

prognostic difference (54). However, this study was statistically underpowered to draw any conclusions.

Ideally, response to chemotherapy should be measured as a continuous variable. No system satisfies the need of accurate pathologic evaluation for the majority of patients who achieve partial or minor response to pre-operative chemotherapy. Rajan et al. proposed that the product of residual tumor size and cellularity might be a more clinically relevant indicator of tumor response than assessing tumor size alone (55). Though it is an interesting proposal, the method needs to be validated in correlation with long-term outcome.

OUTCOME AFTER PRE-OPERATIVE CHEMOTHERAPY AND SURGERY

Several studies have attempted to find more accurate predictors for survival after pre-operative chemotherapy than pCR in the primary tumor. This is because substantial risk of systemic recurrence still remains even if pCR is achieved, whereas substantial patients have excellent prognosis even if pCR is not achieved. If the long-term risk is high, they will be the candidates for clinical trials to determine whether additional aggressive therapy will be of benefit. If a good prognosis is expected even without good response to pre-operative therapy, aggressive chemotherapy might be over-treatment in pre-operative setting.

In the report of retrospective studies from Royal Marsden Hospital and M. D. Anderson Cancer Center, pathologically negative axillary lymph nodes after pre-operative chemotherapy, not pCR in the primary tumor, remained the independent prognostic factor for disease-free survival and overall survival in multivariate analysis adjusted for other prognostic factors (56–58).

It was revealed by a retrospective multivariate analysis of the clinicopathological factors of the 226 patients who had pCR after pre-operative chemotherapy that pre-operative clinical stage IIIB, IIIC, and inflammatory breast cancer, axillary lymph nodes more than 10, and pre-menopausal status were the independent prognostic factors of distant metastasis (59). In another study, only histological grading had an independent prognostic impact on disease-free and overall survival after adjustment for pCR to pre-operative chemotherapy containing doxorubicin (60). Carey et al. found that American Joint Committee on Cancer Tumor-Node-Metastasis staging after pre-operative chemotherapy was useful in prediction of distant disease-free survival and overall survival (61).

Rouzier et al. constructed nomograms combining clinical variables associated with pCR that might accurately predict pCR and distant disease-free survival (62). This was confirmed in an independent dataset within the study. The nomogram included size of residual tumor and the number of metastatic nodes at the time of surgery, histologic grade, estrogen receptor (ER) status and histologic type. On the other hand, biologic markers such as expression of HER2 (63), EGFR (64), p53 (65) or MDR1 gene (66) in tumor specimen before pre-operative chemotherapy, reduction of

expression in topoisomerase II- α (70) or MLH1 (71) after pre-operative chemotherapy are suggested to predict long-term outcome. Although it is not known whether these markers would add to or replace the nomogram, development of more accurate and comprehensive tools for prediction of prognosis is awaited.

PREDICTION OF RESPONSE TO PRE-OPERATIVE CHEMOTHERAPY

The pre-operative setting is ideal to explore molecular predictors of response to therapy. Various clinical and pathologic variables have been studied. Among them, ER status, histologic grade and smaller tumor size seem to be associated with the response to pre-operative chemotherapy (47, 69).

In previous retrospective studies, clinical and pathological responses to pre-operative chemotherapy appear to be lower in invasive lobular carcinoma (ILC) as compared to invasive ductal carcinoma (IDC), and patients with ILC were more likely to receive mastectomy after initial attempt for breast conservation (70–73). However, low pCR rates in ILC have not been translated into survival disadvantage (70–72). These data suggest that different approach should be taken in the clinical management of patients with ILC.

In a biomarker study, ER expression, absence of HER2 and a decrease in Ki67 correlated with good clinical responses subsequent to a pre-operative chemoendocrine therapy (74). Among other biomarkers, bcl-2 and p53 have been studied. bcl-2 has been shown to protect cells from apoptosis induced by chemotherapeutic drugs (75). Although high expression of bcl-2 has been hypothesized to play a role in resistance to chemotherapy, it is still controversial. In one study, higher bcl-2 expression at diagnosis was predictive of pCR in univariate analysis but it did not retain its impact in multivariate analysis (76), while other studies did not find any correlation between bcl-2 expression and the response (77, 78).

p53 is also a potential predictive marker. Active p53 promotes apoptosis in growth-arrested cells whereas loss of p53 function has been reported to enhance cellular resistance to various chemotherapeutics (79). In a clinical setting, in patients treated with single agent epirubicin, mutant p53 was a significant predictor for poor clinical response, but the association was weaker in patients treated with cyclophosphamide/methotrexate/5FU with or without tamoxifen (65). Another study demonstrated that a tumor expressing wild-type p53 was related to resistance to single agent doxorubicin therapy in multivariate analysis (80). *TP53* gene mutation and over expression of p53 were related to epirubicin-containing chemotherapy, but response to paclitaxel seemed to be related to p53-negative tumors (81).

Tumor response and toxicities are different among individual patients. Pharmacogenomic studies aim to elucidate the genetic bases for inter-individual differences and to enable individualization of care. DNA microarray is one of the modern high-throughput biotechnologies that allow

researchers to analyze expression of multiple genes in concert and relate the findings to clinical parameters. In breast cancer, several groups have reported preliminary results suggesting that the gene expression profile of the primary tumor may predict the tumor's response to pre-operative chemotherapy (82–86). One major limitation of microarray studies is overfitting of the predictor: the number of mRNA transcripts far exceed the number of samples (87, 88). The accuracy of the predictive model is low in independent data set (89). More rigorous and critical evidence is necessary before multi-gene predictors can be accepted as a useful and reliable tool in clinical practice.

PRE-OPERATIVE ENDOCRINE THERAPY

The relative benefit of chemotherapy is less in endocrine-responsive disease as compared with endocrine non-responsive disease (1) and recent consensus of the clinical community lays emphasis on the endocrine responsiveness in decision-making of adjuvant systemic therapy (14). Pre-operative endocrine therapy is an attractive alternative for endocrine-responsive disease, because it is easy to perform and can also avoid acute and late side effects caused by cytotoxic chemotherapy, but pre-operative endocrine therapy has not been accepted as the standard therapy because of the slow rate of response (90). We need more accurate measures to select the patients who are most likely to respond to endocrine therapy without compromising the potential benefit of chemotherapy.

APPLICATION TO MOLECULAR-TARGETED THERAPY

Molecular-targeted drugs are anticipated to individualize the therapeutic strategy based on the biology of the tumor. To date, the presence of a target still does not satisfactorily guarantee a response to therapy, but efforts are being made to elucidate the key components of the molecular pathways targeted by a specific agent.

Moshin et al. reported a pre-operative study of trastuzumab as a single agent in HER2-positive locally advanced breast cancer (91). They administered trastuzumab as a single agent for the first 3 weeks, followed by a combination of trastuzumab and docetaxel. Of note, partial response was observed in eight among 35 patients after only 3 weeks of trastuzumab. The accompanying biomarker study suggested that the main mechanism of action of trastuzumab is inhibition of the PI3K/Akt pathway, which results in an increase of apoptosis (79). The clinical role of single-agent trastuzumab in HER2-positive tumors has not been determined, but it is attractive if we can select the responders to trastuzumab as this is usually less toxic than cytotoxic chemotherapy.

A report by Polychronis et al. is unique in respect of testing the efficacy of combination of targeted therapy based on biology-derived hypothesis (92). It was a double-blind placebo controlled phase II randomize trial of pre-operative gefitinib versus gefitinib versus anastrozole in

post-menopausal patients with ER- and EGFR-positive primary breast cancer. The tumors of patients assigned to combination therapy had a greater reduction of Ki67 labeling index than those assigned to gefitinib alone. Although the number of patients in this study was so small that we do not yet know whether reduction in proliferation will be translated into clinical benefit, we foresee a future of individualized therapy.

FUTURE DIRECTIONS

Pre-operative chemotherapy has become the standard of care in management of primary breast cancer. However, we should be aware that a substantial portion of patients may be over-treated by pre-operative chemotherapy because of inaccurate pre-treatment staging. In NSABP-B27 study, addition of docetaxel was beneficial in terms of disease-free survival not in complete responders or non-responders but only in partial responders in a subset analysis according to clinical response after AC. Who needs additional systemic therapy? Who can avoid systemic therapy?

Development of endocrine therapy and trastuzumab has opened the door to important therapeutic advance of 'molecular-targeted therapy'. Transcriptional profiling has revealed that expression levels of these targets, i.e. ER and HER2, are the major genetic determinants of the biology of the disease (93). Thus, we can foresee the future of systemic therapy individualized with endocrine responsiveness and involvement of HER2 signaling pathway. However, to date, the predictive value of screening test for molecular targets remains unsatisfactory.

Identification of clinically useful, prognostic and predictive molecular markers is highly anticipated to optimize therapeutic regimens. The current probability-based therapeutic strategy, 'empiric treatment' so to speak, might give way to biology-based, individualized strategy, 'marker-based treatment', when additional biologic markers are identified that make 'targeted therapy' more targeted and effective. Pharmacogenomic researches that accompany pre-operative therapy might help better understand the biology of breast cancer and thus promote the development of new therapeutic strategies.

Conflict of interest statement

None declared.

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

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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/m², iv on day 1; cyclophosphamide, 500 mg/m², 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/m². 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

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 anti-cancer drugs in breast cancer chemotherapy.

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

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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/m², iv on day 1; cyclophosphamide,

500 mg/m², 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/m² (range, 15–360 mg/m²). 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.

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 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

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— $P = 0.014$)

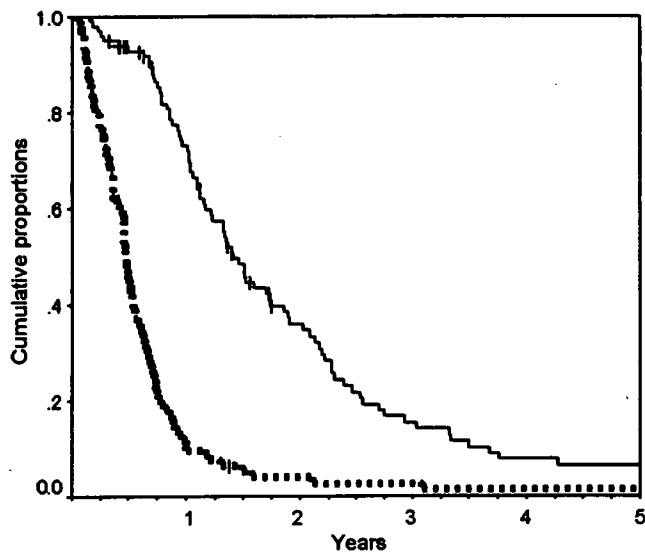


Fig. 1 Kaplan–Meier analysis of time to progression (*dotted line*) and overall survival (*solid line*). Vertical bars indicate censored cases

A total of 482 courses in the 99 patients were assessable for toxicity. The median cumulative dose of doxorubicin for RBC was 240 mg/m² (range, 40–240 mg/m²), and the median total cumulative dose of anthracycline (after conversion to doxorubicin) was 343 mg/m² (range, 102–600 mg/m²). 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 anthracycline-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/m². 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/m² [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 metastatic 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.

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

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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.

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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.

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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 (≥ 100 cigarettes per lifetime, but quit 1 year before diagnosis), and current smokers (≥ 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- μ m 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 μ l 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 μ l 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'-CCTGTCTCTGTGTTCTTGT-3' and 5'-CTGCGGCCAGCCCAGAGGC-3'), exon 19 (5'-CATGTGGCAC CATCTACA-3' and 5'-CCACACAGCAAAGCAGAA AC-3'), and exon 21 (5'-CAGGGTCTTCTCTGTTTCAG-3' and 5'-TAAAGC CACTCCTTACTTT-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 μ l volumes using 1 μ l of template DNA, 7.5 μ l of reaction buffer mix, 0.6 μ l of Primer mix and 0.1 μ l 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_t) were used to interpret the results. Positive results were defined as follows: $C_t \leq 45$ and $F_t \geq 50$. 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

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%) |

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.

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

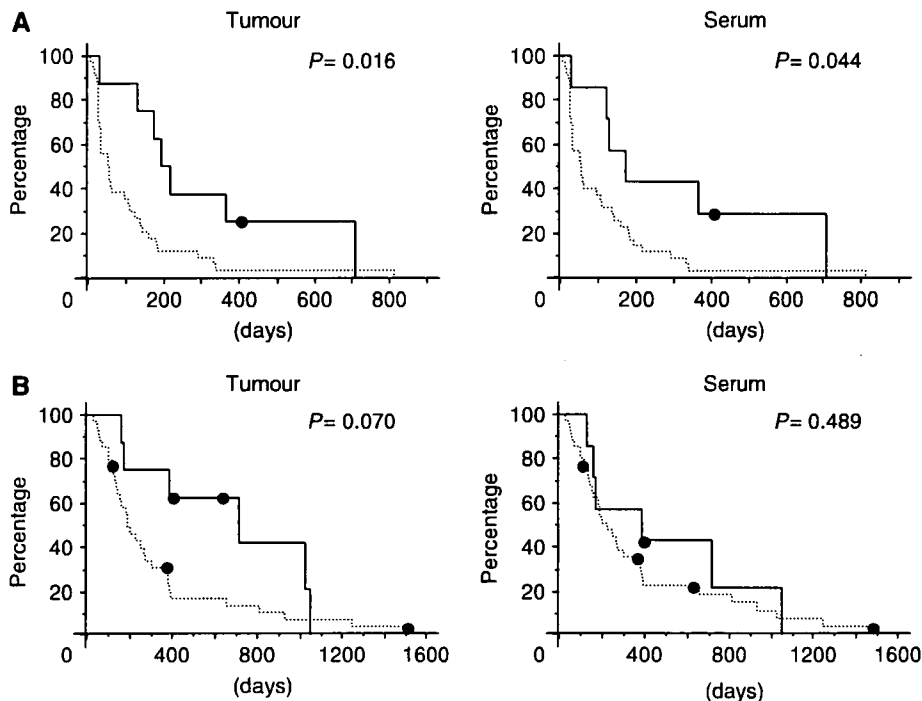


Figure 1 Kaplan–Meier probability of progression-free survival (A) and overall survival (B) with respect to the EGFR mutation status of NSCLC. P-values were calculated by the log-rank test.

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.

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

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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.

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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

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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 tumorigenicity 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% CO₂ 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 Gaithersburg, 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% NaN₃, 1 mM phenylmethylsulfonyl fluoride, 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).