FU group as compared to the 0% CR rate in the CPT-11/5FU group. Although no statistically significant difference in the relative tumor volume on Day 61 was demonstrated between the NK012/5FU and CPT-11/5FU in the case of the HCT-116 tumors ($p = 0.2230$), a trend of superior antitumor effect against these tumors was observed in the NK012/5FU treatment group (Fig. 3*b*). The CR rates for the case of the HCT-116 tumors were 0% in both NK012/5FU and CPT-11/5FU groups.

Specificity of cell cycle perturbation

We studied the differences in the effects between NK012 10 mg/kg and CPT-11 50 mg/kg on the cell cycle (Fig. 4*a*). The data indicated that both NK012 and CPT-11 tended to cause accumulation of cells in the S phase, although the effect of NK012 was stronger and maintained for a more prolonged period than that of CPT-11; the maximal percentage of S-phase cells in the total cell population in the tumors was 34% at 24 hr after the administration of CPT-11, whereas it was 39% at 48 hr after the administration of NK012 (Figs. 4*b*, and 4*c*).

Discussion

Our primary endpoint was to clarify the advantages of NK012 over CPT-11 administered in combination with 5FU. We demonstrated that combined NK012 and 5FU chemotherapy exerts more

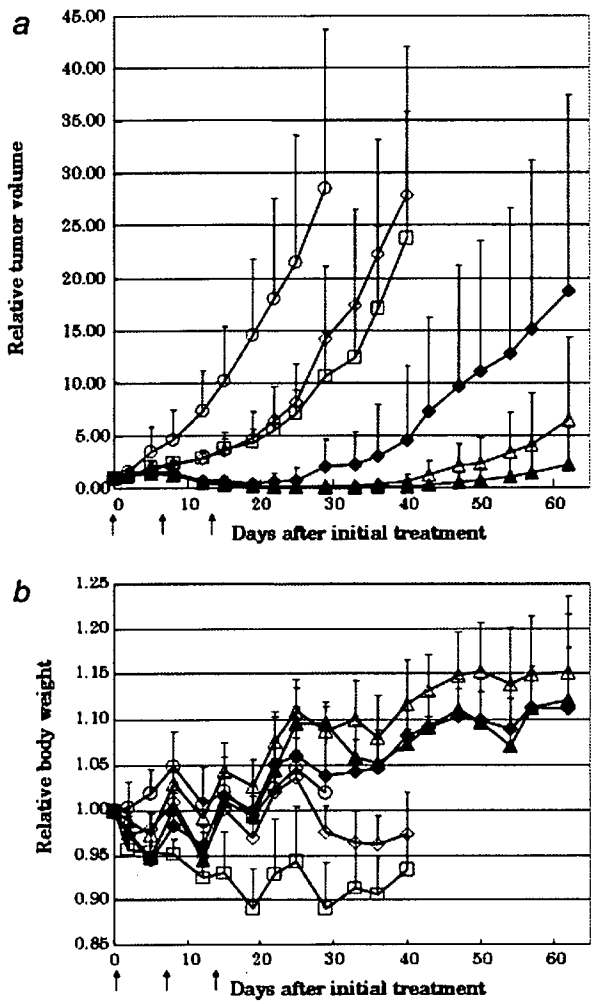

FIGURE 2 – Effect of NK012 alone or NK012 in combination with 5FU against HT-29 tumor-bearing mice. Points, mean; bars, SD. (*a*) Antitumor effect of each regimen on Days 0, 7 and 14. (○) control, (□) 5FU 50 mg/kg alone, (◇) NK012 5 mg/kg alone, (◆) NK012 5 mg/kg 24 hr before 5FU 50 mg/kg, (△) NK012 10 mg/kg alone, (▲) NK012 10 mg/kg 24 hr before 5FU 50 mg/kg. (*b*) Changes in the relative body weight. Data were derived from the same mice as those used in the present study.

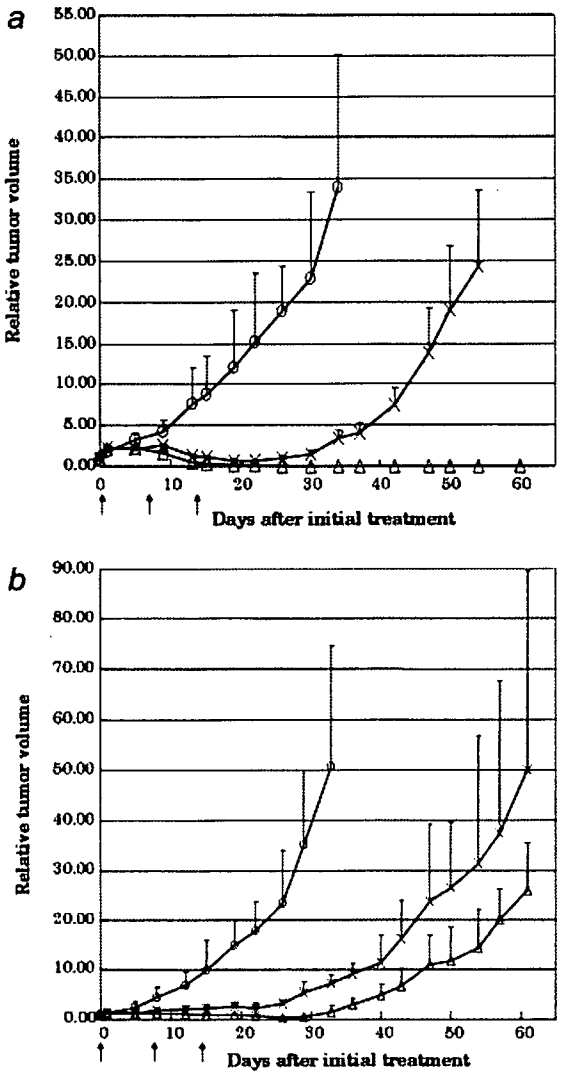

FIGURE 3 – Effect of NK012/5FU as compared with that of CPT11/5FU against HT-29 (*a*) or HCT-116 (*b*) tumor-bearing mice. Antitumor effect of each schedule on Days 0, 7 and 14. (○) control, (×) CPT-11 50 mg/kg 24 hr before 5FU 50 mg/kg, (△) NK012 10 mg/kg 24 hr before 5FU 50 mg/kg. Points, mean; bars, SD.

synergistic activity *in vitro* and significantly greater antitumor activity against human CRC xenografts as compared to CPT-11/5FU. The combination of NK012 and 5FU is considered to hold promise of clinical benefit for patients with CRC.

CPT-11, a topoisomerase-I inhibitor, and 5FU, a thymidilate synthase inhibitor, have been demonstrated to be effective agents for the treatment of CRC. A combination of these 2 drugs has also been demonstrated to be clearly more effective than either CPT-11 or 5FU/LV administered alone *in vivo* and in clinical settings.[1,2,14] Administration of 5FU by infusion with CPT-11 was shown to be associated with reduced toxicity and an apparent improvement in survival as compared to that of administration of the drug by bolus injection with CPT-11.[1,2] This synergistic enhancement may result from the mechanism of action of the 2 drugs; CPT-11 has been reported to cause accumulation of cells in the S phase, and 5FU infusion is known to cause DNA damage specifically in cells of the S phase.[14] On the basis of this background, our results suggesting the more pronounced and more prolonged accumulation of the tumor cells in the S phase caused by NK012 as compared with that by CPT-11 may explain the more effective synergy of the former administered with 5FU infusion. This may be attributable to accumulation of NK012 due to the enhanced permeability and retention (EPR) effect.[9] It is also speculated that NK012 allows sustained release of free SN-38, which may move more freely in the tumor interstitium.[15] Otherwise NK012 itself could internalize into cells to localize in several cytoplasmic organelles as reported by Savic *et al.*[16] These characteristics of NK012 may be responsible for its more potent antitumor activity observed in this study, because CPT-11 has been reported to show time-dependent growth-inhibitory activity against the tumor cells.[17]

The major dose-limiting toxicities of CPT-11 are diarrhea and neutropenia. SN-38, the active metabolite of CPT-11, may cause CPT-11-related diarrhea as a result of mitotic -inhibitory activity.[18] Because it undergoes significant biliary excretion, SN-38 may have a potentially long residence time in the gastrointestinal tract that may be associated with prolonged diarrhea.[19,20] In our previous report, we evaluated the tissue distribution of SN-38 after administration of an equimolar amount of NK012 (20 mg/kg) and CPT-11 (30 mg/kg), and found no difference in the level of SN-38 accumulation in the small intestine.[12] A significant antitumor effect of NK012 with a lower incidence of diarrhea was also dem-

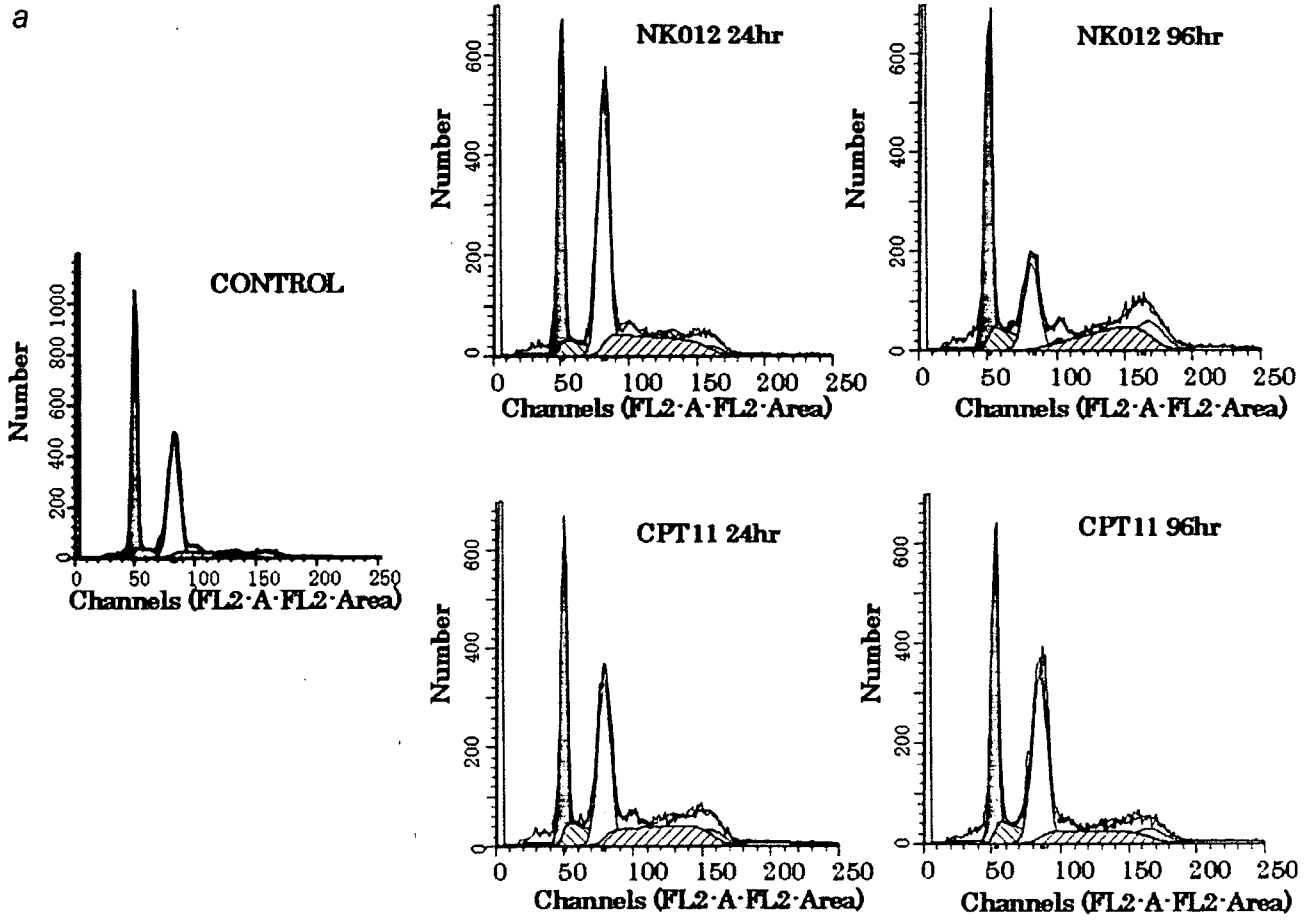

FIGURE 4 – Cell cycle analysis of HT-29 tumor cells collected 24, 48, 72 and 96 hr after administration of NK012 at 10 mg/kg alone or CPT-11 at 50 mg/kg alone using the Modfit program (Verity Software House Topsham, ME). (*a*) Cell cycle analysis of HT-29 tumor cells 24 and 96 hr after administration of NK012 at 10 mg/kg or CPT-11 at 50 mg/kg, respectively. (*b*) Cell cycle distribution of tumor cells 0, 24, 48, 72 and 96 hr after treatment with NK012 at 10 mg/kg. (*c*) Cell cycle distribution of tumor cells 0, 24, 48, 72 and 96 hr after treatment with CPT-11 at 50 mg/kg.

onstrated as compared to that observed with CPT-11 in a rat mammary tumor model.[21] Combined administration of CPT-11 with 5FU/LV infusion appears to be associated with acceptable toxicity in patients with CRC. In addition, no significant difference in the frequency of Grade 3/4 diarrhea was noted between patients treated with FOLFIRI (CPT-11 regimen with bolus and infusional 5FU/LV) and those treated with FOLFOX6 (oxaliplatin regimen with bolus and infusional 5FU/LV).[22,23] Our *in vivo* data actually revealed no severe body weight loss in the NK012/5FU group. Consequently, we expect that the NK012/5FU regimen, especially

with infusional 5FU, may be an attractive arm for a Phase III trial in CRC, with CPT-11/5FU as the control arm. We have already initiated a Phase I trial of NK012 in patients with advanced solid tumors based on the data suggesting higher efficacy and lower toxicity of this preparation than CPT-11 *in vivo*.[12]

In conclusion, we demonstrated that combined NK012 and 5FU chemotherapy exerts significantly greater antitumor activity against human CRC xenografts as compared to CPT-11/5FU, indicating the necessity of clinical evaluation of this combined regimen.

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Matuzumab and cetuximab activate the epidermal growth factor receptor but fail to trigger downstream signaling by Akt or Erk

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Molecular inhibition of the epidermal growth factor receptor (EGFR) is a promising anticancer strategy, and monoclonal antibodies (mAbs) to EGFR are undergoing extensive evaluation in preclinical and clinical trials. However, the effects of anti-EGFR mAbs on EGFR signaling have remained unclear. We have now examined the effects of 2 anti-EGFR mAbs, matuzumab (EMD72000) and cetuximab (Erbitux), both of which are currently under assessment for treatment of various cancers, on EGFR signal transduction and cell survival in nonsmall cell lung cancer cell lines. Similar to EGF, matuzumab and cetuximab each induced phosphorylation of EGFR at several tyrosine phosphorylation sites as a result of receptor dimerization and activation of the receptor tyrosine kinase. In contrast to the effects of EGF, however, EGFR activation induced by these antibodies was not accompanied by receptor turnover or by activation of downstream signaling pathways that are mediated by Akt and Erk and are important for regulation of cell proliferation and survival. In addition, clonogenic survival assays revealed that matuzumab and cetuximab reduced the survival rate of H292 cells, in which they also inhibited the EGF-induced activation of Akt and Erk. Although we have examined only a few cell lines, our results indicate that the antitumor effects of matuzumab and cetuximab depend on inhibition of EGFR downstream signaling mediated by Akt or Erk rather than on inhibition of EGFR itself.

Key words: EGF receptor; signal transduction; matuzumab; cetuximab; nonsmall cell lung cancer

The epidermal growth factor receptor (EGFR, also known as ErbB1), a member of the ErbB family of receptor tyrosine kinases, is a 170-kDa plasma membrane glycoprotein composed of an extracellular ligand binding domain, a transmembrane region and an intracellular tyrosine kinase domain with a regulatory COOH-terminal segment.[1] Binding of ligand to EGFR induces receptor dimerization, activation of the receptor kinase and autophosphorylation of specific tyrosine residues within the COOH-terminal region of the protein.[1] These events trigger intracellular signaling pathways that promote cell proliferation and survival.[2,3]

EGFR is frequently overexpressed in many types of human malignancy, with the extent of overexpression being negatively correlated with prognosis.[4,5] Recognition of the role of EGFR in carcinogenesis has prompted the development of EGFR-targeted therapies that include both small-molecule tyrosine kinase inhibitors (TKIs) that target the intracellular tyrosine kinase domain and monoclonal antibodies (mAbs) that target the extracellular domain.[6–8] Among EGFR-TKIs, gefitinib and erlotinib have been extensively evaluated in nonsmall cell lung cancer (NSCLC), and sensitivity to these drugs has been correlated with the presence of somatic mutations in the EGFR kinase domain or with EGFR gene (*EGFR*) amplification.[9–16] Among anti-EGFR mAbs, cetuximab (Erbitux), a chimeric mouse-human antibody of the immunoglobulin (Ig) G1 subclass, has proved efficacious in the treatment of irinotecan-refractory colon cancer[17] and was recently approved by the U.S. Food and Drug Administration for the treatment of patients with head and neck squamous cell carcinoma.[18] Several clinical studies of anti-EGFR mAbs such as matuzumab (EMD72000, humanized IgG1) and cetuximab are ongoing for other types of cancer including NSCLC.[19–24] Anti-EGFR mAbs bind to the extracellular ligand binding domain of the receptor and are thereby thought to block ligand binding.[18,25] The antitumor effects of these mAbs are thus thought to be attributable to inhibition of EGFR signaling as well as to other mechanisms such as antibody-dependent cellular cytotoxicity.[18,26] However, the detailed effects of anti-EGFR mAbs on EGFR signaling have remained unclear.[27–30]

We have now examined in detail the effects on EGFR signal transduction of 2 anti-EGFR mAbs, matuzumab and cetuximab, both of which are used clinically, to provide insight into the mechanisms of their antitumor effects.

Material and methods

Cell culture and reagents

The human NSCLC cell lines NCI-H292 (H292), NCI-H460 (H460) and Ma-1 were obtained as previously described[31] and were cultured under a humidified atmosphere of 5% CO_2 at 37°C in RPMI 1640 medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum. Matuzumab and cetuximab were kindly provided by Merck KGaA (Darmstadt, Germany) and Bristol Myers (New York, NY), respectively; gefitinib was obtained from AstraZeneca (Macclesfield, UK); and trastuzumab (Herceptin; Genentech, South San Francisco, CA) was obtained from Chugai (Tokyo, Japan). Neutralizing antibodies to EGFR (clone LA1) were obtained from Upstate Biotechnology (Lake Placid, NY).

Immunoblot analysis

Cell lysates were fractionated by SDS-polyacrylamide gel electrophoresis on a 7.5% gel, and the separated proteins were transferred to a nitrocellulose membrane. After blocking of nonspecific sites, the membrane was incubated consecutively with primary and secondary antibodies, and immune complexes were detected with the use of enhanced chemiluminescence reagents, as described previously.[31] Primary antibodies to the specific intracellular phosphorylation sites of EGFR (pY845, pY1068 or pY1173), to Erk, to phospho-Akt and to Akt were obtained from Cell Signaling Technology (Beverly, MA); those to the extracellular domain of EGFR (clone 31G7) were from Zymed (South San Francisco, CA); those to the intracellular domain of EGFR (EGFR 1005) and to phospho-Erk were from Santa Cruz Biotechnology (Santa Cruz, CA); and those to β-actin (loading control) were from Sigma. Horseradish peroxidase-conjugated goat antibodies to mouse or rabbit IgG were obtained from Amersham Biosciences (Little Chalfont, UK).

Chemical cross-linking assay

Cells were incubated first with 1 mM bis(sulfosuccinimidyl) suberate (BS^3; Pierce, Rockford, IL) for 20 min at 4°C and then with

Abbreviations: EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor; mAb, monoclonal antibody; NSCLC, nonsmall cell lung cancer; Ig, immunoglobulin; BS^3, bis(sulfosuccinimidyl) suberate; PE, R-phycoerythrin; PI3K, phosphoinositide 3-kinase.

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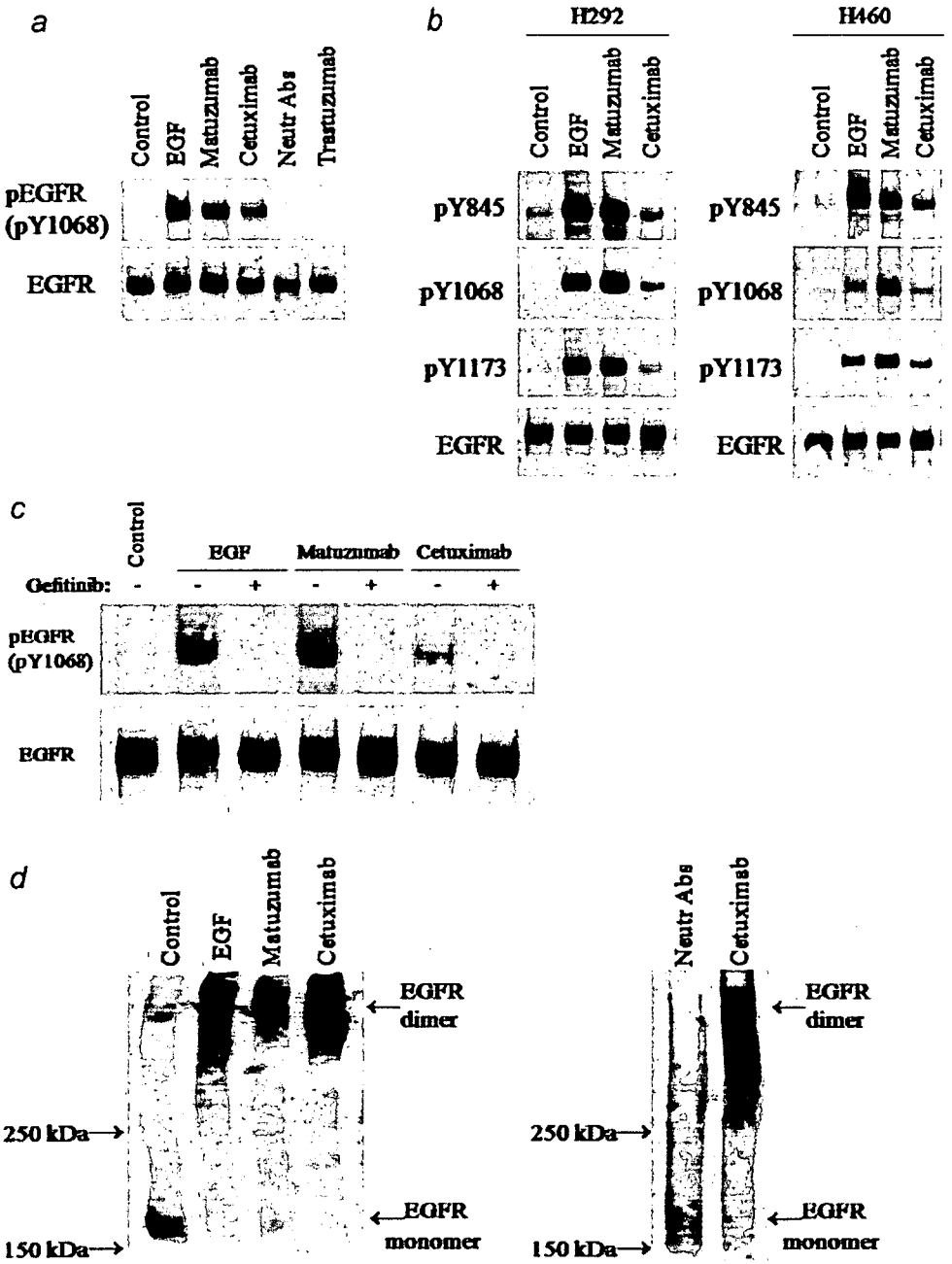

FIGURE 1 – EGFR phosphorylation induced by matuzumab or cetuximab as a result of receptor dimerization and activation of the receptor tyrosine kinase. (*a*) H292 cells were deprived of serum overnight and then incubated for 15 min in the absence (Control) or presence of matuzumab (200 nM), cetuximab (100 nM), neutralizing antibodies to EGFR (80 nM), trastuzumab (50 nM) or EGF (100 ng/ml). Cell lysates were subjected to immunoblot analysis with antibodies to the Y1068-phosphorylated form of EGFR (pY1068) and to total EGFR (the extracellular domain). (*b*) H292 or H460 cells were deprived of serum overnight and then incubated for 15 min in the absence or presence of matuzumab (200 nM), cetuximab (100 nM) or EGF (100 ng/ml). Cell lysates were subjected to immunoblot analysis with antibodies to the Y845-, Y1068- or Y1173-phosphorylated forms of EGFR and to total EGFR (the extracellular domain). (*c*) H292 cells were deprived of serum overnight and then incubated for 15 min in the absence or presence of matuzumab (200 nM), cetuximab (100 nM), EGF (100 ng/ml) or gefitinib (10 μM), as indicated. Cell lysates were subjected to immunoblot analysis with antibodies to the Y1068-phosphorylated form of EGFR and to total EGFR (the extracellular domain). (*d*) H292 cells were deprived of serum overnight and then incubated for 15 min in the absence or presence of matuzumab (200 nM), cetuximab (100 nM), neutralizing antibodies to EGFR (80 nM) or EGF (100 ng/ml). The cells were then washed and exposed to the chemical cross-linker BS^3 after which cell lysates were subjected to immunoblot analysis with antibodies to EGFR (the intracellular domain). The positions of EGFR monomers and dimers as well as of molecular size standards are indicated.

250 mM glycine for 5 min at 4°C to terminate the cross-linking reaction, as described previously.[31] Cell lysates were resolved by SDS-polyacrylamide gel electrophoresis on a 4% gel and subjected to immunoblot analysis with rabbit polyclonal antibodies to the intracellular domain of EGFR (EGFR 1005).

Immunofluorescence analysis

Cells were grown to 50% confluence in 2-well Lab-Tec Chamber Slides (Nunc, Naperville, IL), deprived of serum overnight, and then incubated with 200 nM matuzumab or EGF (100 ng/ml) for 4 hr at 37°C. They were fixed with 4% paraformaldehyde for