81. Kandioler-Exkersberger D, Ludwig C, Rudas M, Kappel S, Janschek E, Wenzel C, et al. TP53 mutation and p53 overexpression for prediction of response to neoadjuvant treatment in breast cancer patients. *Clin Cancer Res* 2000;6:50–6.
82. Chang JC, Wooten EC, Tsimelzon A, Hilsenbeck SG, Gutierrez MC, Elledge R, et al. Gene expression profiling for the prediction of therapeutic response to docetaxel in patients with breast cancer. *Lancet* 2003;362:362–9.
83. Ayers M, Symmans WF, Stec J, Demokosh AI, Clark E, Hess K, et al. Gene expression profiles predict complete pathologic response to neoadjuvant paclitaxel and fluorouracil, doxorubicin, and cyclophosphamide chemotherapy in breast cancer. *J Clin Oncol* 2004;22;2284–93.
84. Chang JC, Wooten EC, Tsimelzon A, Hilsenbeck SG, Gutierrez MC, Tham YL, et al. Patterns of resistance and incomplete response to docetaxel by gene expression profiling in breast cancer patients. *J Clin Oncol* 2005;23:69–77.
85. Fulgueira MA, Carraro DM, Bretani H, Patrao DF, Barbosa EM, Netto MM, et al. Gene expression profile associated with response to doxorubicin-based therapy in breast cancer. *Clin Cancer Res* 2005;11:7434–43.
86. Iwao-Koizumi K, Matoba R, Ueno N, Kim SJ, Ando A, Miyoshi Y, et al. Prediction of docetaxel response in human breast cancer by gene expression profiling. *J Clin Oncol* 2005;23:422–31.
87. Simon R, Radmacher MD, Dobbin K, McShane LM. Pitfalls in the use of DNA microarray data for diagnositic and prognositic classification. *J Natl Cancer Inst* 2003;95:14–8.
88. Ellis M, Ballma K. Trawling for genes that predict response to breast cancer adjuvant therapy. *J Clin Oncol* 2004;22:2267–9.
89. Reid JF, Lusa L, De Cecco L, Coradini D, Veronesi S, Daidone MG, et al. Limits of predictive models using microarray data for breast cancer clinical outcome. *J Natl Cancer Inst* 2005;97:927–30.
90. Wong ZW, Ellis MJ. First-line endocrine treatment of breast cancer: aromatase inhibitor of antiestrogen? *Br J Cancer* 2004;90:20–5.
91. Mohshin SK, Weiss HL, Gutierrez MC, Chamness GC, Schiff R, Digiovanna MP, et al. Neoadjuvant trastuzumab induces apoptosis in primary breast cancers. *J Clin Oncol* 2005; 23:2460–8.
92. Polychronis A, Sinnett HD, Hadijiminas D, Singhal H, Mansi JL, Shivapatham D, et al. Preoperative gefinitib versus gefitinib and anastrozole in postmenopausal patients with oestrogen-receptor positive and epidermal-growth-factor-receptor positive primary breast cancer: a double-blind placebo-controlled phase II randomized trial. *Lancet Oncol* 2005;6:383–91.
93. Sorlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc Natl Acad Sci* 2001;98;10969–10874.

Breast Cancer Res Treat (2007) 103:313–318
DOI 10.1007/s10549-006-9384-8

CLINICAL TRIAL

Prediction of response to repeat utilization of anthracycline in recurrent breast cancer patients previously administered anthracycline-containing chemotherapeutic regimens as neoadjuvant or adjuvant chemotherapy

Kan Yonemori · Noriyuki Katsumata · Masayuki Kaneko · Hajime Uno · Koji Matsumoto · Tsutomu Kouno · Chikako Shimizu · Masashi Ando · Masahiro Takeuchi · Yasuhiro Fujiwara

Received: 14 August 2006 / Accepted: 16 August 2006 / Published online: 25 October 2006

Summary
Purpose: The aim of this study was to identify the predictors of the response to doxorubicin plus cyclophosphamide in patients with recurrent breast cancer (RBC) previously treated with anthracycline-containing regimens in a neoadjuvant or adjuvant setting.
Method: Between December 1993 and October 2005, 664 patients had received combined doxorubicin plus cyclophosphamide chemotherapy (doxorubicin, 40 mg/m^2, iv on day 1; cyclophosphamide, 500 mg/m^2, iv on day 1, every 21 days) for RBC at our institution. In this study, we retrospectively analyzed the efficacy of doxorubicin plus cyclophosphamide in 99 of these 664 RBC patients who had also previously been administered an anthracycline-based chemotherapy in a neoadjuvant or adjuvant setting.
Results: The median cumulative dose of the previously administered anthracycline was 156 mg/m^2. The median disease-free interval (DFI) and median anthracycline-free interval were 33.8 and 43.7 months, respectively. The overall response rate to doxorubicin plus cyclophosphamide therapy was 38.4% (95% CI; range, 28.8–48.0%). The median time to progression and overall survival were 6.2 and 17.5 months, respectively. The results of a multivariate logistic regression analysis revealed a significant association of the response to doxorubicin plus cyclophosphamide therapy with the DFI ($P = 0.02$); human epidermal receptor type 2 (HER2) status also tended to affect the response rate, however the association was not statistically significant ($P = 0.06$).
Conclusion: DFI and HER2 status may be associated with the response to repeat utilization of anthracycline-containing regimens in RBC patients also treated previously with anthracycline-containing chemotherapeutic regimens in a neoadjuvant or adjuvant setting.

Keywords Anthracycline · Anthracycline-free interval · Disease-free interval · Doxorubicin · HER2 status · Prediction reutilization · Recurrent breast cancer

K. Yonemori (✉) · N. Katsumata · K. Matsumoto · T. Kouno · C. Shimizu · M. Ando · Y. Fujiwara
Breast and Medical Oncology Division,
National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku,
Tokyo 104-0045, Japan
e-mail: kyonemor@ncc.go.jp

M. Kaneko · H. Uno · M. Takeuchi
Division of Biostatistics, Kitasato University Graduate School, 5-9-1 Shirokane, Minato-ku,
Tokyo, Japan

Introduction

Breast cancer is known as one of the chemotherapy-sensitive cancers. Neoadjuvant or adjuvant chemotherapy, for eradicating micrometastatic disease, has been shown to improve the survival of patients with early-stage breast cancer [1, 2]. Results of randomized controlled trials and meta-analyses have demonstrated the clinical benefit of anthracyclines in early breast cancer, including in node-positive and node-negative breast cancer, in both pre- and post-menopausal women. Doxorubicin and epirubicin, the two most commonly used anthracyclines, are among the most effective anticancer drugs in breast cancer chemotherapy.

The majority of early breast cancer patients currently receive anthracycline-containing chemotherapy.

However, despite the adjuvant or neoadjuvant chemotherapy, a significant proportion of these patients develop recurrence within the first 5 years. Treatment of patients with recurrent breast cancer (RBC), often poses a difficult therapeutic problem; these patients have already received the most effective therapy as their primary treatment, therefore an alternative agent that might be less effective often needs to be used.

It has been reported that in some chemosensitive tumors, reutilization of potentially active chemotherapeutic agents may be an effective treatment option [3–5]. In the case of ovarian cancer, planning of chemotherapy for recurrent disease after adjuvant chemotherapy is mainly dependent on the platinum-free interval [3]. To the best of our knowledge, there is no information available until date about the optimum anthracycline-free interval (AFI) or predictors of response to repeated utilization of anthracycline-containing regimens in cases of RBC. Similar to their efficacy in the neoadjuvant or adjuvant setting, anthracyclines have also been shown to exhibit clinical efficacy against metastatic breast cancer [6]. Therefore, it may be useful to identify patients of RBC who are likely to show response to repeat utilization of anthracycline-containing regimens.

The objective of this study was to evaluate the efficacy, in terms of the response rate, time to progression and overall survival, of combined doxorubicin plus cyclophosphamide (AC) therapy in RBC patients previously treated with anthracycline-containing chemotherapeutic regimens and to identify patients who are likely to benefit from repeat utilization of anthracycline-based chemotherapy.

Patients and methods

A total of 664 patients with RBC were treated with AC therapy between December 1993 and October 2005 at the National Cancer Center Hospital. We retrospectively selected patients who fulfilled the following selection criteria as the subjects of the present study: (1) previously administered anthracycline-containing chemotherapeutic regimens as neoadjuvant or adjuvant chemotherapy; (2) adequate bone marrow and organ function (neutrophils $\geq 1{,}500\ \mu^{-1}$, platelets $\geq 100{,}000\ \mu^{-1}$, AST $\leq 2.5 \times$ upper limit of normal range (ULN), ALT $\leq 2.5 \times$ ULN, serum creatinine $\leq 1.5 \times$ ULN); (3) availability of written informed consent prior to the start of treatment.

Patients were administered 3 mg of granisetron hydrochloride and 8 mg of dexamethasone intravenously (iv) 30 min prior to the doxorubicin infusion. The dosages of the chemotherapeutic drugs were as follows: doxorubicin, 40 mg/m^2, iv on day 1; cyclophosphamide, 500 mg/m^2, iv on day 1 of each 21-day cycle. Treatment with the AC therapy was continued until evidence of disease progression or of unacceptable toxicity was observed.

Patients with no bidimensionally measurable lesions were considered ineligible for the objective response evaluation. The objective responses were evaluated according to WHO criteria [7]. Patients without measurable lesions were classified as not assessable (NA). Toxicity was re-evaluated according to National Cancer Institute Common Toxicity Criteria (NCI-CTC) ver 2.0.

Statistical analysis

Logistic regression analyses were performed to assess the response to the AC therapy of the RBC patients previously treated with anthracycline-containing chemotherapeutic regimens in a neoadjuvant or adjuvant setting and other factors. Factors with a *P*-value of less than 0.2 in the univariate logistic regression were examined simultaneously with multivariate logistic regression models. A stratified analysis was also performed to assess the effect of the disease-free interval (DFI) and human epidermal receptor type 2 (HER2) status, believed to be factors associated with the response to AC therapy. In regard to the DFI, its validity as a predictor was examined via an ROC analysis, and the cutoff value that yielded 75% sensitivity was selected in order to classify the patients into two categories.

DFI was measured from the date of mastectomy until observation of evidence of the first local, regional, or distant recurrence of the tumor, contralateral breast cancer, or a second primary tumor in addition to the breast tumor. AFI was measured from the last date of administration of anthracycline-containing chemotherapy until the date of re-start of AC therapy. Time to progression was measured from the first day of treatment until disease progression or the final day of follow-up without disease progression, and the overall survival time was measured from the first day of treatment until death or the final day of follow-up. Median time to progression and median overall survival were estimated by the Kaplan–Meier method.

The statistical analysis was performed with SAS, version 9.1.3 (SAS Institute, Cary, NC, USA), and the significance level was set at $P = 0.05$ (two-sided).

Results

Patient characteristics

Of the 664 patients treated with AC therapy for RBC, 99 had also previously received anthracycline-based

chemotherapy in the neoadjuvant or adjuvant setting. The patient characteristics are summarized in Table 1. The median age was 54 years (range, 31–76 years); the median performance status was 0 (range, 0–3). Median number of organs involved was 2 (range, 1–6). Most of patients (91%) had received anthracycline-based chemotherapy in the adjuvant setting while the remaining had received it in a neoadjuvant setting. The median dose of the previously administered anthracycline was 156 mg/m^2 (range, 15–360 mg/m^2). Six patients had received regimens containing anthracycline and taxanes agents as neoadjuvant or adjuvant chemotherapy. Ninety-seven patients had undergone mastectomy and remaining had undergone breast-conserving therapy. Twelve patients had also received adjuvant radiation therapy and 79 patients, adjuvant hormone therapy. The median DFI was 33.8 months (range, 3.8–191.7 months) and the median AFI was 43.7 months (range, 4.7–192.8 months). The majority of these patients (N = 66, 66.6%) had received AC therapy as first-line chemotherapy for RBC. Before AC therapy, remaining patients (N = 33) had received other chemotherapy for RBC, as follows: 21 patients, docetaxel; 13 patients, paclitaxel; 2 patients, CMF; 1 patient, capecitabine; 1 patient, vinorelbine; 1 patient, irinotecan; 1 patient each, vinorelbine and capecitabine.

Table 1 Characteristics of the 99 patients

Characteristics	Value
Median age (range)	54 (31–76)
Side (right/left)	57/42
Median ECOG performance status (range)	0 (0–3)
Median metastatic site (range)	2 (1–6)
Metastatic sites	
Lung	37
Liver	32
Bone	47
Pleural effusion	20
Lymph node	47
Soft tissue	32
No. of previous chemotherapy regimens before treatment with AC	
0	66
1	27
>1	6
Hormone status (ER or PgR)[a]	
Positive	69
Negative	30
HER2 status[a]	
Positive	9
Negative	80
Unknown	10

ECOG Eastern Cooperative Oncology Group, *ER* estrogen receptor, *PgR* progesterone receptor, *HER2* human epidermal receptor type 2

[a] Hormone status and HER2 status were evaluated by immunohistochemical examination

Treatment efficacy and toxicity

A total of 482 courses of AC therapy were administered, and the median number of courses was 6 (range, 1–6). The response rate in the 99 patients was 38.4% (95% CI; range, 28.8–48.0%, 2 CR, 36 PR, 32 SD, 8 NA, and 21 PD). The objective response rates stratified according to the DFI and HER2 status are shown in Table 2. The difference in the response rate between patients with a long DFI (≥2.5 years) and those with a short DFI (<2.5 years) was statistically significant in patients with an HER2-negative status (Chi-Square test— P = 0.014). Although the response rate tended to be higher in the patients with an HER2-positive status, statistical analysis to determine the significance was not performed due to the small sample size. Age and DFI were significantly associated with the response to AC therapy in according to the results of univariate analysis (P = 0.03 and 0.03, respectively). The results of the multivariate logistic regression analysis indicated that DFI as continuous variable significantly affected the response rate to AC therapy (Odds ratio, 1.23; 95% CI: 1.03–1.48, P = 0.02), even after adjusting for the effect of the HER2 status. The HER2 status tended to affect the response rate, however, the association was not statistically significant (Odds ratio, 4.1; 95% CI: 0.94–17.8, P = 0.06). The statistical analysis revealed no significant correlation of other factors, including AFI, with the response to AC therapy. The median time to progression and overall survival were 6.2 months (Fig. 1; 95% CI: 5.6–7.6 months) and 17.5 months (Fig. 1; 95% CI: 14.6–22.2 months), respectively.

Table 2 Objective response rate to doxorubicin plus cyclophosphamide therapy according to the HER2 status and DFI

Profile	No. of patients	Response rate (95% CI)
HER2 positive[a]	9	66.7% (29.9–92.5%)
HER2 negative	90	35.6% (25.7–46.4%)
DFI ≥2.5 years[b]	52	46.2% (31.6–60.7%)
DFI <2.5 years	38	21.1% (6.8–35.3%)
Total	99	38.4% (28.8–48.0%)

[a] The number of patients with HER2 positivity was small. Therefore, Chi-Square test was not performed for the HER2-positive patients according to the DFI

[b] Long DFI was associated with a higher response rate than a short DFI in HER2-negative patients (Chi-Square test— P = 0.014)

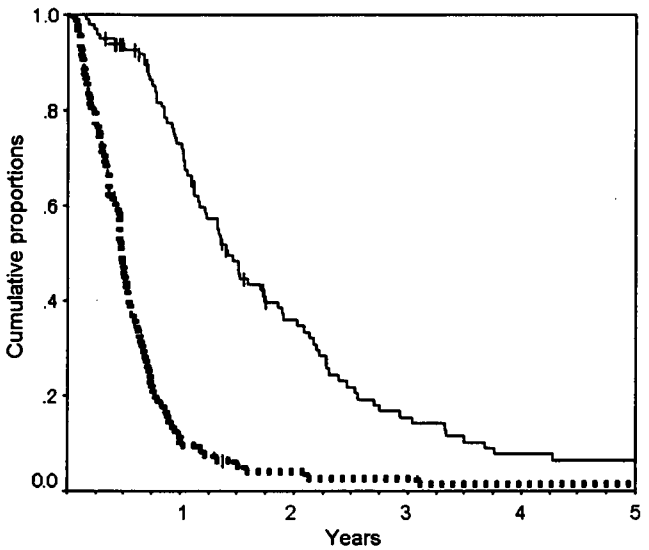

Fig. 1 Kaplan–Meier analysis of time to progression (*dotted line*) and overall survival (*solid line*). *Vertical bars* indicate censored cases

A total of 482 courses in the 99 patients were assessable for toxicity. The median cumulative dose of doxorubicin for RBC was 240 mg/m^2 (range, 40–240 mg/m^2), and the median total cumulative dose of anthracycline (after conversion to doxorubicin) was 343 mg/m^2 (range, 102–600 mg/m^2). The toxicity profile is listed in Table 3. The AC therapy was generally well tolerated and could be managed from an outpatient setting. Grade 3 or 4 neutropenia occurred in 14 patients (14.1%) and 4 patients of these developed febrile neutropenia. No cardiotoxicity was observed. No grade 4 non-hematological toxicity was reported either, and there were no unexpected adverse reactions or treatment-related deaths.

Table 3 Maximum grade (NCI-CTC ver 2.0) toxicity (% of patients)

	Maximum grade % of patients			
	1	2	3	4
Leukopenia	27	24	5	2
Neutropenia	15	21	7	7
Anemia	22	8	1	2
Thrombocytopenia	2	1	0	1
Fatigue	31	2	0	0
Appetite loss	82	7	0	0
Nausea	74	17	0	0
Vomiting	15	9	0	0
Stomatitis	21	1	0	0
Diarrhea	6	0	0	0
Constipation	8	0	0	0
Neurosensory	9	1	0	0

Discussion

This study demonstrated the activity of AC therapy even in RBC patients who had previously received anthracycline-containing chemotherapy in the neoadjuvant or adjuvant setting.

There are few reports of the efficacy of repeat use anthracycline-containing chemotherapy in metastatic or RBC patients. Although repeat use of anthracycline-containing regimens has been reported to yield objective response rates of 30–46%, there were no clear predictive factors of the response to such anthracycline agent-containing chemotherapy in case with RBC [8–12].

The results of the present study demonstrated that patients with a long DFI show favorable response to repeat use of AC as compared with patients with a short DFI. DFI, which reflect the degree of aggressiveness of the disease, had been known as one of the most important prognostic factors in cases of RBC [13]. Anthracyclines are topoisomerase inhibitors, and the topoisomerase II alpha gene has been reported to be associated with anthracycline sensitivity. Tinari et al. reported that anthracycline-sensitive breast cancer had the decreasing changes in topoisomerase II expression after anthracycline-based neoadjuvant chemotherapy and that it is an independent predictor of a long DFI [14]. Therefore, a long DFI may actually indicate inherent sensitivity to anthracycline, and a favorable response.

The role of the HER2 status in predicting the sensitivity to anthracyclines is still under debate. The topoisomerase II alpha gene is closely linked to the HER2 gene on chromosome 17 [15]. Recent studies have suggested that patients with an HER2-positive status might derive greater benefit from adjuvant chemotherapy using anthracycline-containing regimens as compared to that using non-anthracycline-containing regimens [16, 17]. While the HER2 status tended to influence the response rate to AC therapy in this study, the association was not found to be statistically significant. HER2 is generally overexpressed in 20–25% of breast cancers [18]. The relatively low frequency of patients with an HER2-positive status in the present study may also confound the result.

In the present study, all the patients had previously been treated with anthacycline-containing chemotherapeutic regimens, therefore, leukopenia or neutropenia (of any grade) was frequently observed, however, the incidence of febrile neutropenia was approximately equivalent to that reported by a previous randomized multicenter study of doxorubicin plus cyclophosphamide combination chemotherapy as

first-line treatment in cases with metastatic or RBC [19–21]. The retrospective nature of this study did not allow reliable or accurate estimation of the potential doxorubicin-related cardiotoxicity. Even though the incidence of cardiotoxicity may have been underestimated in this study, there were no cases with symptomatic cardiotoxicity or cardiotoxicity requiring treatment. Both the hematological and non-hematological toxicity profiles were similar to those reported by the Japanese randomized multicenter trial using the same standard dose of AC [21].

The use of anthracyclines in clinical practice is limited in most cases by the important drug-associated toxicity, namely, cardiotoxicity. Therefore, extensive research has been directed at the identification of methods capable of ameliorating the threat of anthracycline-related cardiotoxicity. Prevention or reduction of doxorubicin-induced cardiotoxicity would enable continuation of treatment in doxorubicin-responsive patients beyond the limit imposed by potential cardiotoxicity. The risk of anthracycline-related cardiotoxicity can be reduced by setting a cumulative dose limitation, generally in the region of 450–500 mg/m^2. However, it is recognized that injury to the myocardium begins with the first administration of anthracyclines and that cardiotoxicity begins to appear at a total cumulative dose <300 mg/m^2 [22]. In addition, several studies have reported factors that might increase the risk of doxorubicin-induced cardiotoxicity, namely, preexisting heart disease, mediastinal radiation, pediatric age group, and older patients (age >65 years) [23–25]. Also, the weekly schedule of drug administration was less cardiotoxic than the once-in-three weeks schedule [12]. Administration of doxorubicin as a prolonged infusion has been reported to be less cardiotoxic than that by bolus infusion [22, 26]. However, neither the schedule or administration rate recommended above widely accepted, because of the associated inconvenience. Encapsulated doxorubicin by pegylated liposomes is a unique formulation of doxorubicin, which reduces the maximum peak serum level of free doxorubicin and increases the half-life of the drug [27]. In addition, a randomized clinical trial demonstrated that liposomal doxorubicin was associated with a significantly reduced cumulative cardiac toxicity, while providing comparable antitumor efficacy [19]. Dexrazoxane, an iron-chelating agent, has shown the ability to reduce the cardiac toxicity induced by doxorubicin. Recent advances in safety-improvement techniques of may be expected to increase the therapeutic index of anthracyclines and enhance its overall clinical benefit.

Several studies have reported the efficacy of combined anthracycline plus other agent (e.g., taxanes, gemcitabine, vinorelbine) chemotherapy in cases with metastastic or RBC, including those previously administered anthracycline-based adjuvant chemotherapy [10, 12]. For RBC patients, the present study results suggesting the beneficial effects of doxorubicin with dexrazoxane, pegylated liposomal doxorubicin and the aforementioned combination chemotherapies may be significant.

The validity of measurement of the left ventricular ejection fraction by multiple-gated acquisition scan to predict the risk of cardiotoxicity remains controversial [28, 29]. However, at the present time, when considering the reutilization of anthracycline-containing chemotherapeutic regimens, care must be exercised to avoid patients with a high risk of anthracycline-related cardiotoxicity and careful monitoring should be conducted to detect any evidence of cardiotoxicity.

In conclusion, the results of the present study suggest that the treating physician of RBC cases may consider repeat utilization of anthracycline drug in patients likely to show a favorable response based on a long DFI and/or HER2 status. Anthracycline-containing chemotherapy may be administered as a useful chemotherapeutic option in these patients with careful monitoring for any evidence of the onset of cardiotoxicity.

Acknowledgements This study was supported by grants from the Ministry of Health, Labour and Welfare.

References

1. Early Breast Cancer Trialists' Collaborative Group (1998) Polychemotherapy for early breast cancer: an overview of the randomized trials. Lancet 352:930–942
2. Fisher B, Brown A, Mamounas E et al (1997) Effect of preoperative chemotherapy on local-regional disease in women with operable breast cancer: findings from National Surgical Adjuvant Breast and Bowel Project B-18. J Clin Oncol 15:2483–2493
3. Markman M, Markman J, Webster K et al (2004) Duration of response to second-line, platinum-based chemotherapy for ovarian cancer: implications for patient management and clinical trial design. J Clin Oncol 22:3120–3125
4. Beyer J, Kramar A, Mandanas R et al (1996) High-dose chemotherapy as salvage treatment in germ cell tumors: a multivariate analysis of prognostic variables. J Clin Oncol 14:2638–2645
5. Chua YJ, Steer C, Yip D (2004) Recent advances in management of small-cell lung cancer. Cancer Treat Rev 30:521–543
6. Joensuu H, Holli K, Heikkinen M et al (1998) Combination chemotherapy versus single-agent therapy as first- and second-line treatment in metastatic breast cancer: a prospective randomized trial. J Clin Oncol 16:3720–3730
7. World Health Organization (1979) WHO handbook for reporting result of cancer treatment: offset publication 48. World Health Organization, Geneva

8. Buzdar AU, Legha SS, Hortobagyi GN et al (1981) Management of breast cancer patients failing adjuvant chemotherapy with adriamycin-containing regimens. Cancer 47:2798–2802
9. Ro J, Fraschini G, Frye D et al (1989) Reutilization of doxorubicin in patients with progressive metastatic breast cancer. Int J Exp Clin Chemother 2:234–238
10. Martin M, Garcia-Donas J, Casado A et al (2004) Phase II study of pegylated liposomal doxorubicin plus vinorelbine in breast cancer with previous anthracycline exposure. Clin Breast Cancer 5:353–357
11. Overmoyer B, Silverman P, Holder LW et al (2005) Pegylated liposomal doxorubicin and cyclophosphamide as first-line therapy for patients with metastatic or recurrent breast cancer. Clin Breast Cancer 6:150–157
12. Fabi A, Ferretti G, Papaldo P et al (2006) Pegylated liposomal doxorubicin in combination with gemcitabine: a phase II study in anthracycline-naive and anthracycline pretreated metastatic breast cancer patients. Cancer Chemother Pharmacol 57:615–623
13. Yamamoto N, Watanabe T, Katsumata N et al (1998) Construction and validation of a practical prognostic index for patients with metastatic breast cancer. J Clin Oncol 16:2401–2408
14. Tinari N, Lattanzio R, Natoli C et al (2006) Changes of topoisomerase II alpha expression in breast tumors after neoadjuvant chemotherapy predicts relapse-free survival. Clin Cancer Res 12:1501–1506
15. Isola JJ, Tanner M, Holli K et al (2000) Amplification of topoisomerase II alpha is a strong predictor of response to epirubicin-based chemotherapy in HER2/neu positive metastatic breast cancer. In: Abstracts of the 23rd annual San Antonio breast cancer symposium, San Antonio, 6–9 December 2000
16. Di Leo A, Gancberg D, Larsimont D et al (2002) HER2 amplification and topoisomerase II alpha gene aberrations as predictive markers in node-positive breast cancer patients randomly treated either with an anthracycline-based therapy or with cyclophosphamide, methotrexate, and 5-fluorouracil. Clin Cancer Res 8:1107–1116
17. Pritchard KI, Shepherd LE, O'Malley FP et al (2006) HER2 and responsiveness of breast cancer to adjuvant chemotherapy. N Engl J Med 354:2103–2111
18. Salmon DJ, Godolphin W, Jones LA et al (1987) Studies of the HER2/neu proto-oncogene in human breast and ovarian cancer. Science 235:177–182
19. Batist G, Ramakrishnan G, Rao CS et al (2001) Reduced cardiotoxicity and preserved antitumor efficacy of liposomal-encapsulated doxorubicin and cyclophosphamide compared with conventional doxorubicin and cyclophosphamide in a randomized, multicenter trial of metastatic breast cancer. J Clin Oncol 19:1444–1454
20. Biganzoli L, Cufer T, Bruning P et al (2002) Doxorubicin and paclitaxel versus doxorubicin and cyclophosphamide as first-line chemotherapy in metastatic breast cancer: the European organization for research and treatment of cancer 10961 multicenter phase III trial. J Clin Oncol 20:3114–3121
21. Katsumata N, Minami H, Aogi K et al (2005) Phase III trial of doxorubicin/cyclophosphamide, docetaxel, and alternating AC and D as front-line chemotherapy for metastatic breast cancer: Japan Clinical Oncology Group trial. In: Abstracts of the 41st annual meeting of American Society of Clinical Oncology, Orlando, 13–17 May 2005. Proc Am Soc Clin Oncol 23:521A
22. Swain SM, Whaley FS, Gerber MC et al (1997) Delayed administration of dexrazoxane provides cardioprotection for patients with advanced breast cancer treated with doxorubicin-containing therapy. J Clin Oncol 15:1333–1340
23. Von Hoff DD, Layard MW, Basa P et al (1979) Risk factors for doxorubicin-induced congestive heart failure. Ann Intern Med 91:710–717
24. Praga C, Beretta G, Vigo PL et al (1979) Adriamycin cardiotoxicity: a survey of 1273 patients. Cancer Treat Rep 63:827–834
25. Krischer JP, Epstein S, Cuthbertson DD et al (1997) Clinical cardiotoxicity following anthracycline treatment for childhood cancer: the pediatric oncology group experience. J Clin Oncol 15:1544–1552
26. Legha SS, Benjamin RS, Mackay B et al (1982) Reduction of doxorubicin cardiotoxicity by prolonged continuous intravenous infusion. Ann Intern Med 96:133–139
27. Gabizon AA (1994) Liposomal anthracyclines. Hematol Oncol Clin North Am 8:431–450
28. Schwartz RG, McKenzie WB, Alexander J et al (1987) Congestive heart failure and left ventricular dysfunction complicating doxorubicin therapy. Seven-year experience using serial radionuclide angiocardiography. Am J Med 82:1109–1118
29. Swain SM, Whaley FS, Ewer MS (2003) Congestive heart failure in patients treated with doxorubicin: a retrospective analysis of three trials. Cancer 97:2869–2879

British Journal of Cancer (2007) 97, 778–784

www.bjcancer.com

Evaluation of epidermal growth factor receptor mutation status in serum DNA as a predictor of response to gefitinib (IRESSA)

H Kimura*[,1], M Suminoe[2], K Kasahara[1], T Sone[1], T Araya[1], S Tamori[1], F Koizumi[3], K Nishio[3,4], K Miyamoto[2,5], M Fujimura[1] and S Nakao[1]

[1]Department of Respiratory Medicine, Kanazawa University Hospital, Takara-machi 13-1, Kanazawa, Ishikawa 920-8641, Japan; [2]Department of Clinical Pharmacy, Graduate School of Natural Science and Technology, Kanazawa University, Takara-machi 13-1, Kanazawa, Ishikawa 920-8641, Japan; [3]Shien-Lab, National Cancer Center Hospital, Tsukiji 5-1, Chuo-ku, Tokyo 104-0045, Japan; [4]Department of Genome Biology, Kinki University School of Medicine, 377-2 Ohno-Higashi Osaka-Sayama, Osaka 589-8511, Japan; [5]Department of Hospital Pharmacy, School of Medicine, Kanazawa University, Takara-machi 13-1, Kanazawa, Ishikawa 920-8641, Japan

The aim of this study was to evaluate the usefulness of *EGFR* mutation status in serum DNA as a means of predicting a benefit from gefitinib (IRESSA) therapy in Japanese patients with non-small cell lung cancer (NSCLC). We obtained pairs of tumour and serum samples from 42 patients treated with gefitinib. *EGFR* mutation status was determined by a direct sequencing method and by Scorpion Amplification Refractory Mutation System (ARMS) technology. *EGFR* mutations were detected in the tumour samples of eight patients and in the serum samples of seven patients. *EGFR* mutation status in the tumours and serum samples was consistent in 39 (92.9%) of the 42 pairs. *EGFR* mutations were strong correlations between both *EGFR* mutation status in the tumour samples and serum samples and objective response to gefitinib ($P<0.001$). Median progression-free survival time was significantly longer in the patients with *EGFR* mutations than in the patients without *EGFR* mutations (194 *vs* 55 days, $P=0.016$, in tumour samples; 174 *vs* 58 days, $P=0.044$, in serum samples). The results suggest that it is feasible to use serum DNA to detect *EGFR* mutation, and that it's potential as a predictor of response to, and survival on gefitinib is worthy of further evaluation.
British Journal of Cancer (2007) **97,** 778–784. doi:10.1038/sj.bjc.6603949 www.bjcancer.com
Published online 11 September 2007

Keywords: EGFR; mutation; serum; gefitinib

Lung cancer is a major cause of cancer-related mortality worldwide and is expected to remain a major health problem for the foreseeable future (Parkin *et al*, 2005). Most patients have advanced disease at the time of diagnosis. Initial therapy for advanced non-small cell lung cancer (NSCLC) is typically systemic chemotherapy with a two-drug combination regimen, which often includes a platinum agent, but the median survival of patients treated with such regimens has ranged from only 8 to 10 months (Breathnach *et al*, 2001; Kelly *et al*, 2001; Schiller *et al*, 2002). Little improvement in the efficacy of chemotherapy has been made in the last 20 years. A recent report shows that the addition of bevacizumab, a monoclonal antibody against vascular endothelial growth factor, to paclitaxel plus carboplatin in patients with advanced NSCLC has a significant survival benefit, and the median survival was 12.3 months, as compared with 10.3 months in the chemotherapy-alone group (Sandler *et al*, 2006).

Targeting epidermal growth factor receptor (EGFR) is an appealing strategy for the treatment of NSCLC, because EGFR has been found to be expressed, sometimes strongly, in NSCLC (Franklin *et al*, 2002). Gefitinib ('Iressa', AstraZeneca) is a small molecule and selective EGFR tyrosine kinase inhibitor (EGFR-TKI) that has shown antitumour activity in NSCLC patients as a single agent in phase II and III trials (Fukuoka *et al*, 2003; Thatcher *et al*, 2005). An association between mutations in *EGFR* tyrosine kinase sites in NSCLC patients and hyper-responsiveness to gefitinib has recently been reported (Lynch *et al*, 2004; Paez *et al*, 2004). The mutations consisted of small in-frame deletions or substitutions clustered around the ATP-binding site in exons 18–21 of *EGFR*. Some investigators subsequently found that *EGFR* mutations are one of the strong determinants of tumour response to EGFR tyrosine kinase inhibitors (Pao *et al*, 2004; Han *et al*, 2005; Shigematsu *et al*, 2005). The mutation status could be evaluated stably in studies that used surgical tissues to detect the *EGFR* mutations, but most patients who require gefitinib therapy already have advanced disease at the time of diagnosis and therefore are not operated on. It is difficult to obtain sufficient tumour DNA from non-surgical tissue samples, for example, those derived from bronchoscopy that allow detection of *EGFR* mutations by direct sequencing. Actually, translational research in patients with advanced NSCLC in whom gefitinib therapy recommended has been limited by the scarcity of available tumour biopsy tissue, and tumour samples for genetic research were only available for 12.7 and 44.5%, respectively, of patients enrolled in two large phase III clinical studies with EGFR-TKIs (Tsao *et al*, 2005; Hirsch *et al*, 2006). It is therefore important to have sensitive methods for detecting *EGFR* mutations from DNA derived from non-surgical tissue specimens.

*Correspondence: Dr H Kimura;
E-mail: kimura@med3.m.kanazawa-u.ac.jp
Received 2 July 2007; accepted 30 July 2007; published online 11 September 2007

It is well known that the concentration of circulating DNA in plasma or serum has been found to be higher in cancer patients than in cancer-free control subjects, and that significantly higher DNA levels are found in the serum of patients with metastatic disease (Leon *et al*, 1977; Jahr *et al*, 2001; Sozzi *et al*, 2003). The tumour-derived DNA in serum may have been released by a tumour mass that has undergone cell necrosis or tumour cells lysis, or by circulating tumour cells, resulting in a very elevated serum DNA concentration. Some investigators have shown that testing for DNA alterations in peripheral blood has great potential, especially for early detection and diagnosis and for monitoring for a relapse during follow-up (Chen *et al*, 1996; Nawroz *et al*, 1996; Sozzi *et al*, 1999, 2001; Cuda *et al*, 2000; Nunes *et al*, 2001). The same alterations which mean mutations, methylation, and loss of heterozygosity, in genomic DNA have been observed in DNA from both tumour cells in resected and biopsy specimens, and from serum samples in patients with various types of tumours, including NSCLC (Sanchez-Cespedes *et al*, 1998; Esteller *et al*, 1999). Some studies have even reported that genetic aberrations in serum DNA modulate survival in NSCLC patients treated with chemotherapy. Their authors have proposed that the assay used in their studies may obviate the need for tumour tissue analysis (Ramirez *et al*, 2005; de las Penas *et al*, 2006). Serum samples can be obtained safely, with the option of repeat sampling from all NSCLC patients regardless of patient characteristics. The detection of *EGFR* mutations in serum provides a unique and potentially valuable tumour marker for prediction of response and prognosis.

We have previously reported the feasibility of detecting *EGFR* mutations in serum DNA using the Scorpion Amplification Refractory Mutation System (ARMS) method (Kimura *et al*, 2006). The Scorpion ARMS method is one of the most sensitive and fastest methods for specific detection of mutations in DNA (Newton *et al*, 1989; Whitcombe *et al*, 1999). Although *EGFR* mutations were detectable by both PCR direct sequencing, which has generally been used to detect the mutations and the Scorpion ARMS method, mutation status determined with Scorpion ARMS predicted response to gefitinib in our study (Kimura *et al*, 2006). Since the previous study did not clarify the feasibility of using serum DNA as a practical source for detection of *EGFR* mutations, in the present study, we sought to demonstrate that *EGFR* mutation status determined in serum DNA is the same as in actual tumour samples.

The aim of this study was (1) to determine whether the *EGFR* mutations in tumour tissue and serum samples from advanced NSCLC patients are the same, and (2) to identify whether there is a correlation between *EGFR* mutation status detected in serum DNA and both response to gefitinib and survival benefit from gefitinib.

PATIENTS AND METHODS

Patients

The subjects were patients with advanced NSCLC in whom gefitinib therapy was started between July 2002 and February 2006. All patients were treated with gefitinib alone, and 14 patients were treated with gefitinib as initial therapy. The others were treated with gefitinib as second- or third-line therapy. The diagnosis of NSCLC was based on the histological or cytological findings, and the histological type was determined according to WHO criteria (Travis *et al*, 1999). Patients' records consisted of age, gender, smoking habit, and histological tumour type. Patients were divided into three groups according to their smoking status: never-smokers (<100 cigarettes per lifetime), former smokers ($\geqslant 100$ cigarettes per lifetime, but quit 1 year before diagnosis), and current smokers ($\geqslant 100$ cigarettes per lifetime). The response to gefitinib was evaluated in accordance with the 'Response Evaluation Criteria in Solid Tumours (RECIST)' guidelines (Therasse *et al*, 2000). This study was approved by the Institutional Review Board of Kanazawa University Hospital. Written informed consent was obtained from all participants. No research results were entered into the patient's records or released to the patient or the patient's physician.

Tissue preparation and DNA extraction

Tumour specimens were obtained at diagnosis and analysed retrospectively. Twenty-eight tumour samples were collected from the primary cancer (19 via transbronchial lung biopsy, 2 via percutaneous lung biopsy, and 7 surgical specimens). Fourteen tumour samples were from metastatic sites (three from bone, eight lymph nodes, one brain, and one small bowel). All specimens were examined histologically to confirm the diagnosis of NSCLC. The tumour specimens obtained were fixed in formalin and embedded in paraffin wax. Serial sections containing representative malignant cells were deparaffinised in xylene washes and dehydrated in 100% ethanol. DNA was extracted from five serial 10-μm thick sections by using the QIAamp DNA Mini kit (Qiagen, Hilden, Germany) according to the protocol described in the manufacturer's instructions. The DNA obtained was eluted in 50 μl of buffer AE, and the concentration and purity of the extracted DNA were assessed by spectrophotometry. The extracted DNA was stored at -20°C until used.

Blood sample collection and DNA extraction

Blood samples were collected before the start of gefitinib therapy. The volume of each blood sample was 4 ml. Serum was separated within 2 h from the sample collection and stored at -80°C until used. Serum DNA was extracted and purified by using a Qiamp Blood Kit (Qiagen), with the following protocol modifications. One column was used repeatedly until the whole sample had been processed. The resulting DNA was eluted in 50 μl of sterile bi-distilled buffer. The concentration and purity of the extracted DNA were determined by spectrophotometry. The extracted DNA was stored at -20°C until used.

Direct sequencing for detection of *EGFR* mutations

EGFR mutations in exons 18, 19, and 21 were detected by PCR-based direct sequencing. PCR amplification was performed in 10 ng of genomic DNA using the TaKaRa Ex Taq™ Hot Start Version kit (TaKaRa, Tokyo, Japan). The primers (forward and reverse) were: exon 18 (5′-CCTTGTCTCTGTGTTCTTGT-3′ and 5′-CTGCGGCCCAGCCCAGAGGC-3′), exon 19 (5′-CATGTGGCAC CATCTCACA-3′ and 5′-CCACACAGCAAAGCAGAA AC-3′), and exon 21 (5′-CAGGGTCTTCTCTGTTTCAG-3′ and 5′-TAAAGC CACCTCCTTACTTT-3′). DNA was amplified for 35 cycles at 95°C for 30 s, 61°C for 30 s, and 72°C for 60 s followed by 7 min of extension at 72°C. Sequencing was performed with a 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA), and the results were analysed with Sequencer 3.11 software (Applied Biosystems) to compare variations. The sequences were compared with the GenBank human sequence for *EGFR* (accession number AF288738).

Scorpion ARMS for detection of E746_A750del and L858R

An EGFR Scorpion Kit (DxS Ltd, Manchester, UK), which combines two technologies, namely ARMS and Scorpion was to detect mutations in real-time PCR as described previously (Kimura *et al*, 2006). Four scorpion primers for detection of E746_A750del, L858R, and the wild type in both exons 19 and 21 were designed and synthesised by DxS Ltd. All reactions were performed in 25 μl volumes using 1 μl of template DNA, 7.5 μl of reaction buffer mix, 0.6 μl of Primer mix and 0.1 μl of Taq polymerase. All reagents are

included in the kit. Real-time PCR was carried out by using SmartCycler® II (Cepheid, Sunnyvale, CA, USA) under the following conditions: initial denaturation at 95°C for 10 min, 50 cycles of 95°C for 30 s, 62°C for 60 s with fluorescence reading (set to FAM, which allows optical excitation at 480 nm and measurement at 520 nm) at the end of each cycle. Data analysis was performed with Cepheid SmartCycler software (version 1.2b). The cycle threshold (C_t) was defined as the cycle at the highest peak of the second-derivative curve, which represented the point of maximum curvature of the growth curve. Both C_t and maximum fluorescence (F_l) were used to interpret the results. Positive results were defined as follows: $C_t \leqslant 45$ and $F_l \geqslant 50$. These analyses were performed in duplicate for each sample and reviewed by two investigators blinded to any clinical information.

Statistical analyses

Patient characteristics, including gender, tumour histology, smoking habit, and response to gefitinib, were tabulated according to mutation status. Fisher's exact test was used to test for associations between the presence of *EGFR* mutations and the patients' characteristics. Overall survival (OS) and progression-free survival (PFS) according to *EGFR* mutation status were estimated by the Kaplan–Meier method, and compared using the two-sided log-rank test. Overall survival was defined as the interval between the start of gefitinib therapy and death from any cause; patients known to be still alive at the time of the analysis were censored at the time of their last follow-up. Progression-free survival was defined as the interval between the start of gefitinib therapy and the first manifestation of progressive disease (PD) or death from any cause; patients known to be alive and without PD at the time of analysis were censored at the time of their last follow-up.

RESULTS

Patient's characteristics

Forty-two patients were enrolled in this study (Table 1). This study covered a long period. There are two reasons why it took 4 years to assemble the 42 patients enrolled. One is that this study was carried out in Kanazawa University Hospital alone, and was not a multicentre study. The other is that not all patients with NSCLC at the hospital during that period were enrolled in this study, because some were enrolled in other trials or the patients refused. Their median age was 58 years (range, 40–81 years), and there were 14 females (33.3%) and 14 never-smokers (33.3%). The histological and/or cytological diagnosis was adenocarcinoma in 31 patients (73.8%), squamous cell carcinoma in 7 (16.7%), and large-cell carcinoma in 4 (9.5%). The results for response to gefitinib showed that 10 patients (23.8%) had a partial response (PR) and 14 (33.3%) had stable disease (SD). The other 18 patients (42.9%) had PD. Serum DNA was extracted in all 42 samples at a median concentration of 62.0 ng ml^{-1} (range, 0–342.8). The concentrations in 10 samples were below the minimum concentration detectable.

Table 1 Patient characteristics and *EGFR* mutation status

	(n)
No. of patients	42
Age (years)	
Median	58
Range	40–1
Gender	
Male	28 (66.7%)
Female	14 (33.3%)
Smoking habit	
Current	20 (47.6%)
Former	8 (19.1%)
Never	14 (33.3%)
Histology	
Adenocarcinoma	31 (73.8%)
Squamous cell carcinoma	7 (16.7%)
Large-cell carcinoma	4 (9.5%)
Response to gefitinib	
Partial response	10 (23.8%)
Stable disease	14 (33.3%)
Progressive disease	18 (42.9%)

EGFR mutation status detected

Direct sequencing of PCR products from tumour tissues of all patients allowed their mutation status to be determined. Both direct sequencing and Scorpion ARMS allowed mutation status to be determined in the serum samples of all patients. As summarised in Table 2, mutations were identified in 9 (21.4%) of the 42 patients. Mutations in eight patients were detected in tumour samples and seven in serum samples. Five mutations were deletion mutations located in exon 19 (E746_A750del in four and L747_T751del in one). Four mutations were substitution mutations located in exon 21 (L858R), and one was a substitution mutation located in exon 18 (V689L). One patient had double substitution mutations (V689L and L858R). The E746_A750 deletion and L858R substitution mutation were the most common (8 out of 9, 88.9%), and both are well-known hot spot mutations described previously (Kosaka *et al*, 2004; Han *et al*, 2005). There were no T790M mutations identified by direct sequencing on tumour samples or serum samples. Of the nine patients with mutations, six (66.7%) were never-smokers, and five (55.6%) were female patients. Almost all of the patients with mutations had adenocarcinoma (8 out of 9, 88.9%).

Sensitivity and specificity of detection in serum DNA

In six of the patients, the same *EGFR* mutation was detected in both the tumour sample and the serum sample. There were no *EGFR* mutations detected in either the tumour sample or serum sample from 33 of the patients. *EGFR* mutation status was consistent in 39 (92.9%) of the 42 of the pairs (Table 3). In two patients the tumour samples was positive for an *EGFR* mutation and the serum sample was negative. The concentrations of serum DNA in the two patients were below the minimum level of detection by spectrophotometry. In one patient, the serum sample was positive for an *EGFR* mutation and the tumour sample was negative. The tumour sample that contained no mutations from the patient whose serum was positive for a mutation was collected by transbronchial lung biopsy.

Correlation between *EGFR* mutation status and patient characteristics

Detection of *EGFR* mutations occurred significantly more frequently in the serum DNA from the never-smokers (never-smokers 5 out of 14 (35.7%); current/former smokers 2 out of 28 (7.1%); $P = 0.031$) (Table 4). Mutations were more frequently detected in the DNA from tumour samples of never-smokers than of current/former smokers (never-smokers 5 out of 14 (35.7%); current/former smokers 3 out of 28 (10.7%); $P = 0.092$), but the difference was not statistically significant. Mutations were detected more frequently in the samples from females (tumour: females 5 out of 14 (35.7%), males 3 out of 28 (10.7%); serum: females 3 out

Table 2 Patients with *EGFR* mutation

Age	Gender	Histology	Stage	Smoking	Response	EGFR mutation status	
						Tumour tissue	Serum
44	M	Ad	Re	Never	PR	E746_A750del	E746_A750del
79	M	Ad	IV	Former	PR	L858R	L858R
53	M	Ad	IV	Never	PR		V689L, L858R*
59	M	La	IV	Current	PD	E746_A750del	E746_A750del
63	F	Ad	IIIB	Never	PR	L858R	
62	F	Ad	IV	Never	PR	E746_A750del	E746_A750del
56	F	Ad	IV	Never	PR	E746_A750del	E746_A750del
57	F	Ad	IIIB	Former	SD	E746_T751del	
62	F	Ad	IV	Never	PR	L858R	L858R

Ad = adenocarcinoma; del = deletion; EGFR = epidermal growth factor receptor; F = female; La = large-cell carcinoma; M = male; PD = progressive disease; PR = partial response; Re = recurrence after surgery; SD = stable disease. The numbering of the mutation sites was based on NP_005219.2 (amino acid). *L858R was detected both by Scorpion ARMS and direct sequencing. V689L was detected by direct sequencing. All samples detected in serum DNA but the samples (*) were detected by Scorpion ARMS alone.

Table 3 Sensitivity for detection of *EGFR* mutations in serum samples

		Serum	
		+	−
Tumour tissue	+	6	2
	−	1	33

EGFR = epidermal growth factor receptor; + = mutation positive; − = mutation negative.

Table 4 Frequency of *EGFR* mutations

	Tumour tissue			Serum		
	+	−		+	−	
(A) Gender and EGFR mutation status						
Female	5	9		3	11	
Male	3	25	$P=0.092$	4	24	$P=0.669$
(B) Histology and EGFR mutation status						
Ad	7	24		6	25	
Non-Ad	1	10	$P=0.657$	1	10	$P=0.654$
(C) Smoking habit and EGFR mutation status						
Never	5	9		5	9	
Current/former	3	25	$P=0.092$	2	26	$P=0.031$
(D) Response to gefitinib						
PR	6	4		6	4	
SD/PD	2	30	$P<0.001$	1	31	$P<0.001$

Ad = adenocarcinoma; EGFR = epidermal growth factor receptor; PD = progressive disease; PR = partial response; SD = stable disease; + = mutation positive; − = mutation negative. *P*-value: Fisher's exact test.

of 14 (27.2%), males 4 out of 28 (14.3%)) and from patients with adenocarcinoma (tumour: adenocarcinoma 7 out of 31 (22.6%), non-adenocarcinoma 1 out of 11 (9.1%); serum: adenocarcinoma 6 out of 31 (19.4%), non-adenocarcinoma 1 out of 11 (9.1%)), but the differences were not statistically significant. There were no statistically significant differences in demographic characteristics between the patients with *EGFR* deletion mutations and patients with *EGFR* substitution mutations (data not shown).

Correlation between *EGFR* mutation status and response to gefitinib

EGFR mutations were detected significantly more frequently in responders to gefitinib. Seven of the nine patients with mutations had a PR to gefitinib. Comparison between *EGFR* mutation status and response to gefitinib showed that *EGFR* mutation was more frequent in patients with a PR than in patients with SD/PD (Table 4D).

EGFR mutations are associated with increased survival

The median PFS and OS of the patients treated with gefitinib was 60 days (95% CI, 52–68) and 228 days (95% CI, 150–306), respectively. Patients with *EGFR* mutations in both tumour samples and serum samples had a significantly longer median PFS than the patients without *EGFR* mutations (194 *vs* 55 days, $P=0.016$, in tumour samples; 174 *vs* 58 days, $P=0.044$, in serum samples; Figure 1A). The patients with *EGFR* mutations had a longer median OS than the patients without *EGFR* mutations, but the difference was not statistically significant (716 *vs* 193 days, $P=0.070$, in tumour samples; 387 *vs* 228 days, $P=0.489$, in serum samples; Figure 1B). These results suggest that the patients who were serum *EGFR*-mutation-positive had better outcomes of gefitinib therapy in terms of PFS, OS, and response, than patients who were *EGFR*-mutation-negative. In addition smoking status (never-smoker *vs* former/current smoker) was found to be an independent predictor of longer PFS ($P=0.002$) and longer OS ($P=0.035$). Progression-free survival and OS were longer in female patients and patients with adenocarcinoma than in male patients and non-adenocarcinoma patients, respectively, but the differences were not statistically significant.

DISCUSSION

We previously reported detecting *EGFR* mutations in serum DNA by Scorpion ARMS method and that mutation status is useful for predicting response to gefitinib (Kimura *et al*, 2006). The two major findings in the present study provide additional support for the use of serum DNA as an alternative to tumour samples for detection of *EGFR* mutations in patients with advanced NSCLC. First, these results demonstrate that *EGFR* mutation status in serum DNA was the same as in tumour samples in almost every patient. In addition, mutation status in serum DNA predicted for a significantly greater response and time to progression with gefitinib, as well as showing a trend towards increased OS in patients treated with gefitinib. The results confirm the clinical

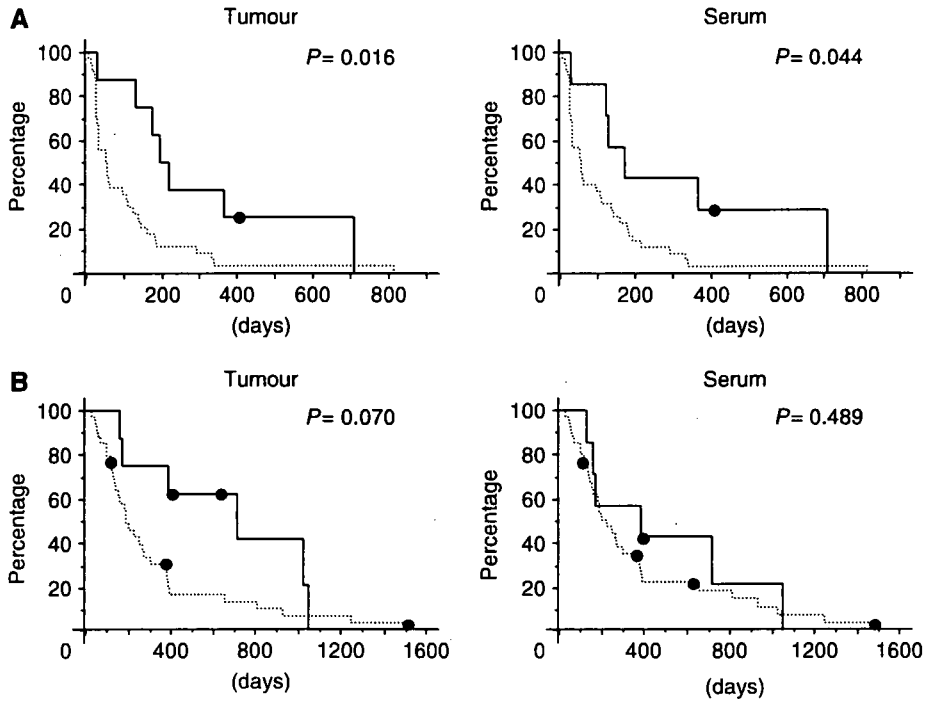

Figure 1 Kaplan–Meier probability of progression-free survival (**A**) and overall survival (**B**) with respect to the EGFR mutation status of NSCLC. *P*-values were calculated by the log-rank test.

reliability of *EGFR* mutation detection in serum DNA as a predictive marker of response to gefitinib.

The sites of the *EGFR* mutations detected in this study are identical to those reported in previous studies (Kosaka *et al*, 2004; Pao *et al*, 2004). The majority mutations were in-frame deletions in exon 19 and the missense mutation L858R in exon 21. The comparison between mutation status and clinical manifestations in this study confirmed the finding in previous studies that *EGFR* mutations are frequently present in small subgroups of NSCLC patients, including females, never-smokers, and patients with adenocarcinoma histology, although these findings were not statistically significant.

EGFR mutations were detected in only 1.0 ml serum samples. The amount of DNA extracted was minute, and its concentration in roughly one-third of patients was below the minimum concentration detectable by spectrophotometry. Moreover, lung cancers are very heterogeneous, and patients' serum also contains DNA derived from normal cells. Direct sequencing seems unable to provide satisfactory results for detection of *EGFR* mutations in samples containing a mixture of mutated and wild-type DNA. Although direct sequencing has generally been used to detect *EGFR* mutations, detection by direct sequencing requires at least 30% of the DNA in the sample to be mutated (Bosari *et al*, 1995; Fan *et al*, 2001). Small amounts and low percentages of mutated DNA in serum can be missed by direct sequencings. When serum is used as the material for detection of *EGFR* mutations, patients with *EGFR* mutations may be diagnosed as having wild-type *EGFR* because of the two limitations described above. In this study, the mutation was detected by direct sequencing in only one patient. The mutation status detected by Scorpion ARMS in serum samples was nearly identical to that in tumour samples. The concentrations of serum DNA in two of seven patients with *EGFR* mutations in serum samples were below the minimum concentration detectable. The high-sensitive method, Scorpion ARMS, completely resolved the problem.

The mutation status in the pairs of samples from three patients (3 out of 42, 7.1%) did not match. The results in the serum DNA of two patients were mutation-negative, whereas mutations were detected in actual tumour samples. The amount of tumour-specific DNA may have been below the threshold of detection with the Scorpion ARMS Kit in the patient with L858R. Little tumour-specific DNA may be circulating in patients, and the quality of the DNA is also a determinant of successful detection. Prolonged storage of serum samples has been reported to result in a decrease in the amount of DNA extracted (Sozzi *et al*, 2005). The other patient had an E746_T751del, and the mutation was not detected with the Scorpion ARMS in the patients. Although we have showed the usefulness of Scorpion ARMS for detection of *EGFR* mutation in serum samples (Kimura *et al*, 2006), Scorpion ARMS is only able to detect mutations targeted by the Scorpion primers designed in advance and in this study was capable of detecting the specific mutation of E746_A750del in exon 19 and L858R in exon 21. E747_P753del insS and L747_T751del are minor variations of deletional mutations in exon 19 and were not detected by this method in a preliminary experiment (data not shown). We do not think that E746_T751del can be detected with Scorpion ARMS. Mutation status in serum DNA was positive (V689L and L858R) in one patient in whom no mutations were detected in actual tumour samples. V689L and L858R are somatic mutations. We concluded that the direct sequencing of DNA from the tumour sample yielded the wrong result. Low rate of tumour-derived DNA in total DNA or impure DNA extracted from tumour samples may have prevented a detection of the mutation by direct sequencing.

On the basis of the results of this study, we conclude that it is feasible to use serum DNA to detect *EGFR* mutation status and evaluate its potential as a predictor of response to EGFR-TKI. The serum assay to detect *EGFR* mutations circumvents the need for tumour tissue and merits further validation of the use of serum DNA to detect *EGFR* mutations as a predictor of response to, and survival on gefitinib in prospective studies.

REFERENCES

Bosari S, Marchetti A, Buttitta F, Graziani D, Borsani G, Loda M, Bevilacqua G, Coggi G (1995) Detection of p53 mutations by single-strand conformation polymorphisms (SSCP) gel electrophoresis. A comparative study of radioactive and nonradioactive silver-stained SSCP analysis. *Diagn Mol Pathol* **4**: 249–255

Breathnach OS, Freidlin B, Conley B, Green MR, Johnson DH, Gandara DR, O'Connell M, Shepherd FA, Johnson BE (2001) Twenty-two years of phase III trials for patients with advanced non-small-cell lung cancer: sobering results. *J Clin Oncol* **19**: 1734–1742

Chen XQ, Stroun M, Magnenat JL, Nicod LP, Kurt AM, Lyautey J, Lederrey C, Anker P (1996) Microsatellite alterations in plasma DNA of small cell lung cancer patients. *Nat Med* **2**: 1033–1035

Cuda G, Gallelli A, Nistico A, Tassone P, Barbieri V, Tagliaferri PS, Costanzo FS, Tranfa CME, Venuta S (2000) Detection of microsatellite instability and loss of heterozygosity in serum DNA of small and non-small cell lung cancer patients: a tool for early diagnosis? *Lung Cancer* **30**: 211–214

de las Penas R, Sanchez-Ronco M, Alberola V, Taron M, Camps C, Garcia-Carbonero R, Massuti B, Queralt C, Botia M, Garcia-Gomez R, Isla D, Cobo M, Santarpia M, Cecere F, Mendez P, Sanchez JJ, Rosell R, On behalf of the Spanish Lung Cancer, G (2006) Polymorphisms in DNA repair genes modulate survival in cisplatin/gemcitabine-treated non-small-cell lung cancer patients. *Ann Oncol* **17**: 668–675

Esteller M, Sanchez-Cespedes M, Rosell R, Sidransky D, Baylin SB, Herman JG (1999) Detection of aberrant promoter hypermethylation of tumor suppressor genes in serum DNA from non-small cell lung cancer patients. *Cancer Res* **59**: 67–70

Fan X, Furnari FB, Cavenee WK, Castresana JS (2001) Non-isotopic silver-stained SSCP is more sensitive than automated direct sequencing for the detection of PTEN mutations in a mixture of DNA extracted from normal and tumor cells. *Int J Oncol* **18**: 1023–1026

Franklin WA, Veve R, Hirsch FR, Helfrich BA, Bunn PA (2002) Epidermal growth factor receptor family in lung cancer and premalignancy. *Semin Oncol* **29**: 3–14

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). *J Clin Oncol* **21**: 2237–2246

Han SW, Kim TY, Hwang PG, Jeong S, Kim J, Choi IS, Oh DY, Kim JH, Kim DW, Chung DH, Im SA, Kim YT, Lee JS, Heo DS, Bang YJ, Kim NK (2005) Predictive and prognostic impact of epidermal growth factor receptor mutation in non-small-cell lung cancer patients treated with gefitinib. *J Clin Oncol* **23**: 2493–2501

Hirsch FR, Varella-Garcia M, Bunn PA, Franklin WA, Dziadziuszko R, Thatcher N, Chang A, Parikh P, Pereira JR, Ciuleanu T, von Pawel J, Watkins C, Flannery A, Ellison G, Donald E, Knight L, Parums D, Botwood N, Holloway B (2006) Molecular predictors of outcome with gefitinib in a phase III placebo-controlled study in advanced non-small-cell lung cancer. *J Clin Oncol* **24**: 5034–5042

Jahr S, Hentze H, Englisch S, Hardt D, Fackelmayer FO, Hesch RD, Knippers R (2001) DNA fragments in the blood plasma of cancer patients: quantitations and evidence for their origin from apoptotic and necrotic cells. *Cancer Res* **61**: 1659–1665

Kelly K, Crowley J, Bunn 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

Kimura H, Kasahara K, Kawaishi M, Kunitoh H, Tamura T, Holloway B, Nishio K (2006) Detection of epidermal growth factor receptor mutations in serum as a predictor of the response to gefitinib in patients with non-small-cell lung cancer. *Clin Cancer Res* **12**: 3915–3921

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

Leon SA, Shapiro B, Sklaroff DM, Yaros MJ (1977) Free DNA in the serum of cancer patients and the effect of therapy. *Cancer Res* **37**: 646–650

Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW, Harris PL, Haserlat SM, Supko JG, Haluska FG, Louis DN, Christiani DC, Settleman J, Haber DA (2004) Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N Engl J Med* **350**: 2129–2139

Nawroz H, Koch W, Anker P, Stroun M, Sidransky D (1996) Microsatellite alterations in serum DNA of head and neck cancer patients. *Nat Med* **2**: 1035–1037

Newton CR, Graham A, Heptinstall LE, Powell SJ, Summers C, Kalsheker N, Smith JC, Markham AF (1989) Analysis of any point mutation in DNA. The amplification refractory mutation system (ARMS). *Nucleic Acids Res* **17**: 2503–2516

Nunes DN, Kowalski LP, Simpson AJG (2001) Circulating tumor-derived DNA may permit the early diagnosis of head and neck squamous cell carcinomas. *Int J Cancer* **92**: 214–219

Paez JG, Janne PA, Lee JC, Tracy S, Greulich H, Gabriel S, Herman P, Kaye FJ, Lindeman N, Boggon TJ, Naoki K, Sasaki H, Fujii Y, Eck MJ, Sellers WR, Johnson BE, Meyerson M (2004) EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science* **304**: 1497–1500

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

Parkin DM, Bray F, Ferlay J, Pisani P (2005) Global cancer statistics, 2002. *CA Cancer J Clin* **55**: 74–108

Ramirez JL, Rosell R, Taron M, Sanchez-Ronco M, Alberola V, de las Penas R, Sanchez JM, Moran T, Camps C, Massuti B, Sanchez JJ, Salazar F, Catot S (2005) 14-3-3σ methylation in pretreatment serum circulating DNA of cisplatin-plus-gemcitabine-treated advanced non-small-cell lung cancer patients predicts survival: The Spanish Lung Cancer Group. *J Clin Oncol* **23**: 9105–9112

Sanchez-Cespedes M, Monzo M, Rosell R, Pifarre A, Calvo R, Lopez-Cabrerizo MP, Astudillo I (1998) Detection of chromosome 3p alterations in serum DNA of non-small-cell lung cancer patients. *Ann Oncol* **9**: 113–116

Sandler A, Gray R, Perry MC, Brahmer J, Schiller JH, Dowlati A, Lilenbaum R, Johnson DH (2006) Paclitaxel-carboplatin alone or with bevacizumab for non-small-cell lung cancer. *N Engl J Med* **355**: 2542–2550

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

Shigematsu H, Lin L, Takahashi T, Nomura M, Suzuki M, Wistuba 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

Sozzi G, Conte D, Leon M, Cirincione R, Roz L, Ratcliffe C, Roz E, Cirenei N, Bellomi M, Pelosi G, Pierotti MA, Pastorino U (2003) Quantification of free circulating DNA as a diagnostic marker in lung cancer. *J Clin Oncol* **21**: 3902–3908

Sozzi G, Conte D, Mariani L, Lo Vullo S, Roz L, Lombardo C, Pierotti MA, Tavecchio L (2001) Analysis of circulating tumor DNA in plasma at diagnosis and during follow-up of lung cancer patients. *Cancer Res* **61**: 4675–4678

Sozzi G, Musso K, Ratcliffe C, Goldstraw P, Pierotti MA, Pastorino U (1999) Detection of microsatellite alterations in plasma DNA of non-small cell lung cancer patients: a prospect for early diagnosis. *Clin Cancer Res* **5**: 2689–2692

Sozzi G, Roz L, Conte D, Mariani L, Andriani F, Verderio P, Pastorino U (2005) Effects of prolonged storage of whole plasma or isolated plasma DNA on the results of circulating DNA quantification assays. *J Natl Cancer Inst* **97**: 1848–1850

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

Therasse P, Arbuck SG, Eisenhauer EA, Wanders J, Kaplan RS, Rubinstein L, Verweij J, Van Glabbeke M, van Oosterom AT, Christian MC, Gwyther SG (2000) New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer,

National Cancer Institute of the United States, National Cancer Institute of Canada. *J Natl Cancer Inst* **92**: 205–216

Travis W, Colby TV, Corrin B (1999) *Histologic Typing of Tumors of Lung and Pleura: World Health Organization International Classification of Tumors (ed 3)*. Springer Verlag: New York, NY

Tsao MS, Sakurada A, Cutz JC, Zhu CQ, Kamel-Reid S, Squire J, Lorimer I, Zhang T, Liu N, Daneshmand M, Marrano P, da Cunha Santos G, Lagarde A, Richardson F, Seymour L, Whitehead M, Ding K, Pater J, Shepherd FA (2005) Erlotinib in lung cancer – molecular and clinical predictors of outcome. *N Engl J Med* **353**: 133–144

Whitcombe D, Theaker J, Guy SP, Brown T, Little S (1999) Detection of PCR products using self-probing amplicons and fluorescence. *Nat Biotechnol* **17**: 804–807

Available online at www.sciencedirect.com
ScienceDirect
Cancer Letters 252 (2007) 225–234
CANCER Letters
www.elsevier.com/locate/canlet

Interaction between CD44 and hyaluronate induces chemoresistance in non-small cell lung cancer cell

Rina Ohashi [a,b,*], Fumiyuki Takahashi [a,b], Ri Cui [a,b], Masakata Yoshioka [a,b], Tao Gu [a,b], Shinichi Sasaki [a], Shigeru Tominaga [a], Kazuto Nishio [c], Kenneth K. Tanabe [d], Kazuhisa Takahashi [a,b]

[a] *Department of Respiratory Medicine, Juntendo University, School of Medicine, 2-1-1 Hongo, Bunkyo-Ku, Tokyo 113-8421, Japan*
[b] *Research Institute for Disease of Old Ages, Juntendo University, School of Medicine, 2-1-1 Hongo, Bunkyo-Ku, Tokyo 113-8421, Japan*
[c] *Pharmacology Division, National Cancer Research Institute, 5-1-1 Tsukiji, Chuo-Ku, Tokyo 104-0045, Japan*
[d] *Division of Surgical Oncology, Department of Surgery, Massachusetts General Hospital, Yawkey 7.924, 55 Fruit Street, Boston, MA 02114, USA*

Received 14 September 2006; received in revised form 18 December 2006; accepted 20 December 2006

Abstract

CD44s is a principle hyaluronate (HA) receptor and has been reported to play an important role in cancer cell invasion and metastasis. The aim of our study is to determine if the interaction between HA and CD44s influences in vitro chemosensitivity of non-small cell lung cancer (NSCLC). NSCLC cell line, H322 cells, transfected with the CD44s gene (H322/CD44s) cultured on HA coated plates were more resistant to cisplatin (CDDP) than that on bovine serum albumin. Multidrug resistance protein2 (MRP2) expression was induced in H322/CD44s cells cultured on HA. MRP2 inhibitor, MK571, not only suppressed MRP2 expression but also reversed CDDP resistance. These results suggest that the interaction between CD44s and HA play a pivotal role in acquired resistance to CDDP in NSCLC and MRP2 could be involved in this potential mechanism.

Keywords: Hyaluronate; Non-small cell lung cancer; Chemoresistance; CD44s; MRP2

1. Introduction

The incidence of patients with lung cancer is increasing and approximately 80% of lung cancer patients have non-small cell lung cancer (NSCLC) [1]. Although surgical resection is the most effective therapy for early stage NSCLC, more than 60% of patients with NSCLC were of the advanced stage of the disease, and cannot be operated at initial presentation [2]. In spite of recent advancements in chemotherapy, the prognosis of patients with stage IV NSCLC still remains poor and most phase III trials have shown a median survival time of 8–10 months and a 1-year survival rate of 30–35%.

Drug resistance remains as a major obstacle in the attainment of favorable outcome or successful

* Corresponding author. Tel.: +81 3 5802 1063; fax: +81 3 5802 1617.
E-mail address: rinaohas@med.juntendo.ac.jp (R. Ohashi).

0304-3835/$ - see front matter © 2007 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.canlet.2006.12.025

use of chemotherapeutic agents especially for treatment of lung cancer. Comparing to hematopoietic cells, NSCLC cells are less chemosensitive and gradually acquire chemoresistance. Various mechanisms for drug resistance have been considered, such as decrease in agent accumulation that crosses the plasma membrane, increased intracellular detoxication and increased DNA repair ability [3]. In fact, Sethi et al. have reported that adhesion of small cell lung cancer cells to the extracellular matrix (ECM) protein; fibronectin, collagen, or laminin, enhances tumorigenecity and confers resistance to chemotherapeutic agents [4]. It has been reported that efficacy of chemotherapeutic agent is variable and dependent upon the targeted organs according to clinical experience. In fact, it has also been reported that leukemic cells acquire chemoresistance through interaction of β_1 integrin with fibronectin on the stroma cell [16]. These findings lead us to hypothesize that the interaction of NSCLC cells and extracellular matrix (ECM) in the microenvironment is responsible in the regulation of chemosensitivity.

Hyaluronate (HA) is a glycosaminoglycan consisting of disaccharide repeats, exists in the ECM, play an important role in cancer cell invasion and metastasis [5]. CD44 is a principle receptor for HA and also has been implicated in tumor invasion and metastasis [6–10]. It consists of various isoforms resulting from alternative splicing of its mRNA. Its low molecular weight CD44 isoform, CD44s, binds to HA to greater extent than the high molecular weight CD44 isoforms. Recently, Bates et al. have revealed that activation of variant CD44 in colon carcinoma cell lines triggers resistance to chemotherapeutic agents [11]. However, the role of HA and its receptor, CD44s, in NSCLC in the resistance against chemotherapeutic agents has not been clarified.

Therefore, the aim of our study is to determine if the interaction of NSCLC cell lines with HA regulates in vitro chemosensitivity against chemotherapeutic agents. In this study, we revealed that interaction between HA and its receptor, CD44s, plays an important role in acquired resistance to CDDP in NSCLC. We also discuss its potential mechanism.

2. Materials and methods

2.1. Reagents, cell lines and monoclonal antibody

The human NSCLC cell line, H322, which originally lacks CD44s expression, was kindly provided by Dr Isaiah Fidler (M.D. Anderson Cancer Center, Houston, TX). Cells were maintained in RPMI1640 (Kohjin Bio, Japan) containing 10% (v/v) fetal calf serum (FCS) at 37 °C in 5% CO_2 atmosphere. The monoclonal antibodies (mAb), BU52, (Binding Site, Inc., San Diego, CA) are directed against epitopes common to all CD44 isoforms and M2 I-4 (ALEXIS, Switzerland) is the monoclonal antibody against multidrug resistance protein 2 (MRP2). For in vitro chemosensitivity experiments, cells were grown in SITA (RPMI 1640 supplemented with 30 nM selenium, 5 μg/ml insulin, 10 μg/ml transferrin and 0.25% (w/v) BSA). Cells were routinely tested for Mycoplasma contamination with MycoAlert Mycoplasma Detection Kit (Cambrex, Rockland, ME).

To evaluate cell viability, the Cell Counting Kit-8 using WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) was purchased from Wako (Osaka, Japan).

2.2. Transfection

Five million H322 cells were electroporated using 10 μg of the expression vector pRC/CMV (Invitrogen, San Diego, CA) containing CD44s cDNA or CD44s cDNA of which the cytoplasmic region was deleted, CD44TR [8]. Briefly, the Cell-Porator (Gibco/BRL Gaithesburg, MD) was set at 800 V/cm and 800 mF; cells were electroporated at 4 °C in Hepes buffered saline. After 3 days of incubation in the complete medium, G418 (Sigma Chemical Co. St. Louis, MI) was added to a final concentration of 500 μg/ml for selection of drug resistant clones. The clones transfected with cDNA for CD44s, CD44TR, and PRC/CMV (no insert) in H322 cells were designated with suffices H322/CD44s, H322/TR and H322/Mock, respectively. Several clones for each transfectant were obtained.

2.3. Western blot

For Western blot analyses, cells were homogenized in lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.02% NaN_3, 1 mM phenylmethylsulfonylfluoride, 1 μg/ml aprotinin, and 1% Triton-X-100). Samples containing equal amounts of protein were separated using non-reducing conditions on acrylamide gels and transferred to a nitrocellulose filter with electroblotting at 4 °C. The filters were blocked for 1 h in PBS containing 10% dry milk, washed in PBS containing 1% dry milk and 0.2% Tween 20, and then incubated with mAb BU52 for 1 h at room temperature. Filters were again washed and then incubated with horseradish-peroxidase-conjugated anti-mouse antibody (Amersham Pharmacia Biotech, Buckinghamshire, England) for 1 h. Filters were then washed in TBST (150 mM NaCl, 10 mM Tris, pH 8, 0.05% Tween 20), and specific proteins were detected using the enhanced chemiluminescence system (Amersham Pharmacia Biotech).

2.4. Adhesion assay

Ninety-six well flat bottom plates (Corning Incorporated, Corning, New York) were coated with 4 mg/ml of hyaluronate or 10 mg/ml BSA in PBS overnight at 4 °C. Following procedures were performed as previously described [7].

2.5. In vitro cell proliferation

Cells were harvested from plates with 0.05% EDTA in PBS, suspended in SITA medium in single suspension and counted. Two thousands cells were added to the 96-well microtiter plate in triplicate, and allowed to grow at 37 °C with 5% CO_2 for 1–3 days. At the indicated time, the cell number was assessed with the Cell Counting Kit-8™ (Wako, Japan) according to the manufacturer's instruction.

2.6. Immunocytochemical staining for the single stranded DNA and TUNEL

To identify apoptotic cells, antibody specific for the single stranded DNA (DAKO Code number: A4506, Kyoto, Japan) was used to identify the cells exhibiting DNA fragmentation. In brief, 2×10^5 H322/CD44s cells were incubated with SITA medium on 9 cm dish coated with HA (4 mg/ml) or BSA (10 mg/ml) at 37 °C for 72 h. The cells were washed with PBS and adjusted to 5×10^3 cells/ml, and fixed on slides with the cytospin method. The slides were fixed with 4% paraformaldehyde, washed with PBS and incubated for 30 min in 10% normal goat serum. Then the anti-single stranded DNA polyclonal antibody was applied at a dilution of 1:400 and incubated overnight at 4 °C. Specific binding was detected with avidine–biotin peroxidase complex formation using the biotin conjugated goat anti-rabbit IgG (Vectastatin ABC kit, Vector, Burlingame, CA) and diaminobendizine (DAB) (Sigma, St. Louis, MI) as the substrate. Staining was absent when isotype-matched immunoglobulin was used as the control.

With other cytospun slides, Transferase-mediated dUTP nick end labeling (TUNEL) staining for apoptosis was also performed with an in situ Cell Death Detection kit (Roche, Indianapolis, IN) according to the manufacturer's instruction. Each experiment conducted with TUNEL reaction mixture without terminal transferase served as the negative control. The fluorescent images were obtained using an epifluorescence microscope.

2.7. In vitro chemosensitivity assay

Cells (1.0×10^4) cultured in 1% FCS/SITA medium were seeded to 96-well microtiter plates coated with HA (4 mg/ml) or BSA (10 mg/ml). Various concentration of chemotherapeutic agents including platinum agents (cis-platinum (II) diammine dichloride; CDDP, LKT Laboratories, MN), gemcitabine (GEM) provided by Eli Lilly (Indianapolis, IN) and vinorelbine (VNB) donated by Kyowa Hakko (Tokyo, Japan) in the absense or presense of 50 μM of (*E*)-3[[[3-[2-(7-chloro-2-qinolinyl)ethenyl]phenyl]-[[3-dimethylamino]-3-oxopropyl]thio]methyl] thio]-propanoic acid, MK571 (Calbiochem, San Diego, CA) were added to each well 1 h after seeding. After 72 h incubation, 10 μl of Cell Counting Kit-8 was added to each well. Four hours later, the optical density was measured at 450 nm with a microplate reader. Results are expressed as the percentage of cell viability (absorbance of exposed cells at 450 nm/absorbance of cells in SITA medium at 450 nm in the absence of platinum agents). Experiments were performed in triplicate in quadruplicate plates.

2.8. Immuno-fluorescence

Cells (1.0×10^6), seeded to 10 cm dish coated with HA or BSA, were cultured in 1% FCS/SITA medium. Incubation was allowed to proceed for 72 h at 37 °C in the absence or presence of 50 μM of MK571. The adherent cells were then detached from plates with 0.05% EDTA in PBS and pellet was fixed with 70% ethanol for 10 min at −20 °C. After permeabilizing in 1X FACS Permeabilizing Solution 2 (Becton and Dickinson, San Jose, CA)/dH_2O, cells were incubated for 5 min in PBS containing 0.5% BSA. Then, the cells (5×10^5) were incubated with mAb M2 I-4 (1 μg/ml) in 1% FCS/PBS at 4 °C for 30 min. Antibody binding was detected with fluorescent-labeled anti-mouse mAb (Sigma Chemical Co.). Fluorescence was analyzed with a FACScan™ (Becton–Dickson Co., Mountain View, CA).

2.9. Statistics

Statistical analysis was performed by analysis of variance. The Kruskal–Wallis method was used to compare the proliferation rate with H322/Mock, H322/CD44s, and H322/TR. All data are presented as means ± SD Differences between means were considered statistically significant at $p < 0.05$. Statview version 5.0 (Abacus corporation) was used for all analyses.

3. Results

3.1. Reintroduction of CD44s to a human non-small cell lung cancer cell line

To determine the role of interaction between HA and its receptor, CD44s, we reintroduced the CD44s cDNA back to the NSCLC cell line, H322, which originally lacks the CD44s isoform with electroporation. We also established the H322/TR and H322/Mock transfectants by reintroducing the CD44s gene encoding for a protein

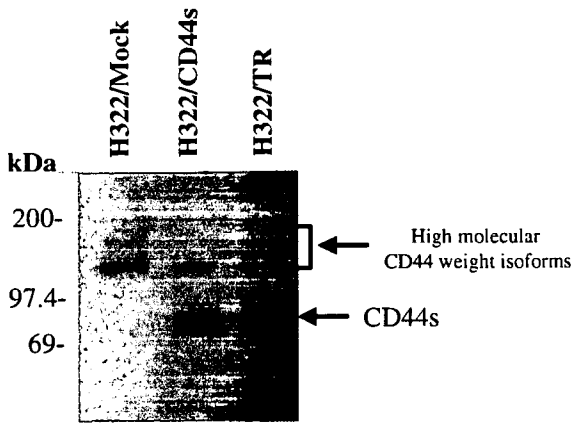

Fig. 1. Establishment of H322 transfected with an empty vector (H322/Mock), CD44s full-length gene (H322/CD44s), and CD44s gene whose cytoplasmic sequence was deleted (H322/TR). Expression of CD44s on H322 CD44s transfectants (H322/CD44s cells), control transfectants (H322/Mock) and CD44s genes whose cytoplasmic sequence is deleted (H322/TR) was assessed with Western blot. The upper arrow indicates endogenous high molecular weight CD44 isoforms. The lower arrow indicates transfected CD44s. Molecular weight markers are indicated on the left.

whose cytoplasmic region was deleted and control expression vector, respectively. G418-resistant clones expressing cell surface CD44s were identified with both Western blot (Fig. 1) and FACS analysis (data not shown). Several clones for CD44s transfectant designated H322/CD44s, H322/TR and H322/Mock were obtained.

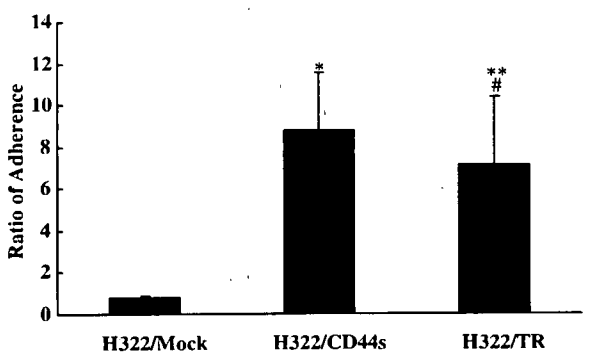

Fig. 2. Adhesion assay. In vitro cell adhesion assay of H322/Mock cells, H322/CD44s cells, or H322/TR cells to BSA or HA. Cells were allowed to adhere to BSA (10 mg/ml) or HA (4 mg/ml) at 4 °C for 1 h. The ratio of adherence (percent specific adhesion to HA/percent specific adhesion to BSA) of H322/CD44s and H322/TR was significantly greater than that of H322/Mock. $^{*}p = 0.0057$ vs. H322/Mock, $^{**}p = 0.5307$ vs. H322/CD44s, $^{\#}p = 0.045$ vs. H322/Mock. Data are presented as the means ± SD in triplicates.

3.2. Adhesion assay

To evaluate the effect of CD44s transfected to H322 cells on HA binding, in vitro cell adhesion assay was performed using H322/CD44s, H322/TR and H322/Mock cells. Cells were investigated for adhesion to either HA or BSA. The ratio of adherence (percent specific adhesion to HA/percent specific adhesion to BSA) of H322/CD44s and H322/TR was significantly greater than that of the H322/Mock (Fig. 2).

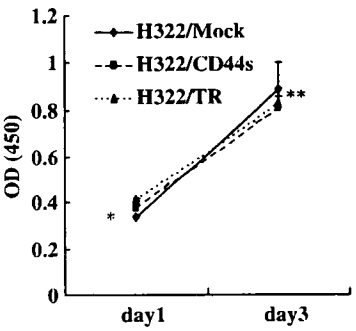

Fig. 3. In vitro cell proliferation assay. Two thousand H322/Mock, H322/CD44s and H322/TR cells were added to the 96-well microtiter plate in triplicate, and allowed to grow for 1–3 days. Cell number was assessed with the cell counting kit-8™. There was no difference in growth speed among the three clones. $^{*}p = 0.2521$, $^{**}p = 0.5611$ among three clones.

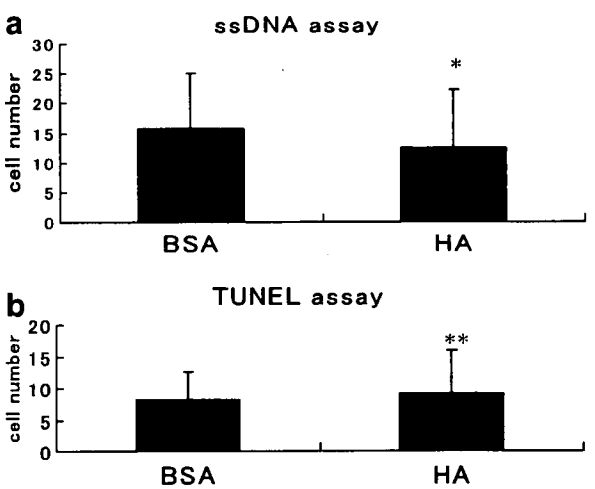

Fig. 4. Immunocytochemical staining for single stranded DNA and TUNEL. Immunocytochemistry for single stranded DNA (ssDNA) (a) and immunofluorescence analysis for Transferase-mediated dUTP Nick-End-Labeling (TUNEL) staining (b) of H322/CD44s cells cultured on HA or BSA coated dish. The number of ssDNA positive cells cultured on HA coated plate was similar to that of the BSA coated dish with both methods. The number of ssDNA-positive cells or TUNEL-positive cells in five fields of cytospin was counted at 400× and presented as means ± SD. $^{*}p = 0.6069$ vs BSA. $^{**}p = 0.7843$ vs BSA.

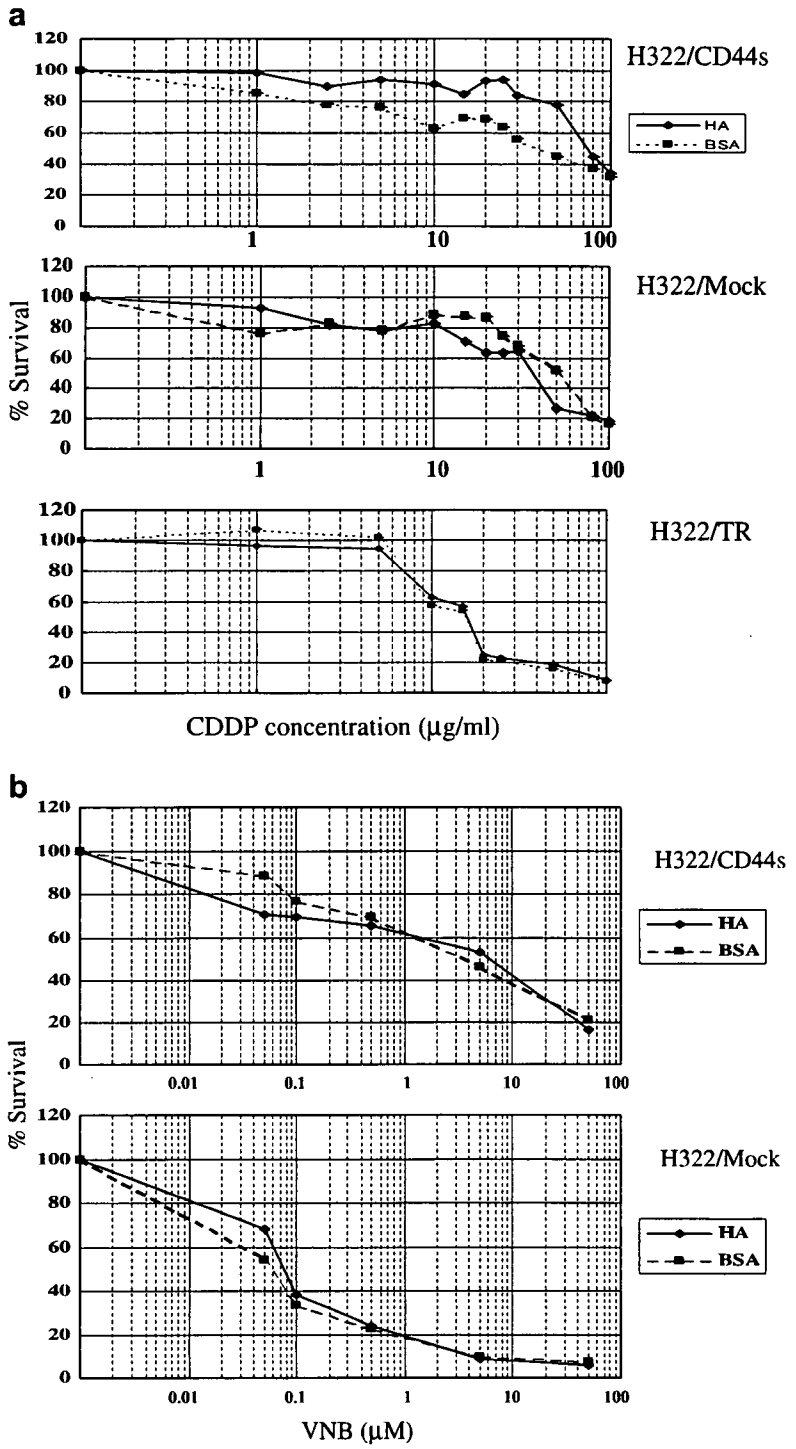

Fig. 5. In vitro chemosensitivity assay. Both H322/CD44s and H322/Mock cells were seeded to the 96-well flat bottom plates coated with HA (4 mg/ml) or BSA (10 mg/ml) and cultured for 72 h in the absence or the presence of various concentration of cisplatin (CDDP; a), vinorelbine (VNB; b), gemcitabine (GEM; c). The in vitro chemosensitivity of each cell line was evaluated by determining the number of surviving cells with the Cell-Counting kit-8. H322/TR cells were also examined in vitro chemosensitivity for CDDP. H322/CD44s cells cultured on HA were more resistant to CDDP than the cells cultured on BSA, while H322/Mock or H322/TR cells cultured on HA did not demonstrate resistance to CDDP (a). Interestingly, there were no resistance revealed for H322/CD44s cells cultured on HA to GEM or VNB compared with the cells cultured on BSA (b and c). As expected, H322/Mock cells cultured on HA did not reveal resistance against GEM or VNB (b and c).

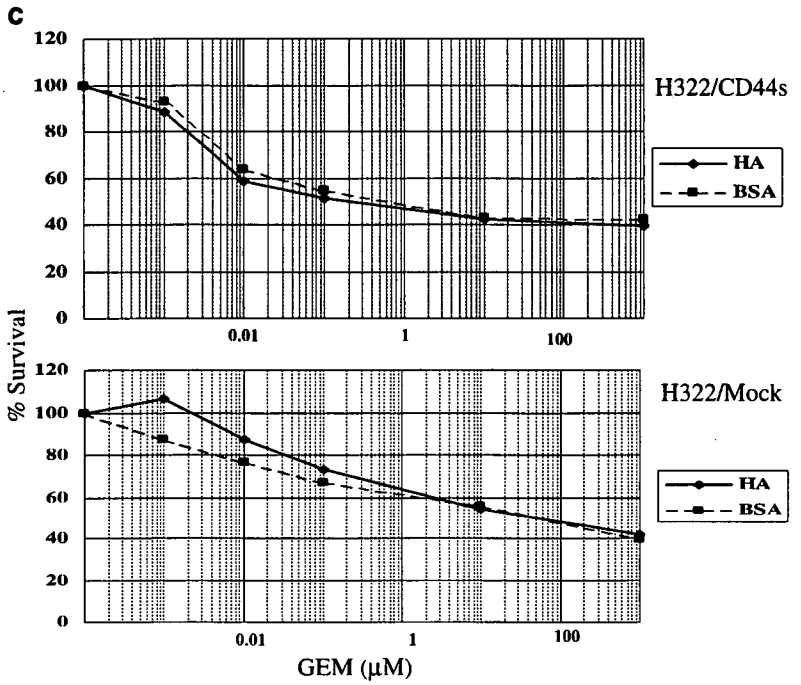

Fig 5. *(continued)*

3.3. The effect of reintroduction of CD44s on in vitro cell growth

To determine whether reintroduction of CD44s to NSCLC cells influences cell growth, the H322/Mock, H322/CD44s, and H322/TR cells were investigated in vitro. As expected reintroduction of CD44s or CD44TR to H322 NSCLC cell line did not influence in vitro cell growth rates (Fig. 3).

3.4. Examination for DNA fragmentation

To investigate whether the binding to HA or BSA influence apoptosis of H322/CD44s cells, we performed both single stranded DNA and TUNEL staining. The number of single stranded DNA positive cells cultured on BSA coated plate was not statistically different in comparison to that of the HA coated plate (Fig. 4a). In the same way, TUNEL positive cells observed in the wells pre-coated with BSA were similar to those of the wells pre-coated with HA (Fig. 4b). These results suggest that the binding of H322/CD44s cells to HA did not influence cell viability.

3.5. In vitro chemosensitivity assay

To evaluate the cytotoxicity of anti-cancer agents, H322 transfectants were exposed continuously to various concentration of CDDP, GEM, VNB for 72 h on either BSA or HA. H322/CD44s cells cultured on HA were more resistant to CDDP than the cells cultured on BSA, while neither H322/Mock cells nor H322/TR cells cultured on HA demonstrated resistance to CDDP (Fig. 5a). At the point of IC 50, H322/CD44s cells cultured on HA was twice more resistant to CDDP than the H322/Mock cells cultured on HA. Interestingly, H322/CD44s cells cultured on HA did not acquire chemoresistance to GEM or VNB compared to the cells cultured on BSA (Fig. 5b and c). H322/Mock cells cultured on HA did not demonstrate resistance to GEM or VNB (Fig. 5b and c). We also tested other clones of H322/CD44s, H322/Mock, and H322/TR. As expected, all clones revealed similar results (data not shown). These data indicate that the interaction between HA and CD44s receptor reduces chemosensitivity against CDDP in H322 cells.

3.6. Induction of MRP2 expression on H322/CD44s stable transfectants cultured on HA

To determine, the mechanism of acquiring chemoresistance, we investigated MRP2 expression on cell surface of H322 transfectants. Strong expression of MRP2 was induced on H322/CD44s cells by culturing on HA, but not on BSA (Fig. 6). In contrast, neither H322/Mock cells nor H322/TR cells cultured on HA induced upregulation of MRP2 expression compared to cells cultured on BSA. Additionally, we also examined whether MRP1 expression