Table III. *Non-hematological toxicity by dose.*

	Grade 2	
	35 mg/m^2 (n=28)	40 mg/m^2 (n=11)
Anorexia	3	1
Fatigue	2	1
Nausea	3	1
Vomiting	2	-
Rash	1	2
Constipation	-	-
Stomatitis	-	1
AST/ALT	-	1
Creatinine	1	-

Table IV. *Response by dose.*

	Dose (mg/m^2)		Total
	35	40	
PR	4	0	4
SD	6	3	9
PD	18	8	26
RR (%)	14.2%	0%	10.2%
DCR (%)	35.7%	27.2%	33.3%

PR: Partial response, SD: stable disease, PD: progressive disease; RR: response rate, DCR: disease control rate.

observed in 2 patients (5%). The non-hematological toxicities are listed in Table III, and amrubicin caused no grade 3 or higher toxicities.

Response to therapy and survival. The objective tumor responses are shown in Table IV. Among the 39 patients, 4 achieved a confirmed partial response and 9 had stable disease, for an overall response rate of 10.2% (95% CI, 2.9 to 24.2%) and disease control rate of 33.3% (95% CI, 19.1 to 50.2%). The patients receiving the 35 mg/m^2 dose had a response rate of 14.2% and a disease control rate of 35.7%, while those receiving the 40 mg/m^2 dose had no response and a disease control rate of 27.2% and overall survival data are shown in Figure 1. The overall median survival time (MST) and one-year survival rate were 5.7 months (95% CI, 3.4 to 7.9 months) and 28%, respectively. The overall progression-free survival was 1.5 months (95% CI, 1.2 to 1.7 months).

Discussion

It has not been demonstrated whether third-line or more chemotherapy would actually increase survival in patients with second-line failure. It was reported that among 43 patients (extracted from over 700 patient records with

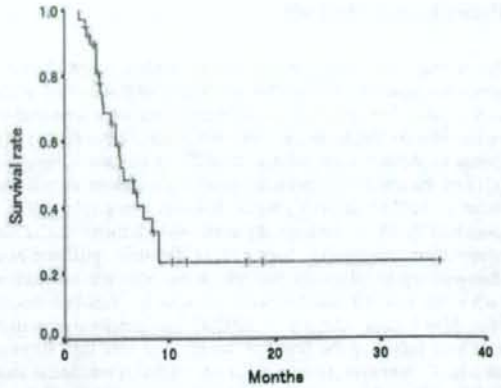

Figure 1. *Survival curve. Median survival time was 5.7 months (95% confidence interval, 3.4 to 7.9 months).*

recurrent NSCLC) who had received two prior chemotherapy regimens including platinum and docetaxel for recurrent NSCLC, the response rates decreased with each line of treatment (third-line, 2.3%; fourth-line, 0%), the disease control rate also decreased (third-line, 30.2%; and fourth-line, 21.4%) and the median overall survival time from the start of the last treatment was 4 months (14). The results of the presented study indicated that amrubicin monotherapy had variable clinical outcomes (overall response rate, MST, and progression-free survival were 10.2%, 5.7 months and 1.5 months, respectively) and acceptable toxicity profiles among the NSCLC patients with failure of second-line or subsequent therapy, including docetaxel and EGFR-TKI.

This study could serve as a foundation in regard to the investigation of the antitumor activity of amrubicin in the aforementioned setting. Additionally, the safety profile of the drug, such as tolerable hematological toxicity and slight non-hematological toxicity beyond the second-line setting, is certainly valuable. Neutropenia has been recognized as the principal toxicity of amrubicin monotherapy (9, 10). In this study, the incidence of severe neutropenia was less frequent in the patients receiving 35 mg/m^2 in comparison with those receiving 40 mg/m^2, and the clinical response of the patients receiving 35 mg/m^2 was not inferior to that of the patients receiving 40 mg/m^2. The information presented in the study serves to strengthen the rationale for use of the drug at 35 mg/m^2 in the third-line setting and beyond.

In conclusion, amrubicin is an active agent against NSCLC that may play a prominent role in third-line treatment and beyond. The information presented in this study might provide a new direction for clinical research on the treatment of recurrent NSCLC in the third-line setting. However, because of the retrospective nature of this study,

the conclusions cannot be completely definitive and a prospective study aiming at elucidating the efficacy of this agent for recurrent NSCLC is proposed.

References

1 Schiller JH, Harrington D, Belani CP *et al*: Comparison of four chemotherapy regimens for advanced non-small cell lung cancer. N Engl J Med *346*: 92-98, 2002.

2 Ohe Y, Ohashi Y, Kubota K *et al*: Randomized phase III study of cisplatin plus irinotecan *versus* carboplatin plus paclitaxel, cisplatin plus gemcitabine, and cisplatin plus vinorelbine for advanced non-small cell lung cancer: Four-Arm Cooperative Study in Japan. Ann Oncol *18*: 317-323, 2007.

3 Shepherd FA, Dancey J, Ramlau R *et al*: Prospective randomized trial of docetaxel *versus* best supportive care in patients with non-small cell lung cancer previously treated with platinum-based chemotherapy. J Clin Oncol *18*: 2095-3013, 2000.

4 Roszkowski K, Pluzanska A, Krzakowski M *et al*: A multicenter, randomized phase III study of docetaxel plus best supportive care *versus* best supportive care in chemotherapy-naïve patients with metastatic or nonresectable localized non-small cell lung cancer (NSCLC). Lung Cancer *27*: 145-157, 2000.

5 Shepherd FA, Rodrigues Pereira J *et al*: Erlotinib in previously treated non-small cell lung cancer. N Engl J Med *353*: 123-132, 2005.

6 Douillard J-Y, Kim ES, Hirsh V *et al*: Phase III, randomized, open-label, parallel-group study of oral gefitinib (IRESSA™) *versus* intravenous docetaxel in patients with locally advanced or metastatic non-small cell lung cancer who have previously received platinum-based chemotherapy (INTEREST). Eur J Cancer *5*: 2007 (suppl.).

7 Thatcher N, Chang A, Parikh P *et al*: Gefitinib plus best supportive care in previously treated patients with refractory advanced non-small cell lung cancer: results from a randomised, placebo-controlled, multicentre study (Iressa™ Survival Evaluation in Lung Cancer). Lancet *366*: 1527-1537, 2005.

8 Ando M, Okamoto I, Yamamoto N *et al*: Predictive factors for interstitial lung disease, antitumor response, and survival in non-small cell lung cancer patients treated with gefitinib. J Clin Oncol *24*: 2549-56, 2006.

9 Sawa T, Yana T, Takada M *et al*: Multicenter phase II study of amrubicin, 9-amino-anthracycline, in patients with advanced non-small cell lung cancer (Study 1): West Japan Thoracic Oncology Group (WJTOG) trial. Invest New Drugs *24*: 151-158, 2006.

10 Takeda K, Takifuji N, Negoro S *et al*: Phase II study of amrubicin, 9-amino-anthracycline, in patients with advanced non-small cell lung cancer: a West Japan Thoracic Oncology Group (WJTOG) study. Invest New Drugs *25*: 377-383, 2007.

11 Onoda S, Masuda N, Seto T *et al*: Phase II trial of amrubicin for treatment of refractory or relapsed small cell lung cancer: Thoracic Oncology Research Group Study 0301. J Clin Oncol *24*: 5448-53, 2006.

12 Igawa S, Yamamoto N, Ueda S *et al*: Evaluation of the recommended dose and efficacy of amrubicin as second- and third-line chemotherapy for small cell lung cancer. J Thorac Oncol 2: 741-4, 2007.

13 Cancer Therapy Evaluation Program. Common Terminology Criteria for Adverse Events (CTCAE). Version 3.0, DCTD, NCI, NIH, DHHS. August 9, 2006.

14 Massarelli E, Andre F, Liu DD *et al*: A retrospective analysis of the outcome of patients who have received two prior chemotherapy regimens including platinum and docetaxel for recurrent non-small cell lung cancer. Lung Cancer *39*: 55-61, 2003.

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ORIGINAL ARTICLE

Efficacy of S-1 in heavily pretreated patients with metastatic breast cancer: cross-resistance to capecitabine

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Abstract
Background It is not clear what the optimal treatment of chemotherapy is for patients with heavily treated metastatic breast cancer (MBC). We have retrospectively examined the efficacy and safety of S-1 in patients with MBC who had been previously treated with anthracycline, taxane, and capecitabine.
Methods Patients with MBC who had been administered S-1, an oral modulated compound containing a fluoropyrimidine derivative, between November 2001 and June 2003 at the Cancer Institute Hospital were retrospectively reviewed. S-1 at a standard dose of 50 mg/body was administered twice daily for four weeks, followed by a two-week rest period. This was repeated every six weeks until disease progression or unacceptable toxicity.
Results Thirty-five patients were assessed. The patients were heavily pretreated with anthracycline (100%), taxane (paclitaxel or docetaxel) (100%), capecitabine (100%), vinorelbine (71%), and mitomycin (69%). Median follow-up time of patients was 9.6 months (range, 1.2–26.6). ORR was 3% (95% confidence interval: 0–9%), and CBR was 20% (95% confidence interval: 6–33%). Time to treatment failure was 2.8 months. Overall survival was 21.4 months. Grade 1 or 2 adverse events were observed in 17% and 13%, respectively. Grade 3 events occurred as anorexia (9%), nausea (9%), vomiting (9%), diarrhea (14%), fatigue (3%), and elevation of AST/ALT (3%). No grade 3 was seen as hand-foot syndrome. Neither grade 3 nor 4 was observed in bone marrow suppression.
Conclusions S-1 was fairly well tolerated, but demonstrated very limited activity in capecitabine-pretreated patients who had already been exposed to anthracycline and taxane. It was suggested that S-1 clinically exhibited cross-resistance to capecitabine.

Keywords S-1 · Capecitabine · Taxane · Anthracycline · Metastatic breast cancer

Introduction

Many active agents have been used to treat metastatic breast cancer (MBC), which is defined as breast cancer with any distant metastasis. However, it is difficult to achieve an absolute cure. Endocrine treatment, chemotherapy or molecular targeted agents are useful for controlling MBC. Hormone-insensitive or life-threatening MBC favors chemotherapy. Anthracycline, taxane, and trastuzumab play a central role in the chemotherapy of MBC. A durable response with less toxicity may prolong survival with a better quality of life (QOL) [1]. However, prior exposure to anthracycline and taxane limits the chance of choosing subsequent treatments. In such cases, third-line agents such as capecitabine [2], vinorelbine [9–11], gemcitabine [12–14], irinotecan [15] and ixabepilone [16–18] may be tried. However, it is not clear from the data which drug is optimal.

Capecitabine, a fluoropyrimidine derivative, is a common third-line drug [2–8]. Capecitabine is an oral

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fluoropyrimidine carbonate, which is converted to 5FU selectively in tumors through a cascade of three enzymes. Based on the differential distribution of these three enzymes in different tissues, this drug is designed to yield more 5FU in cancer cells than in bone marrow cells or gastrointestinal epithelial cells [19, 20]. Capecitabine produces a response rate of approximately 20% with a duration of 3–6 months [2–8]. The convenient oral delivery of capecitabine gives mild gastrointestinal toxicity and myelosuppression without hair loss. The major toxicity of capecitabine is hand-foot syndrome [2–8].

S-1 is also one of the derivatives and produces a response rate of approximately 20% in patients who did not receive capecitabine [21]. S-1 is an orally administered agent containing 1M tegafur (FT) and two classes of a modulator, 5-chloro-2,4-dihydroxypyrimidine (CDHP) and potassium oxonate (Oxo), at a molar ratio of FT:CDHP:Oxo = 1:0.4:1. One phase II study of S-1 for MBC patients in a heterogeneous population demonstrated that the response rate was 42% among 108 patients [21]. The common toxicities were neutropenia, anemia, stomatitis, or nausea/vomiting. Hand-foot syndrome was rarely seen. Another phase II study was conducted in patients with refractory MBC. In 55 patients who had received taxane, the response rate was 21.8%. The common toxic profile was similar to the previous study [22].

Since S-1 has the same final active metabolites in its mechanism of action as capecitabine, cross-resistance is presumed to exist between the drugs. However, there is no clinical data on the activity of S-1 in capecitabine-pretreated patients with MBC. Here we have retrospectively examined the usefulness of S-1 in patients who were pretreated with anthracycline, taxane and capecitabine. We have focused on whether S-1 is cross-resistant to prior treatment with capecitabine.

Materials and methods

Patients

Patients with MBC who had been given S-1 between November 2001 and June 2003 at the Cancer Institute Hospital were retrospectively reviewed. The eligibility criteria were: (1) histologically confirmed MBC; (2) prior treatment with anthracycline, taxane, and capecitabine; (3) absolute neutrophil count >2,000μ/L; (4) serum bilirubin <1.25 × upper normal limit (UNL) of range; (5) transaminase <2.5 × UNL (in cases of hepatic metastasis <5 × UNL); (6) serum creatinine <1.5 × UNL (7) measurable lesion(s) according to the *Response Evaluation Criteria in Solid Tumors* guidelines; (8) performance status of 0, 1, or 2 on the Eastern Cooperative Oncology Group scale; (9) written informed consent from each patient.

Treatment plan

When body surface area (BSA) was between 1.25 and 1.5 m^2, S-1 was administered orally at a dose of 50 mg/body, twice daily, for four weeks followed by a two-week rest period. This was repeated every six weeks until disease progression or unacceptable toxicity. S-1 was given 60 mg/body when BSA >1.5 m^2 and 40 mg/body for BSA <1.25 m^2. In patients with HER2-positive cancer (HER2 protein scored as 3+ in immunohistochemistry or HER2 gene was amplified twofold or greater in fluorescence in situ hybridization), trastuzumab was administered intravenously at an initial loading dose of 4 mg/kg, followed by 2 mg/kg weekly. Treatment interruption and/or individual dose adjustment of S-1 was considered when patients experienced any adverse events assessed at grade 2 or more as defined by the National Cancer Institute, Common Toxicity Criteria, version 3.0. Patients with an objective response or stable disease (SD) continued to receive treatment until progressive disease (PD) or unacceptable toxicity developed.

Evaluation of efficacy and safety

Efficacy was evaluated by intention-to-treat analysis. Responses were assessed according to the *Response Evaluation Criteria in Solid Tumors* guidelines. Complete response (CR) was defined as the disappearance of all known lesions for at least four weeks. Partial response (PR) was defined as a reduction of the sum of all measurable lesions by at least 30%. PD was defined as an increase of the sum of all measurable lesions by greater than 20%, or as the appearance of a new lesion. Stable disease (SD) was defined as neither CR, PR, nor PD. Long SD was defined as SD lasting for more than 24 weeks.

Objective response rate (ORR) was defined as the sum of the CR and PR rates. Clinical benefit rate (CBR) was defined as the sum of the CR, PR, and long SD rates. Time-to-treatment failure (TTF) was defined as the period from the commencement of S-1 to the discontinuation of S-1 and/or trastuzumab due to PD or unacceptable toxicity.

All adverse events and laboratory parameters were graded according to the National Cancer Institute, Common Toxicity Criteria, version 3.0.

Statistical analysis

TTF were calculated by the Kaplan–Meier method, performed to analyze censored data. Confidence intervals (CI) were set at the 95% level.

Results

Patient characteristics

Thirty-five patients were assessed in the present study. Median follow-up time of patients was 9.6 months, and the range was 1.2–26.6 months. All patients were Japanese women. The demographic characteristics of the present study population are presented in Table 1. Median age was 54 years (range 31–83).

The patients in the present study had advanced disease. More than half of the patients (57%) had three or more metastatic organs, visceral metastasis of the lung (18%), or of the liver (19%). The patients were heavily pretreated with anthracycline (100%), taxane (paclitaxel or docetaxel) (100%), capecitabine (100%), vinorelbine (71%), and mitomycin (69%). More than five prior chemotherapie courses for MBC had been administered to 57% of the patients. In terms of hormonal status, 60% were positive to both estrogen and/or progesterone receptors. With regard to HER2 status, 17% of the patients were HER2 protein 3+ in immunohistochemistry or HER2 gene-amplified in FISH.

Table 1 Patient characteristics ($n = 35$)

Characteristics	No. of patients	Percentage (%)
Mean age (range)	54 (31–83)	
Performance status		
0	12	34
1	14	40
2	9	26
Estrogen receptor/progesterone receptor status		
+/+	19	54
+/−	2	6
−/−	12	34
Unknown	2	6
HER2 status		
Positive (IHC 3+ or FISH+)	6	17
Negative (IHC 0, 1+ or FISH−)	28	80
Unknown	1	3
Recurrent or stage IV		
Recurrent	26	74
Stage IV	9	26
No. of metastases		
Median (range)	3 (1–5)	
1	7	20
2	8	23
3	11	31
4	8	23
5	1	3
Sites of metastases		
Lymph node	23	25
Chest wall/skin	11	12
Lung	17	18
Pleura	8	9
Bone	16	17
Liver	18	19
No. of prior chemotherapy courses for MBC		
Median (range)	5 (1–8)	
1–2	5	14
3–4	10	29
5 onwards	20	57
Agents used in prior chemotherapy		
Anthracycline	35	100
Pre- or postoperative usage	11	31
Taxane (paclitaxel or docetaxel)	35	100
Pre-or postoperative usage	3	9
Capecitabine	35	100
Vinorelbine	25	71
Mitomycin	24	69

Efficacy

Out of 35 patients, the response was assessable in 28. One patient achieved PR (3%). Eight patients obtained SD (23%), and six of those eight patients were long SD (17%) Therefore, ORR was 3% (95% CI; 0–9%). CBR was 20% (95% CI; 6–33%) (Table 2). Median TTF was 2.8 months (Fig. 1). Median overall survival was 21.4 months (Fig. 2).

Among 21 patients in whom the disease progressed during treatment with capecitabine, one PR and five SD (four long SD) were obtained. ORR was 5% and CBR was 23%. A PR was observed after progression to capecitabine preceding stable disease for seven months. In seven patients who had discontinued capecitabine due to their toxicities (four hand-foot syndrome, one thrombocytopenia, one cystitis, one eruption), two SD (one long SD) was observed. In six patients with HER2-positive cancer who were treated with S-1 combined with trastuzumab, two long SD were obtained.

Safety

Grade 1 or 2 adverse events were observed in 17 or 13%, respectively (Table 3). Grade 3 was rarely seen (3% of patients). One grade 4 toxicity of anorexia was observed. Common toxicities at any grade were anorexia (54%), nausea (49%), vomiting (34%), diarrhea (52%), and hand-foot syndrome (35%). Grade 3 events occurred as anorexia (9%), nausea (9%), vomiting (9%), diarrhea (14%), fatigue (3%), and elevation of AST/ALT (3%). No grade 3 was seen as hand-foot syndrome. Neither grade 3 nor 4 was observed in bone marrow suppression. There was no serious organ toxicity.

Table 2 Response

	All ($n = 35$)		Capecitabine-resistant ($n = 21$)		Discontinued capecitabine due to toxicities ($n = 7$)	
	n	%	*n*	%	*n*	%
Response						
Partial response	1	3	1	5	0	0
Stable disease	8	23	5	24	2	28
Long stable disease	6	17	4	19	1	14
Progressive disease	19	54	13	62	2	28
Not evaluable	7	20	2	10	3	43
Objective response rate	1	3	1	5	0	0
Clinical benefit rate	7	20	5	23	1	14

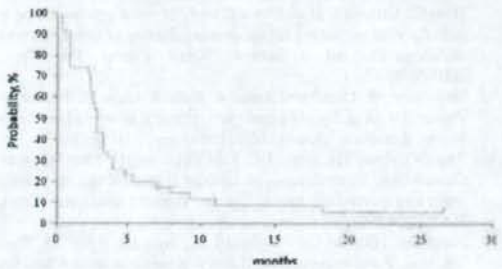

Fig. 1 Time to treatment failure ($n = 35$)

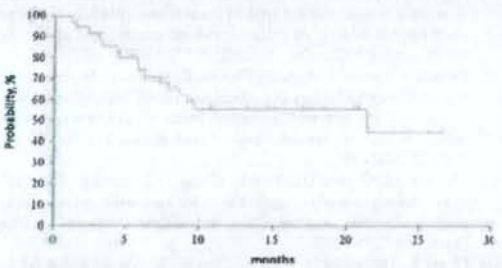

Fig. 2 Overall survival ($n = 35$)

In four patients who had discontinued capecitabine because of hand-foot syndrome, three patients experienced no hand-foot syndrome, but one patient had grade 2. One patient who had discontinued capecitabine due to hemorrhagic cystitis experienced grade 3 diarrhea without other gastrointestinal complaints. One patient who had discontinued capecitabine due to upper abdominal pain experienced grade 2 anorexia without pain. In ten patients (29%), S-1 was discontinued due to toxicities such as diarrhea (four cases), deterioration of PS (3), hand-foot syndrome (1), conjunctivitis (1), and enhancement neuropathy of concomitant phenytoin (1).

Table 3 Adverse events ($n = 35$)

	Total		Grade 1		Grade 2		Grade 3		Grade 4	
	n	%	*n*	%	*n*	%	*n*	%	*n*	%
Anorexia	19	54	12	34	3	9	3	9	1	3
Fatigue	14	40	9	26	11	13	1	3	0	0
Nausea	17	49	6	17	8	23	3	9	0	0
Vomiting	12	34	1	3	8	23	3	9	0	0
Diarrhea	18	51	11	31	2	6	5	14	0	0
Hand-foot syndrome	9	35	4	11	5	14	0	0	0	0
Hair loss	0	0	0	0	0	0	NA	NA	NA	NA
Leukopenia	12	34	10	29	2	6	0	0	0	0
Neutropenia	8	23	5	14	3	9	0	0	0	0
Anemia	16	46	11	31	14	21	0	0	0	0
Thrombocytopenia	3	9	1	3	2	6	0	0	0	0
AST elevation	13	37	8	23	4	11	1	3	0	0
ALT elevation	10	29	5	14	4	11	1	3	0	0
Total bilirubin elevation	5	14	5	14	0	0	0	0	0	0
Creatinine elevation	2	6	2	6	0	0	0	0	0	0
All events	158	30	90	17	66	13	17	3	1	0.2

NA not applicable

Discussion

S-1 or capecitabine is active in MBC patients who have been previously treated with anthracycline and taxane. Both drugs may exhibit cross-resistance because of their shared final active metabolite. The present study showed that S-1 demonstrated 3% ORR and 20% CBR in patients who were heavily pretreated with anthracycline, taxane, and capecitabine. The median of 2.8 months TTF was relatively short (Fig. 1). These results suggest that S-1 has very limited activity in such heavily treated patients. However, for the minority, the disease may stabilize. It was suggested that S-1 demonstrated almost complete cross-resistance to capecitabine.

The safety profile was fairly good. Grade 4 toxicity was rare. Common toxicities were anorexia, nausea/vomiting, diarrhea, and hand-foot syndrome. All toxicities were manageable. However, several patients with poor PS could not continue treatment with moderate toxicities. No severe diarrhea (grade 4) was seen. Interestingly, it is likely that there is a lower incidence of hand-foot syndrome with S-1, even in patients who have suffered from hand-foot syndrome with capecitabine. In the majority, this well-tolerated profile may contribute to maintaining QOL in heavily treated patients.

In managing patients with MBC, it is still controversial as to whether a single agent or combination chemotherapy is superior. For instance, concurrent treatment with

docetaxel and capecitabine produced a longer survival than sequential treatment with each drug [23]. However, more patients receiving capecitabine plus docetaxel required dose reductions because of adverse events [24]. Recently, concurrent usage with capecitabine and ixabepilone was reported to give superior results in terms of progression-free survival than single administration of capecitabine, but overall survival data are not available [18]. There are no conclusive data on the superiority of concurrent treatment because of a lack of data comparing the sequential single usage of each agent. There is also no information on S-1 including other active agents such as vinorelbine, gemcitabine, or irinotecan. Sequential treatment has the potential advantage of yielding fewer adverse events. The strong safety profile with single usage of S-1 may be desirable in heavily treated MBC patients.

This study is both small and retrospective. There is no standard treatment in MBC pretreated with anthracycline, taxane and capecitabine. Well-designed clinical trials or palliative care would be recommended in this setting. It is not clear whether S-1 is active against HER2-positive MBC. Further investigations should be carried out on the clinical effectiveness of the upfront usage of S-1.

In conclusion, S-1 is fairly well tolerated but demonstrates very limited activity in capecitabine-pretreated patients who have already been exposed to anthracycline and taxane. It is suggested that S-1 clinically exhibits cross-resistance to capecitabine.

References

1. Gonzalez-Angulo AM, Morales-Vasquez F, Hortobagyi GN. Overview of resistance to systemic therapy in patients with breast cancer. Adv Exp Med Biol. 2007;608:1–22.
2. Talbot DC, Moiseyenko V, Van Belle S, O'Reilly SM, Alba Conejo E, Ackland S, et al. Randomised, phase II trial comparing oral capecitabine (Xeloda®) with paclitaxel in patients with metastatic/advanced breast cancer pretreated with anthracyclines. Br J Cancer. 2002;86:1367–72.
3. Blum JL, Jones SE, Buzdar AU, LoRusso PM, Kuter I, Vogel C, et al. Multicenter phase II study of capecitabine in paclitaxel-refractory metastatic breast cancer. J Clin Oncol. 1999;17:485–93.
4. Blum JL, Dieras V, Lo Russo PM, Horton J, Rutman O, Buzdar A, et al. Multicenter, phase II study of capecitabine in taxane-pretreated metastatic breast carcinoma patients. Cancer. 2001;92:1759–68.
5. Reichardt P, Von Minckwitz G, Thuss-Patience PC, Jonat W, Kolbl H, Janicke F, et al. Multicenter phase II study of oral capecitabine (Xeloda®) in patients with metastatic breast cancer relapsing after treatment with a taxane-containing therapy. Ann Oncol. 2003;14:1227–33.
6. Fumoleau P, Largillier R, Clippe C, Dieras V, Orfeuvre H, Lesimple T, et al. Multicentre, phase II study evaluating capecitabine monotherapy in patients with anthracycline- and taxane-pretreated metastatic breast cancer. Eur J Cancer. 2004;40:536–42.
7. Wist EA, Sommer HH, Ostenstad B, Risberg T, Bremnes Y, Mjaaland I. Oral capecitabine in anthracycline- and taxane-pretreated advanced/metastatic breast cancer. Acta Oncol. 2004;43:186–9.
8. Osako T, Ito Y, Takahashi S, Tokudome N, Iwase T, Hatake K. Intermittent capecitabine monotherapy with lower dose intensity in heavily pretreated patients with metastatic breast cancer. Tumori. 2007;93:129–32.
9. Nisticò C, Garufi C, Milella M, Vaccaro A, D'Ottavio AM, Fabi A, et al. Weekly schedule of vinorelbine in pretreated breast cancer patients. Breast Cancer Res Treat. 2000;59:223–9.
10. Zelek L, Barthier S, Riofrio M, Fizazi K, Rixe O, Delord JP, et al. Weekly vinorelbine is an effective palliative regimen after failure with anthracyclines and taxanes in metastatic breast carcinoma. Cancer. 2001;92:2267–72.
11. Toi M, Saeki T, Aogi K, Sano M, Hatake K, Asaga T, et al. Late phase II clinical study of vinorelbine monotherapy in advanced or recurrent breast cancer previously treated with anthracyclines and taxanes. Jpn J Clin Oncol. 2005;35:310–5.
12. Smorenburg CH, Bontenbal M, Seynaeve C, van Zuylen C, de Heus G, Verweij J, et al. Phase II study of weekly gemcitabine in patients with metastatic breast cancer relapsing or failing both an anthracycline and a taxane. Breast Cancer Res Treat. 2001;66:83–7.
13. Spielmann M, Llombart-Cussac A, Kalla S, Espié M, Namer M, Ferrero JM, et al. Single-agent gemcitabine is active in previously treated metastatic breast cancer. Oncology. 2001;60:303–7.
14. Rha SY, Moon YH, Jeung HC, Kim YT, Sohn JH, Yang WI, et al. Gemcitabine monotherapy as salvage chemotherapy in heavily pretreated metastatic breast cancer. Breast Cancer Res Treat. 2005;90:215–21.
15. Perez EA, Hillman DW, Mailliard JA, Ingle JN, Ryan JM, Fitch TR, et al. Randomized phase II study of two irinotecan schedules for patients with metastatic breast cancer refractory to an anthracycline, a taxane, or both. J Clin Oncol. 2004;22:2849–55.
16. Thomas E, Tabernero J, Fornier M, Conté P, Fumoleau P, Lluch A, et al. Phase II clinical trial of ixabepilone (BMS-247550), an epothilone B analog, in patients with taxane-resistant metastatic breast cancer. J Clin Oncol. 2007;25:3399–406.
17. Perez EA, Lerzo G, Pivot X, Thomas E, Vahdat L, Bosserman L, et al. Efficacy and safety of ixabepilone (BMS-247550) in a phase II study of patients with advanced breast cancer resistant to an anthracycline, a taxane, and capecitabine. J Clin Oncol. 2007;25:3407–14.
18. Thomas ES, Gomez HL, Li RK, Chung HC, Fein LE, Chan VF, et al. Ixabepilone plus capecitabine for metastatic breast cancer progressing after anthracycline and taxane treatment. J Clin Oncol. 2007;25:5210–7.
19. Hirata K, Horikoshi N, Aiba K, Okazaki M, Denno R, Sasaki K, et al. Pharmacokinetic study of S-1, a novel oral fluorouracil antitumor drug. Clin Cancer Res. 1999;5:2000–5.
20. Nukatsuka M, Fujioka A, Nakagawa F, Oshimo H, Kitazato K, Uchida J, et al. Antimetastatic and anticancer activity of S-1, a new oral dihydropyrimidine-dehydrogenase-inhibiting fluoropyrimidine, alone and in combination with paclitaxel in an orthotopically implanted human breast cancer model. Int J Oncol. 2004;25:1531–6.
21. Saeki T, Takashima S, Sano M, Horikoshi N, Miura S, Shimizu S, et al. A phase II study of S-1 in patients with metastatic breast cancer—a Japanese trial by the S-1 Cooperative Study Group, Breast Cancer Working Group. Breast Cancer. 2004;11:194–202.
22. Hino M, Saeki T, Sato Y, Sano M, S-1 Cooperative Study Group, Breast Cancer Working Group. Late phase II study of S-1 in patients with taxane resistant breast cancer (ASCO Annual Meeting Proceedings). J Clin Oncol. 2004;22(14S):745.

23. O'Shaughnessy J, Miles D, Vukelja S, Moiseyenko V, Ayoub JP, Cervantes G, et al. Superior survival with capecitabine plus docetaxel combination therapy in anthracycline-pretreated patients with advanced breast cancer: phase III trial results. J Clin Oncol. 2002;20:2812–23.

24. Leonard R, O'Shaughnessy J, Vukelja S, Gorbounova V, Chan-Navarro CA, Maraninchi D, et al. Detailed analysis of a randomized phase III trial: can the tolerability of capecitabine plus docetaxel be improved without compromising its survival advantage? Ann Oncol. 2006;17:1379–85.

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Minireview

Emerging ethnic differences in lung cancer therapy

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Although global clinical trials for lung cancer can enable the development of new agents efficiently, whether the results of clinical trials performed in one population can be fully extrapolated to another population remains questionable. A comparison of phase III trials for the same drug combinations against lung cancer in different countries shows a great diversity in haematological toxicity. One possible reason for this diversity may be that different ethnic populations may have different physiological capacities for white blood cell production and maturation. In addition, polymorphisms in the promoter and coding regions of drug-metabolising enzymes (e.g., CYP3A4 and UGT1A1) or in transporters (e.g., ABCB1) may vary among different ethnic populations. For example, epidermal growth factor receptor (EGFR) inhibitors are more effective in Asian patients than in patients of other ethnicities, a characteristic that parallels the incidence of EGFR-activating mutations. Interstitial lung disease associated with the administration of gefitinib is also more common among Japanese patients than among patients of other ethnicities. Although research into these differences has just begun, these studies suggest that possible pharmacogenomic and tumour genetic differences associated with individual responses to anticancer agents should be carefully considered when conducting global clinical trials.
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Lung cancer is the most common malignancy worldwide. Approximately 1.2 million people are diagnosed with lung cancer annually (accounting for 12.3% of all cancers); the second most common malignancy is breast cancer (10.4%), followed by colorectal cancer (9.4%). As lung cancer almost invariably has a poor prognosis, it is the largest single cause of death from cancer in the world, with a mortality of 1.1 million annually (Stewart and Kleihues, 2003). Only 15% of lung cancer patients have a disease that is confined to the lung and are candidates for surgical resection; most patients with this disease have distant metastases or pleural effusion at the time of their initial diagnosis. These patients can be treated with systemic chemotherapy, but the efficacy of currently available anticancer agents is limited and patients with advanced diseases rarely live long.

As the development of new anticancer agents and chemotherapeutic regimens is both time and money consuming, clinical trials need to be as efficient as possible. One effort in this direction has been the adoption of global clinical trials for new agents that involve trial centres on more than one continent; this strategy enables adequate sample sizes to be obtained in a relatively short-time period and eliminates the need for redundant clinical trials with similar objectives conducted in different countries. However, whether the results of clinical trials performed in one population can be fully extrapolated to other populations remains questionable because of potential differences in trial designs, study-specific criteria, patient demographics, frequency of monitoring, and population-related pharmacokinetics, pharmacodynamics and pharmacogenomics. Recently, these genetic and physiologic factors influencing cancer chemotherapy have been increasingly examined and reported.

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CLINICAL OBSERVATIONS OF TOXICITY DURING CYTOTOXIC CHEMOTHERAPY

A comparison of phase III trials for the same drug combinations against non-small cell lung cancer conducted in different countries shows a great diversity in toxicity (Sekine *et al*, 2006). Among trials studying the combination of carboplatin and paclitaxel, the dose of carboplatin was fixed in all the trials, but the dose of paclitaxel was 200 mg m^{-2} in Japanese and European trials and 225 mg m^{-2} in American trials. Grades 3–4 neutropenia was noted in 88% of the patients in the Japanese trial, 15–51% of the patients in the European trials, and 6–65% of the patients in the American trials. Meanwhile, grades 3–4 febrile neutropenia was encountered in 16% of the patients in the Japanese trial, 0–9% of the patients in the European trials, and 2–4% of the patients in the American trials (Table 1). For combinations of cisplatin and docetaxel (Table 1) and cisplatin and vinorelbine (Table 2), the incidences of grades 3–4 neutropenia and febrile neutropenia were almost the same between phase III trials performed in different areas, but the doses of docetaxel and vinorelbine in the Japanese trials were lower than those in the European and American trials. Thus, neutropenia in patients receiving a combination of platinum and antimicrotubule agents may be more severe in Japanese than in Europeans and Americans. A higher frequency of grades 3–4 neutropenia in Japanese patients than in American patients was associated with combinations of cisplatin and irinotecan (65 *vs*

Table 1 Toxicity associated with a combination of platinum and taxane

Research group	Chemotherapy dose: Platinum	Chemotherapy dose: Taxane	No. of patients	Grades 3–4 toxicity (%): NP	Grades 3–4 toxicity (%): FNP	Reference
A combination of carboplatin and paclitaxel						
Japan	6 (AUC)	200 ($mg\,m^{-2}$)	145	88	16	Ohe *et al* (2007)
Greece	6 (AUC)	200 ($mg\,m^{-2}$)	252	15	0	Kosmidis *et al* (2002)
EU	6 (AUC)	200 ($mg\,m^{-2}$)	309	51	4	Rosell *et al* (2002)
ECOG	6 (AUC)	225 ($mg\,m^{-2}$)	290	63	4	Schiller *et al* (2002)
SWOG	6 (AUC)	225 ($mg\,m^{-2}$)	206	57	2	Kelly *et al* (2001)
SWOG	6 (AUC)	225 ($mg\,m^{-2}$)	182	—	3	Gandara *et al* (2004)
USA	6 (AUC)	225 ($mg\,m^{-2}$)	190	65	—	Belani *et al* (2005)
USA	6 (AUC)	225 ($mg\,m^{-2}$)	345	6	—	Herbst *et al* (2004)
A combination of cisplatin and docetaxel						
Japan	80 ($mg\,m^{-2}$)	60 ($mg\,m^{-2}$)	151	74	2	Ohe *et al* (2007)
ECOG	75 ($mg\,m^{-2}$)	75 ($mg\,m^{-2}$)	289	69	11	Schiller *et al* (2002)
USA	75 ($mg\,m^{-2}$)	75 ($mg\,m^{-2}$)	408	75	5	Fossella *et al* (2003)

NP, neutropenia; FNP, febrile neutropenia.

Table 2 Toxicity associated with a combination of cisplatin and vinorelbine

Research group	Chemotherapy dose ($mg\,m^{-2}$): Cisplatin	Chemotherapy dose ($mg\,m^{-2}$): Vinorelbine	No. of patients	Grades 3–4 toxicity (%): NP	Grades 3–4 toxicity (%): FNP	Reference
Japan	80 (day 1)	25 (days 1, 8)	145	88	18	Ohe *et al* (2007)
Greece	80 (day 8)	30 (days 1, 8)	204	37	11	Georgoulias *et al* (2005)
France	100 (day 1)	30 (weekly)	156	83	22	Pujol *et al* (2005)
EU	120 (day 1)	30 (weekly)	206	79	4	Le Chevalier *et al* (1994)
SWOG	100 (day 1)	25 (weekly)	202	76	1	Kelly *et al* (2001)
USA	100 (day 1)	25 (weekly)	404	79	5	Fossella *et al* (2003)

NP, neutropenia; FNP, febrile neutropenia.

32%, $P<0.001$) and cisplatin and etoposide (92 *vs* 66%, $P<0.001$) for the treatment of extensive small-cell lung cancer (Lara *et al*, 2007).

How can this ethnic difference in the severity of neutropenia be explained? One possibility is that the physiological capacity of the white blood cell production and maturation may vary among different ethnic populations. An asymptomatic reduction in neutrophils (benign neutropenia) is more commonly observed in individuals of African descent than in Caucasians, and no data on this phenomenon are available for Asians (Hsieh *et al*, 2007). The mechanisms are unclear, but a lower bone marrow reserve, an intrinsic marrow difference, an abnormal cytokine response, or any combination of these factors have been suggested (Hsieh *et al*, 2007). The lower neutrophil counts were associated with higher levels of IL-8 and granulocyte colony-stimulating factor in African volunteers. Thus, these cytokines are considered to compensate for the relatively low neutrophil counts in this population (Mayr *et al*, 2007). A recent report showed that ethnicity-related low neutrophil counts were associated with neutrophil elastase (ELA2) polymorphisms (C-199A), but not with serum cytokine levels (Grann *et al*, 2007).

ETHNIC DIFFERENCES IN DRUG METABOLISING ENZYMES

An explanation for the ethnic differences in haematological toxicity may be the varying activities of drug-metabolising enzymes and transporters that are mainly associated with polymorphisms in the promoter and coding regions of these enzymes (Fujita and Sasaki, 2007). The haematological toxicity of docetaxel monotherapy was associated with the clearance of this agent in Asian patients, a phenomenon that can be largely explained by CYP3A4 activity (Yamamoto *et al*, 2000). A study conducted in the Netherlands showed that docetaxel clearance was associated with the homozygous C1236T polymorphism in the ABCB1 (p-glycoprotein) gene (ABCB1*8) but was not associated with any CYP3A4 gene polymorphisms (Bosch *et al*, 2006). In contrast, docetaxel pharmacokinetics were not associated with the percent decrease in neutrophil counts nor with any polymorphisms in the CYP3A4 and ABCB1 genes in American patients (Lewis *et al*, 2007). Another example of ethnic differences in drug-metabolising enzymes is the association between polymorphisms in genes involved in irinotecan metabolism and irinotecan-induced neutropenia. Among the patients who received irinotecan with or without another anticancer agent, grade 4 neutropenia was noted in 40–57% of the patients with UDP-glucuronosyltransferase (UGT) 1A1*28 (a polymorphism in the promoter region of the UGT1A1 gene) homozygosity, whereas neutropenia was only observed in 15% or less of the patients with wild-type alleles. This association was consistent in both Asian and Caucasian patients, although the frequency of homozygosity was about 10% in Caucasians and much lower in Asians. The UGT1A1*6 allele is another polymorphism at exon 1 that is associated with defective glucuronidating function and is found almost exclusively in Asian individuals with a frequency as high as 20% (Fujita and Sasaki, 2007). UGT1A1*6 is significantly linked to polymorphisms of UGT1A7 and UGT1A9. A haplotype including UGT1A1*6 and UGT1A7*3, noted in as many as 15% of Japanese patients, and UGT1A1*6 homozygosity, noted in 7% of Korean patients, were significantly associated with decreased glucuronosyltransferase activity for SN-38 and severe neutropenia (Han *et al*, 2006; Fujita

et al, 2007). In 177 Japanese patients treated with irinotecan-including chemotherapy, a homozygous or double heterozygous genotype for UGT1A1*6 and UGT1A1*28 (*6/*6, *28/*28 or *6/*28) was significantly associated with severe neutropenia (Minami *et al*, 2007). In addition, patients with a homozygous C3435T polymorphism in the ABCB1 gene are four-fold more likely to develop grade 3 diarrhoea when treated with a combination of cisplatin and irinotecan (Lara *et al*, 2007).

Data on associations between polymorphisms in genes coding drug-metabolising enzymes and therapeutic efficacy remain scarce. A recent prospective study in 250 patients with metastatic colorectal cancer showed a significantly higher response rate (67 *vs* 40%) and a nonsignificant survival advantage (hazard ratio (HR): 0.81; 95% confidence interval (CI): 0.45–1.44) in patients homozygous for UGT1A1*28, compared with those with wild-type alleles; these outcomes were associated with a higher exposure to SN-38 (Toffoli *et al*, 2006). In a study of 81 NSCLC patients, those who were homozygous for UGT1A1*6 had a lower response rate (0 *vs* 50%, $P=0.038$) and a poorer MST (7.6 *vs* 17.7 months, $P=0.017$) as well as greater toxicities than the other patients (Han *et al*, 2006). The most plausible explanation for the negative effects of UGT1A1*6 on treatment outcome may be that the dose intensity or cycle number might have been reduced in patients with UGT1A1*6 because of polymorphism-associated toxicities (Fujita and Sasaki, 2007).

These pharmacogenetic analyses have been rather preliminary. Data on genotyping, pharmacokinetics, and pharmacodynamics collected from a large number of patients with different ethnic backgrounds are needed to demonstrate the cause of ethnic differences in chemotherapy-associated toxicity.

EFFICACY OF EPIDERMAL GROWTH FACTOR RECEPTOR TYROSINE KINASE INHIBITORS

Epidermal growth factor receptor (EGFR), a cell membrane receptor with tyrosine kinase activity, is expressed in most patients with NSCLC and plays a role in cellular proliferation, inhibition of apoptosis, angiogenesis, metastatic potential, and chemoresistance. Small-molecule inhibitors of EGFR, such as gefitinib and erlotinib, have shown antitumor activity and have alleviated symptoms in NSCLC patients who were previously treated with standard chemotherapy. Two randomized phase II studies, IDEAL (Iressa Dose Evaluation in Advanced Lung Cancer)-1 (involving 210 patients and conducted in Europe, Australia, South Africa, and Japan) and IDEAL-2 (involving 216 patients and conducted in the USA), have evaluated the efficacy of gefitinib at a dose of either 250 mg daily or 500 mg daily in patients with advanced NSCLC in whom earlier platinum-based chemotherapy had failed. No difference in the response rates between the doses was noted, but an increased response rate was recorded for never smokers, women, and those with an adenocarcinoma histology, compared with patients who did not have these characteristics. In addition, the response rate was 28% in Japanese patients but only 9–12% in patients of other ethnicities (Fukuoka *et al*, 2003; Kris *et al*, 2003). A randomized phase III trial, ISEL (Iressa Survival Evaluation in Lung Cancer), of gefitinib *vs* a placebo in 1692 NSCLC patients who had been previously treated with one or two chemotherapeutic regimens failed to show any survival benefit of gefitinib; in the overall population, the median survival times (MSTs) in the gefitinib and placebo arms were 5.6 and 5.1 months, respectively (HR: 0.89; 95% CI: 0.78–1.03). A subgroup analysis, however, showed that the MST was longer in Asian patients receiving gefitinib than in those receiving the placebo (MST: 9.5 *vs* 5.5 months; HR: 0.66; 95% CI: 0.48–0.91). Similar results were seen for never smokers: patients receiving gefitinib survived longer than those receiving the placebo (MST: 8.9 *vs* 6.1 months; HR: 0.67, 95% CI: 0.49–0.91) (Thatcher *et al*, 2005).

A similar association between objective responses and ethnicity was observed in studies on erlotinib monotherapy for previously treated advanced NSCLC. In an American phase II trial of this agent in 57 advanced NSCLC patients with disease progression or relapse after platinum-based chemotherapy, the response rate was 12% and the MST was 8.4 months (Perez-Soler *et al*, 2004). In contrast, the combined data of two Japanese phase II trials of erlotinib in similar patient populations showed objective responses in 30 of 106 (28%) patients and an MST of 13.8 months. Among the responders, significantly higher proportions of females (50%) than males (17%) ($P=0.0009$) and of never smokers (51%) than smokers (14%) were observed ($P<0.0001$) (Tamura *et al*, 2007). A phase III trial of erlotinib or a placebo in 731 NSCLC patients previously treated with one or two chemotherapy regimens showed that the response rate in Asian patients was higher than that in patients of other ethnicities (28 *vs* 10%, $P=0.02$) (Shepherd *et al*, 2005).

These results of phases II and III trials consistently suggest that EGFR tyrosine kinase inhibitors may be more effective in Asian patients than in patients of other ethnicities.

In April 2004, the activating mutations of the EGFR gene were identified in NSCLC specimens, and cancers with these mutations were reported to be highly sensitive to gefitinib. The populations with higher responses to gefitinib (females, non-smokers and patients with an adenocarcinoma histology) also have higher incidences of EGFR mutations (Kosaka *et al*, 2004; Pao *et al*, 2004; Shigematsu *et al*, 2005). The incidence of EGFR mutations in surgically resected tissue samples is summarised in Table 3 (Kosaka *et al*, 2004; Pao *et al*, 2004; Marchetti *et al*, 2005; Qin *et al*, 2005; Shigematsu *et al*, 2005; Soung *et al*, 2005; Tokumo *et al*, 2005; Yang *et al*, 2005; Sasaki *et al*, 2006). The incidence varies from one report to another, but EGFR mutations tend to be more common among patients with an adenocarcinoma histology and among non-smokers. Among Asian patients, the average incidences of EGFR mutations were 31% overall, 47% among patients with adenocarcinoma, and 56% among non-smokers; among other ethnic populations, however, the average incidences were 7–8% overall, 13–15% among patients with adenocarcinoma, and 34–35% among non-smokers (Table 3). Thus, the percentage of responders to gefitinib or erlotinib almost paralleled the percentage of patients with EGFR mutations.

The mechanism responsible for the high frequency of EGFR mutations in Asian patients is a subject of great interest, and polymorphisms in the regulatory sequence of the EGFR gene have been vigorously investigated. The CA simple sequence repeat 1 (CA-SSR1), a highly polymorphic locus containing 14–21 CA dinucleotide repeats, is located at the 5′ end of intron 1 of the EGFR gene. Studies of CA-SSR1 repeat length and EGFR expression in breast cancer tissues have shown a constant decline in EGFR expression with increasing repeat length (Buerger *et al*, 2000, 2004). In addition, a shorter repeat length was associated with an elevated risk of lung cancer (Zhang *et al*, 2007) and poor survival in NSCLC patients (Dubey *et al*, 2006). The CA-SSR1 repeat length distribution varies according to ethnicity, with Asians tending to have longer repeats than Americans (Liu *et al*, 2003). Two single-nucleotide polymorphisms in the promoter region of the EGFR gene (−219G/T and −191C/A) were also associated with promoter activity and EGFR expression (Liu *et al*, 2005), and their polymorphic types (associated with low EGFR expression) were more common among Asians than among other ethnicities (Nomura *et al*, 2007). These observations suggest that many Asians have polymorphic types that lead to a decreased intrinsic production of EGFR protein. If a certain critical level of EGFR is required to drive the cell toward a malignant phenotype, another mechanism including activating mutations of EGFR and/or the autonomous activation of downstream signalling may be required for the development of lung cancer among Asians (Nomura *et al*, 2007).

Table 3 Incidence of EGFR mutations in surgically resected specimens

		All cases		Adenocarcinoma		Non-smokers	
Author	Country	Total N	Mutation N (%)	Total N	Mutation N (%)	Total N	Mutation N (%)
Western areas							
Shigematsu	USA	80	11 (14)	44	11 (25)	26	7 (27)
Pao	USA	96	11 (11)	72	11 (15)	15	7 (47)
Yang	USA	219	26 (12)	164	25 (15)	34	12 (35)
Marchetti	Italy	860	39 (5)	375	39 (10)	103[a]	23 (22)
	Subtotal	1255	87 (7)	655	86 (13)	75	26 (35)
Asian areas							
Shigematsu	Japan	263	71 (27)	154	67 (44)	78	47 (60)
Kosaka	Japan	277	111 (40)	224	110 (49)	112[a]	76 (68)
Tokumo	Japan	120	38 (32)	82	37 (45)	36	25 (69)
Sasaki	Japan	95	35 (37)	71	32 (45)	36	25 (69)
Shigematsu	Taiwan	93	32 (34)	55	31 (56)	55	27 (49)
Qin	China	41	10 (24)	17	7 (41)	21	6 (29)
Soung	Korea	153	30 (20)	69	26 (38)	54	25 (46)
Shigematsu	Others	361	107 (30)	214	102 (48)	135	76 (56)
	Subtotal	1403	434 (31)	886	412 (47)	415	231 (56)
Other areas							
Shigematsu	Australia	83	6 (7)	36	5 (14)	7	4 (57)
Shigematsu	Others	158	13 (8)	75	12 (16)	31	9 (29)
	Subtotal	241	19 (8)	111	17 (15)	38	13 (34)
	Toatl	2899	540 (19)	1652	515 (31)	528	270 (51)

[a]Including only patients with adenocarcinoma histology.

INTERSTITIAL LUNG DISEASE ASSOCIATED WITH GEFITINIB AND ERLOTINIB

The frequencies of grades 3–4 common toxicities after the administration of gefitinib, including diarrhoea, skin rash, and elevated liver transaminase levels, have been similar among study populations, but the incidence of severe interstitial lung disease (ILD) associated with the administration of gefitinib differs between patients in Japan and those in other countries. In the IDEAL studies, two Japanese patients developed grades 3–4 ILD (2%), whereas no patients outside of Japan experienced ILD (Fukuoka *et al*, 2003; Kris *et al*, 2003). A retrospective study of 1976 consecutive patients treated with gefitinib at 84 institutions showed that the incidence of ILD was 3.5% and the mortality rate was 1.6%. Several risk factors for the development of gefitinib-induced ILD were identified in the Japanese population: a history of pulmonary fibrosis, a history of smoking, a poor performance status, and a male sex (Ando *et al*, 2006). A similar incidence of ILD (4.6%) was also noted in association with erlotinib chemotherapy in Japanese phase II trials (Tamura *et al*, 2007).

The association between ILD and anticancer treatment is a major topic in Japan because (1) the diagnosis of ILD can be difficult and a consensus among physicians is sometimes not reached, (2) the risk factors for ILD have not been fully established, 3) an effective treatment for ILD has not been established and the condition is often fatal, and (4) the low frequency of this complication makes it difficult to conduct pertinent clinical trials. Gefitinib-induced ILD seems to be more common among Japanese patients than among other patients, but the reasons for this ethnic difference are totally unknown.

CONCLUSION

The findings discussed here suggest that considerable variations in the toxicity and efficacy of anticancer agents may exist among patients of different ethnicities. Although research into these differences has just begun, these studies suggest that possible pharmacogenomic and tumour genetic differences associated with individual responses to anticancer agents should be carefully considered when conducting global clinical trials.

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REFERENCES

Ando M, Okamoto I, Yamamoto N, Takeda K, Tamura K, Seto T, Ariyoshi Y, Fukuoka M (2006) Predictive factors for interstitial lung disease, antitumor response, and survival in non-small-cell lung cancer patients treated with gefitinib. *J Clin Oncol* 24: 2549–2556

Belani CP, Lee JS, Socinski MA, Robert F, Waterhouse D, Rowland K, Ansari R, Lilenbaum R, Natale RB (2005) Randomized phase III trial comparing cisplatin-etoposide to carboplatin-paclitaxel in advanced or metastatic non-small cell lung cancer. *Ann Oncol* 16: 1069–1075

Bosch TM, Huitema AD, Doodeman VD, Jansen R, Witteveen E, Smit WM, Jansen RL, van Herpen CM, Soesan M, Beijnen JH, Schellens JH (2006) Pharmacogenetic screening of CYP3A and ABCB1 in relation to population pharmacokinetics of docetaxel. *Clin Cancer Res* **12**: 5786–5793

Buerger H, Gebhardt F, Schmidt H, Beckmann A, Hutmacher K, Simon R, Lelle R, Boecker W, Brandt B (2000) Length and loss of heterozygosity of an intron 1 polymorphic sequence of egfr is related to cytogenetic alterations and epithelial growth factor receptor expression. *Cancer Res* **60**: 854–857

Buerger H, Packeisen J, Boecker A, Tidow N, Kersting C, Bielawski K, Isola J, Yatabe Y, Nakachi K, Boecker W, Brandt B (2004) Allelic length of a CA dinucleotide repeat in the egfr gene correlates with the frequency of amplifications of this sequence – first results of an inter-ethnic breast cancer study. *J Pathol* **203**: 545–550

Dubey S, Stephenson P, Levy DE, Miller JA, Keller SM, Schiller JH, Johnson DH, Kolesar JM (2006) EGFR dinucleotide repeat polymorphism as a prognostic indicator in non-small cell lung cancer. *J Thorac Oncol* **1**: 406–412

Fossella F, Pereira JR, von Pawel J, Pluzanska A, Gorbounova V, Kaukel E, Mattson KV, Ramlau R, Szczesna A, Fidias P, Millward M, Belani CP (2003) Randomized, multinational, phase III study of docetaxel plus platinum combinations versus vinorelbine plus cisplatin for advanced non-small-cell lung cancer: the TAX 326 study group. *J Clin Oncol* **21**: 3016–3024

Fujita K, Ando Y, Nagashima F, Yamamoto W, Eodo H, Araki K, Kodama K, Miya T, Narabayashi M, Sasaki Y (2007) Genetic linkage of UGT1A7 and UGT1A9 polymorphisms to UGT1A1*6 is associated with reduced activity for SN-38 in Japanese patients with cancer. *Cancer Chemother Pharmacol* **60**: 515–522

Fujita K, Sasaki Y (2007) Pharmacogenomics in drug-metabolizing enzymes catalyzing anticancer drugs for personalized cancer chemotherapy. *Curr Drug Metab* **8**: 554–562

Fukuoka M, Yano S, Giaccone G, Tamura T, Nakagawa K, Douillard JY, Nishiwaki Y, Vansteenkiste J, Kudoh S, Rischin D, Eek R, Horai T, Noda K, Takata I, Smit E, Averbuch S, Macleod A, Feyereislova A, Dong RP, Baselga J (2003) Multi-institutional randomized phase II trial of gefitinib for previously treated patients with advanced non-small-cell lung cancer (The IDEAL 1 Trial) [corrected]. *J Clin Oncol* **21**: 2237–2246

Gandara DR, Ohe Y, Kubota K, Nishiwaki Y, Ariyoshi Y, Saijo N, Williamson S, Lara PN, Crowley J, Fukuoka M (2004) Japan-SWOG common arm analysis of paclitaxel/carboplatin in advanced stage non-small cell lung cancer (NSCLC): a model for prospective comparison of cooperative group trials. *Proc ASCO* **22**: 618s

Georgoulias V, Ardavanis A, Tsiafaki X, Agelidou A, Mixalopoulou P, Anagnostopoulou O, Ziotopoulos P, Toubis M, Syrigos K, Samaras N, Polyzos A, Christou A, Kakolyris S, Kouroussis C, Androulakis N, Samonis G, Chatzidaki D (2005) Vinorelbine plus cisplatin versus docetaxel plus gemcitabine in advanced non-small-cell lung cancer: a phase III randomized trial. *J Clin Oncol* **23**: 2937–2945

Grann V, Bowman N, Wei Y, Horwitz M, Joseph C, Abdul K, Abdul K, Barlatier H, Sandoval R, Jacobson J, Hershman D (2007) Ethnic neutropenia among women of European, African, and Caribbean backgrounds. *J Clin Oncol* **25**(Suppl): 343s (abstract 6587)

Han JY, Lim HS, Shin ES, Yoo YK, Park YH, Lee JE, Jang IJ, Lee DH, Lee JS (2006) Comprehensive analysis of UGT1A polymorphisms predictive for pharmacokinetics and treatment outcome in patients with non-small-cell lung cancer treated with irinotecan and cisplatin. *J Clin Oncol* **24**: 2237–2244

Herbst RS, Giaccone G, Schiller JH, Natale RB, Miller V, Manegold C, Scagliotti G, Rosell R, Oliff I, Reeves JA, Wolf MK, Krebs AD, Averbuch SD, Ochs JS, Grous J, Fandi A, Johnson DH (2004) Gefitinib in combination with paclitaxel and carboplatin in advanced non-small-cell lung cancer: a phase III trial – INTACT 2. *J Clin Oncol* **22**: 785–794

Hsieh MM, Everhart JE, Byrd-Holt DD, Tisdale JF, Rodgers GP (2007) Prevalence of neutropenia in the US population: age, sex, smoking status, and ethnic differences. *Ann Intern Med* **146**: 486–492

Kelly K, Crowley J, Bunn Jr PA, Presant CA, Grevstad PK, Moinpour CM, Ramsey SD, Wozniak AJ, Weiss GR, Moore DF, Israel VK, Livingston RB, Gandara DR (2001) Randomized phase III trial of paclitaxel plus carboplatin versus vinorelbine plus cisplatin in the treatment of patients with advanced non-small-cell lung cancer: a Southwest Oncology Group trial. *J Clin Oncol* **19**: 3210–3218

Kosaka T, Yatabe Y, Endoh H, Kuwano H, Takahashi T, Mitsudomi T (2004) Mutations of the epidermal growth factor receptor gene in lung cancer: biological and clinical implications. *Cancer Res* **64**: 8919–8923

Kosmidis P, Mylonakis N, Nicolaides C, Kalophonos C, Samantas E, Boukovinas J, Fountzilas G, Skarlos D, Economopoulos T, Tsavdaridis D, Papakostas P, Bacoyiannis C, Dimopoulos M (2002) Paclitaxel plus carboplatin versus gemcitabine plus paclitaxel in advanced non-small-cell lung cancer: a phase III randomized trial. *J Clin Oncol* **20**: 3578–3585

Kris MG, Natale RB, Herbst RS, Lynch Jr TJ, Prager D, Belani CP, Schiller JH, Kelly K, Spiridonidis H, Sandler A, Albain KS, Cella D, Wolf MK, Averbuch SD, Ochs JJ, Kay AC (2003) Efficacy of gefitinib, an inhibitor of the epidermal growth factor receptor tyrosine kinase, in symptomatic patients with non-small cell lung cancer: a randomized trial. *JAMA* **290**: 2149–2158

Lara P, Redman M, Lenz H, Gordon M, Shibata T, Fukuda H, Tamura T, Saijo N, Natale R, Gandara D (2007) Cisplatin (Cis)/etoposide (VP16) compared to cis/irinotecan (CPT11) in extensive-stage small cell lung cancer (E-SCLC): pharmacogenomic (PG) and comparative toxicity analysis of JCOG 9511 and SWOG 0124. *J Clin Oncol* **25**(Suppl): 390s (abstract 7524)

Le Chevalier T, Brisgand D, Douillard JY, Pujol JL, Alberola V, Monnier A, Riviere A, Lianes P, Chomy P, Cigolari S, Gottfried M, Ruffie P, Panizo A, Gaspard MH, Ravaioli A, Besenval M, Besson F, Martinez A, Berthaud P, Tursz T (1994) Randomized study of vinorelbine and cisplatin versus vindesine and cisplatin versus vinorelbine alone in advanced non-small-cell lung cancer: results of a European multicenter trial including 612 patients. *J Clin Oncol* **12**: 360–367

Lewis LD, Miller AA, Rosner GL, Dowell JE, Valdivieso M, Relling MV, Egorin MJ, Bies RR, Hollis DR, Levine EG, Otterson GA, Millard F, Ratain MJ (2007) A comparison of the pharmacokinetics and pharmacodynamics of docetaxel between African-American and Caucasian cancer patients: CALGB 9871. *Clin Cancer Res* **13**: 3302–3311

Liu W, Innocenti F, Chen P, Das S, Cook Jr EH, Ratain MJ (2003) Interethnic difference in the allelic distribution of human epidermal growth factor receptor intron 1 polymorphism. *Clin Cancer Res* **9**: 1009–1012

Liu W, Innocenti F, Wu MH, Desai AA, Dolan ME, Cook Jr EH, Ratain MJ (2005) A functional common polymorphism in a Sp1 recognition site of the epidermal growth factor receptor gene promoter. *Cancer Res* **65**: 46–53

Marchetti A, Martella C, Felicioni L, Barassi F, Salvatore S, Chella A, Camplese PP, Iarussi T, Mucilli F, Mezzetti A, Cuccurullo F, Sacco R, Buttitta F (2005) EGFR mutations in non-small-cell lung cancer: analysis of a large series of cases and development of a rapid and sensitive method for diagnostic screening with potential implications on pharmacologic treatment. *J Clin Oncol* **23**: 857–865

Mayr FB, Spiel AO, Leitner JM, Firbas C, Kliegel T, Jilma B (2007) Ethnic differences in plasma levels of interleukin-8 (IL-8) and granulocyte colony stimulating factor (G-CSF). *Transl Res* **149**: 10–14

Minami H, Sai K, Saeki M, Saito Y, Ozawa S, Suzuki K, Kaniwa N, Sawada J, Hamaguchi T, Yamamoto N, Shirao K, Yamada Y, Ohmatsu H, Kubota K, Yoshida T, Ohtsu A, Saijo N (2007) Irinotecan pharmacokinetics/pharmacodynamics and UGT1A genetic polymorphisms in Japanese: roles of UGT1A1*6 and *28. *Pharmacogenet Genomics* **17**: 497–504

Nomura M, Shigematsu H, Li L, Suzuki M, Takahashi T, Estess P, Siegelman M, Feng Z, Kato H, Marchetti A, Shay JW, Spitz MR, Wistuba II, Minna JD, Gazdar AF (2007) Polymorphisms, mutations, and amplification of the EGFR gene in non-small cell lung cancers. *PLoS Med* **4**: e125

Ohe Y, Ohashi Y, Kubota K, Tamura T, Nakagawa K, Negoro S, Nishiwaki Y, Saijo N, Ariyoshi Y, Fukuoka M (2007) Randomized phase III study of cisplatin plus irinotecan versus carboplatin plus paclitaxel, cisplatin plus gemcitabine, and cisplatin plus vinorelbine for advanced non-small-cell lung cancer: Four-Arm Cooperative Study in Japan. *Ann Oncol* **18**: 317–323

Pao W, Miller V, Zakowski M, Doherty J, Politi K, Sarkaria I, Singh B, Heelan R, Rusch V, Fulton L, Mardis E, Kupfer D, Wilson R, Kris M, Varmus H (2004) EGF receptor gene mutations are common in lung cancers from 'never smokers' and are associated with sensitivity of tumors to gefitinib and erlotinib. *Proc Natl Acad Sci USA* **101**: 13306–13311

Perez-Soler R, Chachoua A, Hammond LA, Rowinsky EK, Huberman M, Karp D, Rigas J, Clark GM, Santabarbara P, Bonomi P (2004) Determinants of tumor response and survival with erlotinib in patients with non-small-cell lung cancer. *J Clin Oncol* **22**: 3238–3247

Pujol JL, Breton JL, Gervais R, Rebattu P, Depierre A, Morere JF, Milleron B, Debieuvre D, Castera D, Souquet PJ, Moro-Sibilot D, Lemarie E, Kessler R, Janicot H, Braun D, Spaeth D, Quantin X, Clary C (2005) Gemcitabine-docetaxel versus cisplatin-vinorelbine in advanced or metastatic non-small-cell lung cancer: a phase III study addressing the case for cisplatin. *Ann Oncol* 16: 602–610

Qin BM, Chen X, Zhu JD, Pei DQ (2005) Identification of EGFR kinase domain mutations among lung cancer patients in China: implication for targeted cancer therapy. *Cell Res* 15: 212–217

Rosell R, Gatzemeier U, Betticher DC, Keppler U, Macha HN, Pirker R, Berthet P, Breau JL, Lianes P, Nicholson M, Ardizzoni A, Chemaissani A, Bogaerts J, Gallant G (2002) Phase III randomised trial comparing paclitaxel/carboplatin with paclitaxel/cisplatin in patients with advanced non-small-cell lung cancer: a cooperative multinational trial. *Ann Oncol* 13: 1539–1549

Sasaki H, Shimizu S, Endo K, Takada M, Kawahara M, Tanaka H, Matsumura A, Iuchi K, Haneda H, Suzuki E, Kobayashi Y, Yano M, Fujii Y (2006) EGFR and erbB2 mutation status in Japanese lung cancer patients. *Int J Cancer* 118: 180–184

Schiller JH, Harrington D, Belani CP, Langer C, Sandler A, Krook J, Zhu J, Johnson DH (2002) Comparison of four chemotherapy regimens for advanced non-small-cell lung cancer. *N Engl J Med* 346: 92–98

Sekine I, Nokihara H, Yamamoto N, Kunitoh H, Ohe Y, Saijo N, Tamura T (2006) Common arm analysis: one approach to develop the basis for global standardization in clinical trials of non-small cell lung cancer. *Lung Cancer* 53: 157–164

Shepherd FA, Rodrigues Pereira J, Ciuleanu T, Tan EH, Hirsh V, Thongprasert S, Campos D, Maoleekoonpiroj S, Smylie M, Martins R, van Kooten M, Dediu M, Findlay B, Tu D, Johnston D, Bezjak A, Clark G, Santabarbara P, Seymour L (2005) Erlotinib in previously treated non-small-cell lung cancer. *N Engl J Med* 353: 123–132

Shigematsu H, Lin L, Takahashi T, Nomura M, Suzuki M, Wistuba II, Fong KM, Lee H, Toyooka S, Shimizu N, Fujisawa T, Feng Z, Roth JA, Herz J, Minna JD, Gazdar AF (2005) Clinical and biological features associated with epidermal growth factor receptor gene mutations in lung cancers. *J Natl Cancer Inst* 97: 339–346

Soung YH, Lee JW, Kim SY, Seo SH, Park WS, Nam SW, Song SY, Han JH, Park CK, Lee JY, Yoo NJ, Lee SH (2005) Mutational analysis of EGFR and K-RAS genes in lung adenocarcinomas. *Virchows Arch* 446: 483–488

Stewart B, Kleihues P (2003) The global burden of cancer. In *World Cancer Report. International Agency for Research on Cancer*, Stewart B, Kleihues P (eds), pp 11–19. IARC Press: Lyon

Tamura T, Nishiwaki Y, Watanabe K, Nakagawa K, Matsui K, Takahashi T, Segawa Y, Ichinose Y, Fukuoka M, Saijo N (2007) Evaluation of efficacy and safety of erlotinib as monotherapy for Japanese patients with advanced non-small cell lung cancer (NSCLC); integrated analysis of two Japanese phase II studies. *J Thorac Oncol* 2(Suppl): s742

Thatcher N, Chang A, Parikh P, Rodrigues Pereira J, Ciuleanu T, von Pawel J, Thongprasert S, Tan EH, Pemberton K, Archer V, Carroll K (2005) Gefitinib plus best supportive care in previously treated patients with refractory advanced non-small-cell lung cancer: results from a randomised, placebo-controlled, multicentre study (Iressa Survival Evaluation in Lung Cancer). *Lancet* 366: 1527–1537

Toffoli G, Cecchin E, Corona G, Russo A, Buonadonna A, D'Andrea M, Pasetto LM, Pessa S, Errante D, De Pangher V, Giusto M, Medici M, Galon F, Sandri P, Galligioni E, Bonura S, Boccalon M, Biason P, Frustaci S (2006) The role of UGT1A1*28 polymorphism in the pharmacodynamics and pharmacokinetics of irinotecan in patients with metastatic colorectal cancer. *J Clin Oncol* 24: 3061–3068

Tokumo M, Toyooka S, Kiura K, Shigematsu H, Tomii K, Aoe M, Ichimura K, Tsuda T, Yano M, Tsukuda K, Tabata M, Ueoka H, Tanimoto M, Date H, Gazdar AF, Shimizu N (2005) The relationship between epidermal growth factor receptor mutations and clinicopathologic features in non-small cell lung cancers. *Clin Cancer Res* 11: 1167–1173

Yamamoto N, Tamura T, Kamiya Y, Sekine I, Kunitoh H, Saijo N (2000) Correlation between docetaxel clearance and estimated cytochrome P450 activity by urinary metabolite of exogenous cortisol. *J Clin Oncol* 18: 2301–2308

Yang SH, Mechanic LE, Yang P, Landi MT, Bowman ED, Wampfler J, Meerzaman D, Hong KM, Mann F, Dracheva T, Fukuoka J, Travis W, Caporaso NE, Harris CC, Jen J (2005) Mutations in the tyrosine kinase domain of the epidermal growth factor receptor in non-small cell lung cancer. *Clin Cancer Res* 11: 2106–2110

Zhang W, Weissfeld JL, Romkes M, Land SR, Grandis JR, Siegfried JM (2007) Association of the EGFR intron 1 CA repeat length with lung cancer risk. *Mol Carcinog* 46: 372–380

EphA4 promotes cell proliferation and migration through a novel EphA4-FGFR1 signaling pathway in the human glioma U251 cell line

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Abstract

The Eph receptor tyrosine kinases and their ephrin ligands form a unique cell-cell contact-mediated bidirectional signaling mechanism for regulating cell localization and organization. High expression of Eph receptors in a wide variety of human tumors indicates some roles in tumor progression, which makes these proteins potential targets for anticancer therapy. For this purpose, we did gene expression profiling for 47 surgical specimens of brain tumors including 32 high-grade glioma using a microarray technique. The analysis, focused on the receptor tyrosine kinases, showed that EphA4 mRNA in the tumors was 4-fold higher than in normal brain tissue. To investigate the biological significance of EphA4 overexpression in these tumors, we analyzed EphA4-induced phenotypic changes and the signaling mechanisms using human glioma U251 cells. EphA4 promoted fibroblast growth factor 2-mediated cell proliferation and migration accompanied with enhancement of fibroblast growth factor 2-triggered mitogen-activated protein kinase and Akt phosphorylation. In addition, active forms of Rac1 and Cdc42 increased in the EphA4-overexpressing cells. Furthermore, we found that EphA4 formed a heteroreceptor complex with fibroblast growth factor receptor 1 (FGFR1) in the cells and that the EphA4-FGFR1 complex potentiated FGFR-mediated downstream signaling. Thus, our results indicate that EphA4 plays an important role in malignant phenotypes of glioblastoma by enhancing cell proliferation and migration through accelerating a canonical FGFR signaling pathway. [Mol Cancer Ther 2008;7(9):2768–78]

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Note: Microarray analyses were carried out using BRB Array Tools developed by Dr. Richard Simon and Amy Peng. K. Nishio and T. Itakura designed the research, J. Fukai and H. Yokote did the research, R. Yamanaka and T. Arao contributed to data analysis, and J. Fukai wrote the article.

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Introduction

The Eph receptors represent the largest family of receptor protein tyrosine kinases and interact with their ligands, ephrins. The Eph receptors and ephrins are divided into two subclasses, A and B, based on their homologies, structures, and binding affinities (1). Fourteen Eph receptors and eight ephrin ligands have been identified thus far in mammals (2–4). Ephrin-A ligands are anchored to the plasma membrane by a glycosylphosphatidylinositol modification. Ephrin-B ligands have a transmembrane domain and a short cytoplasmic tail. The Eph-ephrin system relays a direct cell-cell contact-mediated bidirectional signaling pathway (5, 6). This bidirectional signaling is fundamentally involved in developmental processes (7–9) or in the remodeling of blood vessels (10, 11). Eph-ephrin signaling mainly affects the cell shape and motility by regulating cytoskeletal organization and cell adhesion and also influences cell proliferation and cell-fate determination (3). Therefore, it is speculated that Eph signaling could play some roles in tumorigenesis as one of their possible consequences.

More recently, the genes for Eph receptors and ephrins have been recognized to be differentially expressed in various human tumors including malignant melanoma, glioma, prostate cancer, breast cancer, small cell lung cancer, endometrial cancer, esophageal cancer, gastric cancer, and colorectal cancer (12–18). Profound distortion of expression patterns could be correlated with altered tumor behavior such as increased invasiveness or increased metastatic potential and consequently with poor patient outcome. Despite the widely observed phenomenon of Eph receptor overexpression, their role has not been fully elucidated in the process of malignant phenotypes. However, recent bodies of evidence have implicated Eph involvement in the tumor progression of human cancers including malignant gliomas (19, 20).

The fibroblast growth factor receptor (FGFR) also belongs to a family of receptor protein tyrosine kinases and consists of four members, FGFR1, FGFR2, FGFR3, and FGFR4. At the present, 23 FGF ligands have been cloned and known to bind to and activate FGFRs with their different affinities (21). The FGFR-associated substrate 2α (FRS2α) is a docking

protein that plays an important role in FGFR signaling. FRS2α binds constitutively to FGFR through its PTB domain at the NH_2 terminus and six tyrosine residues at the COOH terminus are phosphorylated by activated FGFR. Once FRS2α is phosphorylated, it recruits adaptor molecules such as Grb2 and Shp2 and relays the signals to the Ras/mitogen-activated protein kinase (MAPK) or phosphatidylinositol 3-kinase/Akt pathway (22, 23). In addition, FGFR is known to direct cytoskeletal reorganization through regulating small GTPases, although its precise signaling mechanism has not been clarified (24).

The FGF growth factor family mediates a wide range of biological activities by binding to and activating the FGFR family (25). Aberrant FGFR signaling leads to diverse human pathologies including carcinogenesis. In gliomas, malignant progression from low to high grade appears to involve up-regulation of FGFR expression (26). FGFR2 is expressed abundantly in normal white matter and in all low-grade astrocytomas but is repressed in malignant astrocytomas. Conversely, FGFR1 expression is almost absent in normal white matter but is sufficiently expressed to be detected in malignant astrocytomas (27). Glioblastomas also express an alternatively spliced form of FGFR1 containing two immunoglobulin-like disulfide loops (FGFR1β), whereas normal human adult expresses FGFR1α, a form of the receptor containing three immunoglobulin-like loops. Intermediate grades of astrocytomas exhibit a gradual loss of FGFR2 and a shift in expression from FGFR1α to FGFR1β as they progress (28). Thus, FGFR1 signaling is suggested to be associated closely with malignant progression of astrocytes.

In this article, we would like to report that our gene expression study showed aberrant expression of the EphA4 receptor in high-grade astrocytic tumors and that the overexpressed EphA4 contributed to the malignant phenotype of the tumor cells through enhancing proliferation and migration by its interaction with FGFR1.

Materials and Methods

Microarray Gene Expression Analysis

Tumor specimens were obtained from 47 patients who underwent therapeutic removal under approved protocols from the institutional review board. Histologic diagnosis was made by light microscopic evaluation of the sections stained with H&E. The classification of human brain tumors was based on the WHO criteria for tumors of the nervous system (29). The 47 brain tumors consisted of 32 high-grade glioma and 15 low-grade glioma. Total RNA was extracted from tissue samples and assessed for quality. Amplified RNA from tumor and normal brain tissue was labeled with Cy5 and Cy3 (Amersham Biosciences), respectively. Hybridization target probes were prepared from total RNA and hybridized to the CodeLink Agilent Human I Bioarray chip according to the manufacturer's instructions (Amersham Biosciences). Microarray images were processed using Gene Spring software (Silicon Genetics). Intensity values were normalized using lowess normalization (30). Average fold differences in gene expression between tumor and normal brain were calculated. Basic data visualization, data filtering, and hierarchical clustering were done. The microarray raw data and clinical features has been submitted to Gene Expression Omnibus[5] (accession no. GSE4381) in our previous report (31).

Cell Culture

GP2-293 cells (Clontech) were cultured and maintained with DMEM with 10% fetal bovine serum and used as packaging cells for pseudo-retrovirus production. RPMI 1640 supplemented with 10% fetal bovine serum was used for each culture of U251 cells (human malignant astrocytoma cell line; Japanese Collection of Research Bioresources Cell Bank).

Expression Vector Construction and Viral Production

The expression constructs of EphA4 and FGFR1 were generated as follows. The full-length cDNA fragment encoding human EphA4 or FGFR1 was obtained from the U251 cells with the reverse transcription-PCR method using the primers EphA4-P1-F (CGGATCCAC-CATGGCTGGGATTTTCTATTTC), EphA4-R (GAAGCTT-GACGGGAACCATTCTGCCGTGCATC), FGFR1-F (CGGATCCGAAATGTGGAGCTGGTGACCCCAGCA), and FGFR1-R (GAAGCTTGCGGCGTTTGAGTCCGC-CATTGGCA). The DNA fragment for EphA4 DN lacking both juxtamembrane and kinase domains was generated with the recombinant PCR method using the following primers EphA4-P1-F, EphA4-P2-R (ACTGCTTGGTTGG-GATCTTCATTCAAATGTTTCTCTTCAT), EphA4-P3-F (ATGAAGAGAAACATTTGAATGAAGATCCCAAC-CAAGCAGT), EphA4-P4-R (GAAGCTTGTTGCTGTT-CACCAGGATGTTCC). First, two DNA fragments of EphA4 were amplified using P1-F/P2-R and P3-F/P4-R primer sets. Next, the recombined DNA fragment for EphA4 DN was amplified using the two PCR products as templates and the P1-F/P4-R primer set. The DNA fragment for FGFR1 DN lacking cytoplasmic domain was amplified with the primers, FGFR1-F and FGFR1(TM)-R (CAAGCTTCTTGTAGACGATGACCGACCCCAC). The sequences of all of the PCR-amplified DNAs were confirmed by sequencing after cloning into a pCR-Blunt II-TOPO cloning vector according to the manufacturer's instructions (Invitrogen). HA and myc tag were added at the COOH-terminal ends of EphA4 and FGFR1 constructs, respectively. All DNA fragments were cut out and transferred into a pQCLIN retroviral vector (BD Biosciences Clontech) together with enhanced green fluorescent protein (EGFP) following internal ribosome entry site sequence to monitor the expression of the inserts indirectly. A pVSV-G vector (Clontech) for constitution of the viral envelope and the pQCXIX constructs were cotransfected into the GP2-293 cells using a FuGENE6 transfection reagent (Roche Diagnostics). Briefly, 80% confluent cells

[5] http://www.ncbi.nlm.nih.gov/geo

cultured on a 10 cm dish were transfected with 2 μg pVSV-G plus 6 μg pQCXIX vectors. Forty-eight hours after transfection, the culture medium was collected and the viral particles were concentrated by centrifugation at 15,000 × *g* for 3 h at 4°C. The viral pellet was resuspended in fresh RPMI 1640. The viral vector titer was calculated by counting EGFP-positive cells, which were infected by serial dilution with virus-containing medium, and the multiplicity of infection was determined. These EphA4 and FGFR1 constructs are illustrated schematically in Fig. 2A.

Antibodies and Reagents

Recombinant human FGF2 and mouse ephrin-A1/Fc chimera were purchased from R&D Systems. Antibodies used for the study were as follows: anti-MAPK rabbit polyclonal, anti-phospho-MAPK rabbit polyclonal, anti-Akt rabbit polyclonal, anti-phospho-Akt rabbit polyclonal, and horseradish peroxidase-linked second antibodies (Cell Signaling); anti-HA and anti-myc monoclonal antibodies (Roche Diagnostics); anti-FRS2 rabbit polyclonal, anti-EphA4 rabbit polyclonal, anti-FGFR1 rabbit polyclonal, and anti-RhoA mouse monoclonal (Santa Cruz); anti-phosphotyrosine mouse monoclonal and anti-Rac1 and anti-Cdc42 mouse monoclonal (BD Biosciences). Rac GTPase-specific inhibitor was purchased from Calbiochem EMD Biosciences.

Cell Proliferation Assay

Cell proliferation was assessed using a CellTiter96 Aqueous One Solution Cell Proliferation Assay kit (Promega) according to the manufacturer's instructions. Briefly, cells (1×10^3) serum-starved overnight were seeded on 96-well plastic plates with 200 μL culture medium supplemented with or without 0.5 μg/mL ephrin-A1/Fc or 20 ng/mL FGF2. After 72 h incubation at 37°C, 40 μL 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt reagent was added followed by further incubation for 1 h. The absorbance at 490 nm was measured using a microplate reader.

Cell Migration Assay

The U251 cells were cultured in 12-well plates until confluence was reached. The monolayer cells were scratched out using a fine pipette tip after serum starvation overnight. To monitor the migration, pictures were taken at 0, 12, and 24 h after addition of ephrin-A1 or FGF2 using a fluorescence microscope (Keyence Biozero). The areas occupied by the cells were calculated with NIH image software (NIH). The ratio of the increased area by cell migration after 12 and 24 h to that at 0 h was calculated to quantitate the extent of migration.

Immunoblot Analysis

Cells that were serum starved for 24 h were treated with or without ephrin-A1/Fc or FGF2. The cells were washed with cold PBS and harvested with Lysis A buffer containing 50 mmol/L HEPES buffer, 1% Triton X-100, 5 mmol/L EDTA, 50 mmol/L sodium chloride, 10 mmol/L sodium pyrophosphate, 50 mmol/L sodium fluoride, 1 mmol/L sodium orthovanadate, and protease inhibitor mix, Complete (Roche Diagnostics). The lysate was clarified by centrifugation for protein analysis. The proteins separated with SDS-PAGE were transferred onto polyvinylidene difluoride membranes. After blocking with 3% bovine serum albumin in TBS (pH 8.0) with 0.1% Tween 20, the membrane was probed with the first antibody. After rinsing twice with TBS, the membrane was incubated with the horseradish peroxidase-conjugated second antibody (Cell Signaling) followed by visualization using a ECL detection system (Amersham Biosciences).

Pull-Down Assay

GTP-bound RhoA and Rac1/Cdc42 were pulled down from the cell lysate using agarose-conjugated GST-fused Rhotekin binding domain and GST-fused PAK-1 binding domain (Upstate), respectively, followed by quantitative detection by immunoblotting. Briefly, the cells were treated with or without ephrin-A1/Fc or FGF2 and lysed in magnesium-containing lysis buffer (25 mmol/L HEPES buffer, 150 mmol/L sodium chloride, 1% Triton X-100, 10% glycerol, 25 mmol/L sodium fluoride, 10 mmol/L $MgCl_2$, 1 mmol/L EDTA, 1 mmol/L sodium orthovanadate, 10 μg/mL leupeptin, and 10 μg/mL aprotinin). After clarification, an equal amount of lysate was incubated with 20 μg agarose-conjugated GST-fused Rhotekin binding domain or GST-fused PAK-1 binding domain at 4°C for 3 h. The agarose beads were washed three times with magnesium-containing lysis buffer and the samples were analyzed with SDS-PAGE followed by immunoblotting.

Immunoprecipitation

The total protein extracted with Lysis A buffer (as described above) was incubated with the antibody at 4°C overnight. The antibody was immobilized with protein A agarose by incubation for a further 2 h. The immunoprecipitates were collected by centrifugation at 3,000 × *g* for 30 s. After the immunoprecipitates were washed four times with Lysis A buffer, the samples were analyzed with SDS-PAGE followed by immunoblotting.

Statistics

Comparisons were made using Student's *t* test. $P < 0.05$ was considered statistically significant.

Results

High Expression of EphA4 mRNA in Malignant Glioma

We analyzed the expression of 13,156 clones from Incyte's human cDNA library genes using Agilent human cDNA microarrays (Agilent Technologies) for 32 malignant gliomas consisting of 10 anaplastic astrocytomas and 22 glioblastomas. We did hierarchical clustering of 103 tyrosine kinases (Fig. 1A). The histograms to determine the fold difference in the measured expression levels between tumors and normal brain are shown in Fig. 1B. Among several receptors with increased expression, we found the mRNA levels of the EphA4 receptor were significantly higher in glioma tissues (>4-fold) than those in normal brain tissues. There was a difference between anaplastic astrocytomas and glioblastomas with regard to EphA4 expression. The level in glioblastomas was higher than that in anaplastic astrocytomas. In low-grade gliomas, EphA4 expression level was also elevated but lower than

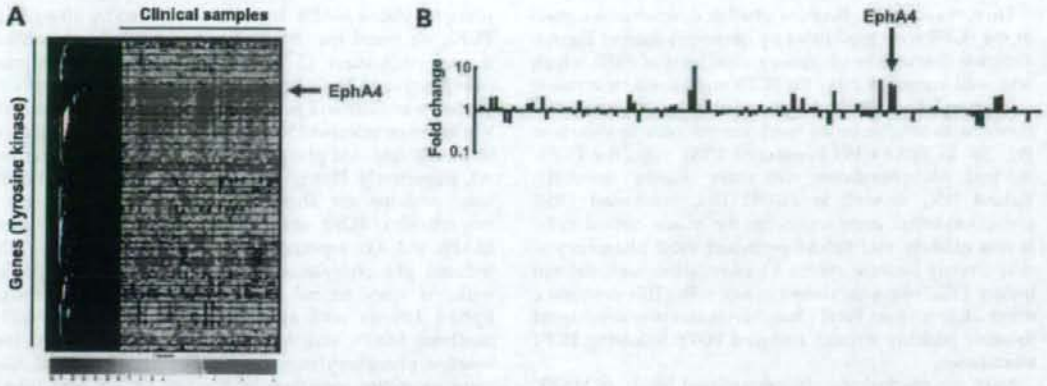

Figure 1. Profiles of 103 tyrosine kinases mRNA expression in 47 surgical samples of brain tumors. *Arrows*, EphA4 mRNA expression. **A**, hierarchical clustering of 103 tyrosine kinases. Each row and column shows a single gene and a tissue sample, respectively. The order of the clinical samples (*X axis*) corresponds to the WHO criteria from high grade to low. **B**, histogram of gene expression. The mRNA level of the EphA4 gene was significantly higher in glioma tissues (>4-fold) than in normal brain tissues.

that in high grade. EphA4 expression correlated with increasing tumor grade. The other transcripts with significantly higher expression were indicated in Supplementary Table.[6] Based on these results, we focused on the high expression level of EphA4 and examined the biological significance of EphA4 in gliomas.

Design of EphA4 and FGFR1 Constructs Used in This Study

Based on the previous evidence that EphA4 interacts with FGFR (32) and that, among the FGFR family, FGFR1 is most closely associated with the malignant progression of human glioma as described in Introduction, we sought to investigate how EphA4 affected FGFR signaling using human glioma U251 cells. For this study to explore the role of EphA4-FGFR1 interaction in glioma cell biology, it is vital to select glioma cell lines that express high levels of both EphA4 and FGFR1. However, these cell lines were not available, so we selected U251 cells, which have increased expression of FGFR1 (33), introduced EphA4 into U251 cells, and did our experiments using the transfected U251 cells.

Expression constructs of EphA4 and FGFR1 are summarized schematically in Fig. 2A. We generated a dominant-negative form of EphA4 (EphA4 DN), which lacked its juxtamembrane domain and COOH-terminal half of the kinase domain. It has been reported that EphA4 transphosphorylates FGFR through their direct interaction and that the NH_2-terminal region of the EphA4 kinase is necessary to interact with the FGFR (32). The juxtamembrane domain is postulated to close its interaction portion constitutively when EphA4 kinase is inactive. Therefore, this juxtamembrane-deleted EphA4 mutant can expose its binding site to FGFR although lacking transphosphorylation activity. In addition, this kinase-inactive mutant can also work as an EphA4 inhibitor against ephrin-ligand stimulation. On the contrary, a dominant-negative form of FGFR1 (FGFR1 DN) lacks the whole cytoplasmic domain, which inhibits the activation of intrinsic FGFR1 by FGF stimulation. These wild-type and dominant-negative constructs of both EphA4 and FGFR1 were retrovirally introduced into human glioma U251 cells and their protein expression was checked as seen in Fig. 2B. As the expression unit included an internal ribosomal entry site sequence followed by EGFP, we could monitor the expression by fluorescence microscopy in Fig. 2C.

EphA4 Promotes FGF2-Mediated Proliferation and Enhances FRS2, MAPK, and Akt Phosphorylation in U251 Cells

We evaluated the effect of EphA4 on cell proliferation of the U251 cells using the ectopic expression of EphA4 WT retrovirally. EphA4 WT did not affect cell proliferation significantly without FGF stimulation but promoted cell proliferation significantly in the presence of FGF2 at 20 ng/mL for 72 h compared with a mock control (Fig. 3A). Inversely, EphA4 DN expression as well as FGFR1 DN inhibited FGF2-triggered proliferation. FGF2-triggered proliferation of FGFR1 DN was higher than that with no ligand stimulation. The reason might be that FGFR1 DN could not completely block FGF2-mediated FGFR1 activation. However, FGF2-induced cellular responses were clearly inhibited in FGFR1 DN compared with those in the other cells. Ephrin-A1 stimulation seemed slightly to promote cell proliferation for EphA4 WT cells, FGFR1 DN-expressing cells, and mock control cells, but it was not statistically significant. These results suggested some strong correlation of EphA4 with FGFR signaling.

[6] Supplementary materials for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

Then, we sought to examine whether downstream signals of the FGFR were modulated by overexpression of EphA4. First, we checked the phosphorylation level of FRS2, which was well known to relay the FGFR signals via its tyrosine phosphorylation. FRS2 phosphorylation by FGF2 stimulation was detectable in the mock control cells as shown in Fig. 3B. In EphA4 WT-introduced U251 cells, the FGF2-induced phosphorylation was more evident. Inversely, EphA4 DN, as well as FGFR1 DN, weakened FRS2 phosphorylation compared with the mock control cells. It was unlikely that EphA4 promoted FRS2 phosphorylation directly because ephrin-A1 stimulation itself did not induce FRS2 phosphorylation in any cells. This promotive effect of EphA4 on FRS2 phosphorylation was considered to occur possibly through activated FGFR following FGF2 stimulation.

Next, we checked the phosphorylation levels of MAPK and Akt, which were well known to affect cell proliferation and survival, respectively. Those were further downstream signaling molecules of FGFR and were also relayed via FRS2 phosphorylation. From time-course analysis of phosphorylated MAPK and Akt stimulated by 20 ng/mL FGF2, we found that their phosphorylation level reached a peak at around 15 min. Although MAPK was not phosphorylated by Ephrin-A1 stimulation, Akt-phosphorylation was clear and peaked at 30 min (data not shown). We therefore selected 15 and 30 min to evaluate the degree of MAPK and Akt phosphorylation by FGF2 and ephrin-A1, respectively. Phosphorylated MAPK and Akt and their total proteins are shown in Fig. 3C (*left* and *right*, respectively). FGF2 stimulation clearly phosphorylated MAPK and Akt; especially, EphA4 WT augmented FGF2-induced phosphorylation of MAPK and Akt compared with the mock control cells (Fig. 3D). On the contrary, EphA4 DN as well as FGFR1 DN attenuated FGF2-mediated MAPK and Akt phosphorylation. Notably, the baseline phosphorylation levels of both MAPK and Akt were moreover increased in the EphA4 WT-introduced U251 cells without ligand stimulation but inhibited in the EphA4 DN-introduced cells. Ephrin-A1 stimulation showed an almost undetectable increase of phospho-MAPK but a small increase of phospho-Akt. EphA4 DN

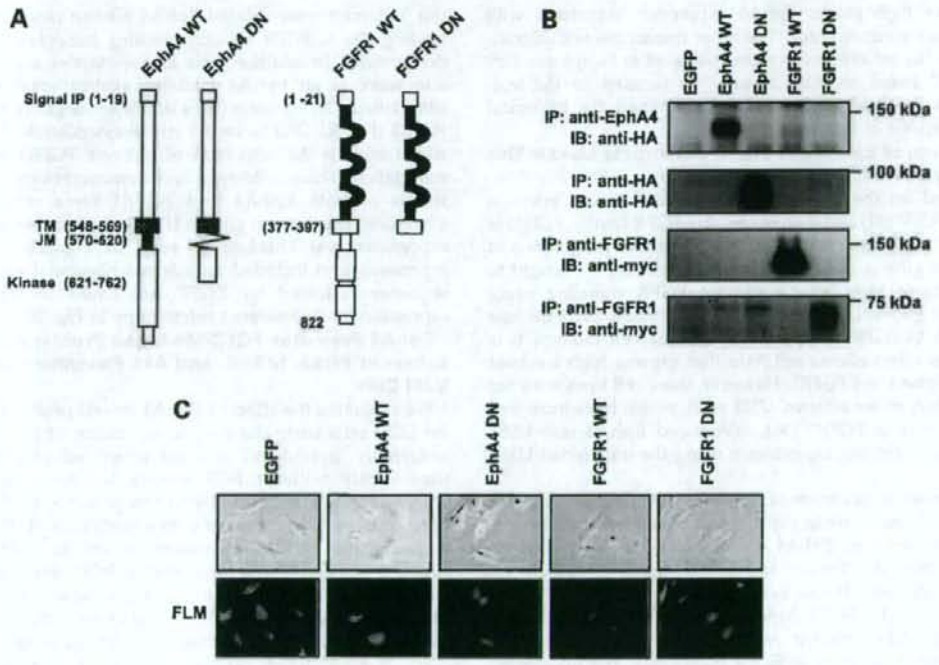

Figure 2. Construction of expression vectors for either the wild-type (*WT*) or the dominant-negative (*DN*) form of EphA4 or FGFR1. **A**, schematic representation of EphA4 and FGFR1 constructs. *Numbers*, amino acids. EphA4 DN is a deletion mutant lacking the juxtamembrane (570-620) and COOH-terminal half of EphA4 kinase domain (763-986) amino acids. FGFR1 DN is also a deletion mutant without whole cytoplasmic domain. *TM*, transmembrane domain; *JM*, juxtamembrane domain. **B**, checking the expression of EphA4 and FGFR1 constructs in U251 cells. EphA4 and FGFR1 constructs were tagged with HA and myc, respectively, at the COOH terminus. Each lysate (500 μg) was immunoprecipitated and detected by immunoblotting with the HA or myc antibody. *IB*, immunoblotting; *IP*, immunoprecipitation. **C**, microscopic imaging of EGFP coexpressing with the introduced proteins of interest. *FM*, fluorescent microscopy.

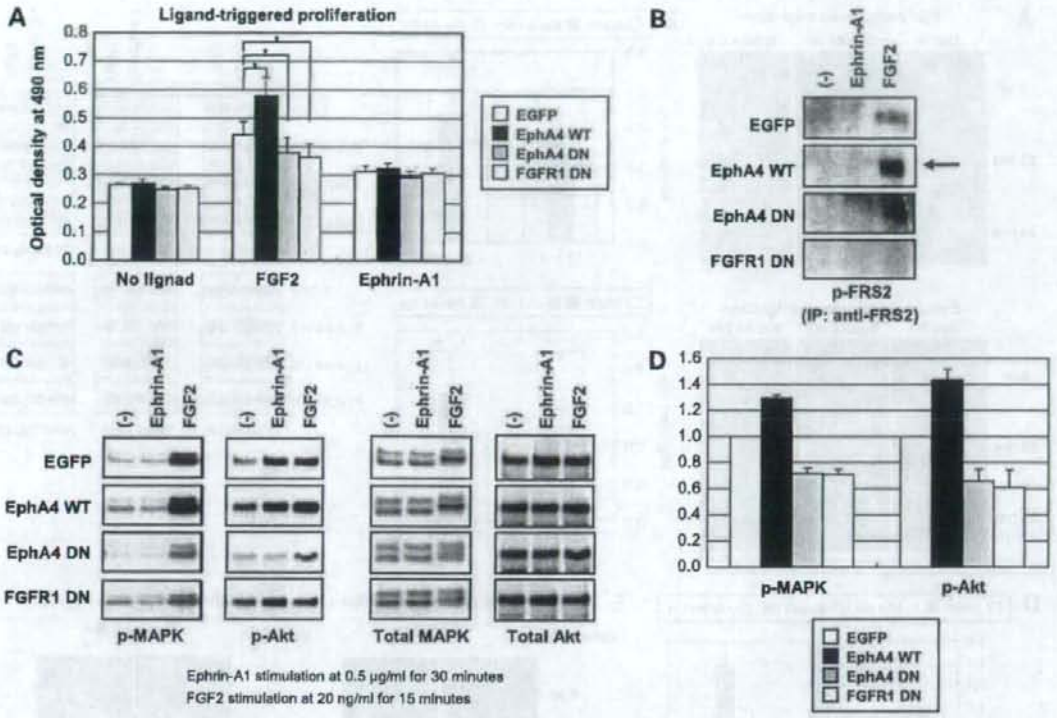

Figure 3. Effects of EphA4 WT on proliferation in the U251 cells. **A,** cell proliferation judged by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt assay. Serum-starved cells (1×10^3) were plated on 96-well plates in the absence or presence of ligands (20 ng/mL FGF2 or 0.5 μg/mL ephrin-A1). The absorbance at 490 nm of each well was measured after 72 h incubation. *Bars,* SD. *, $P < 0.05$ versus the EGFP cells. **B,** effect of EphA4 on FRS2 phosphorylation. Serum-starved cells were treated with 20 ng/mL FGF2 for 15 min or 0.5 μg/mL ephrin-A1 for 30 min. The immunoprecipitated samples using an anti-FRS2 antibody were checked by immunoblotting with an anti-phosphotyrosine antibody. **C,** effect of EphA4 on phosphorylation of MAPK and Akt. Each cell lysate (10 μg) treated with 20 ng/mL FGF2 for 15 min or 0.5 μg/mL ephrin-A1 for 30 min was analyzed by immunoblotting. *Left,* probing with anti-phospho-MAPK and anti-phospho-Akt antibodies; *right,* reprobing with anti-total MAPK and anti-total Akt antibodies. **D,** relative densitometric units of the phospho-MAPK and phospho-Akt bands in each cell stimulated by FGF2. The densities of the EGFP bands are set arbitrarily at 1.0. *Bars,* SD.

blocked this increase, but FGFR1 DN did not, suggesting that ephrin-A1-triggered Akt phosphorylation was not mediated by FGFR1.

EphA4 Also Promotes FGF2-Mediated Migration Accompanied with Increased Active Rac1/Cdc42

To study the effect of EphA4 on cell migration, we did a scratch wound assay using the U251 cells ectopically expressing EGFP, EphA4 WT, EphA4 DN, or FGFR DN and checked the extent of their migration after scratching by monitoring EGFP for 24 h (Fig. 4A). If the incubation time is so long that migrating cells are able to proliferate, the migration rate could be affected by proliferation. Therefore, we analyzed the migration rate before proliferation might have a significant effect on this analysis (Fig. 4B). It was evident that EphA4 WT promoted FGF2-stimulated cell migration to cover the scratched area compared with the mock control and EphA4 DN (Fig. 4A, *top*). Under the same experimental condition, FGF2 stimulation caused a small increase in cell proliferation and the difference of proliferation was quite limited among the transfected cells (Supplementary Fig. S1).[6] These results imply that the wound closing process is due to cell migration activity triggered by FGF2. To evaluate this quantitatively, we assayed in triplicate and calculated the migration area (by square inches) using NIH imaging software and illustrated this graphically (Fig. 4B, *top*). EphA4 WT-expressing cells significantly migrated more than EGFP-expressing, EphA4 DN-expressing, or FGFR1 DN-expressing cells for 24 h. Interestingly, the promotion of cell migration by EphA4 WT was also observed under ephrin-A1 stimulation (Fig. 4A, *bottom*). As for ephrin-A1 treatment, cell proliferation was slightly induced in the transfected cells (Fig. 3A). Therefore, promoted wound closure means increased activity of cell migration triggered by ephrin-A1.

As Rho family GTPases have been known to play key roles in cell migration by regulating actin dynamics, we