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Phase II study of weekly irinotecan and cisplatin for refractory or recurrent non-small cell lung cancer

Yuichi Takiguchi[a,*], Tetsuro Moriya[a,b], Yoshiko Asaka-Amano[a], Tatsuo Kawashima[c], Katsushi Kurosu[a], Yuji Tada[a], Keiichi Nagao[a], Takayuki Kuriyama[a]

[a] *Department of Respirology (B2), Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan*
[b] *Department of Respiratory Medicine, Matsudo City Hospital, 4005 Kamihongo, Matsudo 271-8511, Japan*
[c] *Department of Internal Medicine, Toho University Sakura Medical Center, 564-1 Shimoshizu, Sakura 285-8741, Japan*

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Summary Even with the standard first-line chemotherapy, advanced non-small cell lung cancer (NSCLC) recurs in most cases. The purpose of this study is to develop a new chemotherapeutic regimen for patients with NSCLC that has relapsed or was refractory to previous chemotherapy. Patients with proven NSCLC refractory or recurrent after previous single-regimen chemotherapy, PS of 0–2, age of 15 years or older, adequate organ functions and measurable lesions were treated with irinotecan at 60 mg/m^2 and cisplatin at 25 mg/m^2 with 1000 ml hydration on day 1. This administration, considered as one cycle, was repeated every week without rest unless encountering defined skip and dose-reduction criteria. The treatment was administered for six cycles over a 49-day period, both median values, to 48 patients, with a response rate of 26%, progression free and median survival times of 3 and 11 months, respectively, and a 1-year survival rate of 46%. The most frequent grade 3 or 4 toxicities were neutropenia, anaemia and nausea, which were manageable. Subset analyses suggested that the response rate was independent of response to the first-line chemotherapy. In conclusion, second-line chemotherapy of weekly irinotecan and cisplatin with minimum hydration seemed effective, with tolerable toxicity, and is potentially useful irrespective of the outcome of previous chemotherapy.

1. Introduction

Current standard first-line chemotherapies for advanced NSCLC are four to six cycles of two-drug combinations consisting of a platinum and one of the relatively newly developed agents [1]. Despite the role of first-line chemotherapy, a substantial proportion of thusly treated patients will in due time become troubled by progression of

* Corresponding author. Tel.: +81 43 226 2577; fax: +81 43 226 2176.
E-mail address: takiguchi@faculty.chiba-u.jp (Y. Takiguchi).

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the disease, and seek second-line treatment. Challenges to the second-line treatment for NSCLC include chemotherapy with a single agent of either cytotoxic or molecular-target drug, and combination chemotherapy [2–5]. Despite a body of reported phase II studies, only monotherapies with docetaxel [6] and erlotinib [2] have been evaluated in phase III studies, and have been proven to prolong the survival of patients with front-line chemotherapy-refractory and/or relapsed NSCLC over best supportive care (BSC). In addition, a randomized study comparing a new cytotoxic agent, pemetrexed, with docetaxel for this situation was conducted, revealing comparable survival data with preferable adverse effects with pemetrexed, adding this agent to the list of candidates for standard second-line chemotherapy for NSCLC [7].

Clinical relevance of irinotecan for advanced NSCLC has been suggested by phase II studies [8–14]. Its conventional administration schedule for NSCLC, in Japan, is at a dose of 60 mg/m^2 on days 1, 8, and 15, combined with 80 mg/m^2 of cisplatin on day 1, every 4 weeks.

On the other hand, cisplatin in combination chemotherapy for NSCLC evidently contributes to improving the survival of patients despite its relatively low response rate as a single agent [15]. This unique characteristic may give us hope that it might still have power in a second-line therapy if it is combined with another active agent, even for patients who had been previously treated with cisplatin. It is usually administered to patients with NSCLC at a dose of 60–80 mg/m^2 every 3–4 weeks; however, its weekly-fractionated administration was shown to be active [16–18]. Weekly administration of cisplatin may require only a minimal hydration volume that would be convenient and advantageous for the patient's quality of life.

Based on these facts, a phase II study of weekly irinotecan and cisplatin as a second-line chemotherapy for previously treated patients with NSCLC was conducted.

2. Patients and methods

2.1. Eligibility

Patients meeting all the following criteria were enrolled into this multicentre trial: proven NSCLC refractory or recurrent after previous single-regimen chemotherapy, interval of more than 4 weeks from the last chemotherapy/radiotherapy, PS (ECOG) of 0–2, age of 15 years or older, adequate organ functions (leucocytes $\geq$3000 mm^{-3}, neutrophils $\geq$2000 mm^{-3}, haemoglobin $\geq$9.0 g/dl, platelets $\geq$100,000 mm^{-3}, total bilirubin $\leq$1.5 g/dl, AST/ALT < twice of normal value, creatinine $\leq$1.5 mg/dl, creatinine clearance $\geq$40 ml/min, and PaO_2 $\geq$70 Torr), measurable lesions by Response Evaluation Criteria in Solid Tumours (RECIST), life expectancy exceeding 8 weeks, and written informed consent. Patients with any of the following conditions were ineligible: previous treatment with irinotecan and/or surgery, requirement of thoracic irradiation, interstitial lung disease, pleural effusion or ascites requiring treatment, pericardial effusion, symptomatic brain metastasis, concomitant malignancy or other inadequate condition.

2.2. Pretreatment evaluation

The baseline evaluation included history with complete record regarding the front-line treatment and concomitant medical conditions, physical examinations, PS, complete blood counts, serum chemistries and electrolytes, urinalysis, chest radiogram, chest CT, abdominal CT, brain MRI with contrast medium enhancement otherwise contraindicated, and bone scintigram. These examinations were to be performed within 1 month prior to enrollment.

2.3. Drug administration

Irinotecan (60 mg/m^2 in 500-ml electrolyte solution, on day 1) and cisplatin (25 mg/m^2 in 500-ml saline, on day 1, without further hydration) with prophylactic anti-emesis agents consisting of either oral or intravenous 5-HT_3 agonist and dexamethasone (8–16 mg) constitute one cycle of the regimen and were administered every week for at least six cycles unless encountering defined skip and dose-reduction criteria. Post-treatment was withheld until evident disease progression, followed by no restriction afterward.

2.4. Evaluation during chemotherapy

Symptoms, physical examination, complete blood counts, and serum chemistries were checked twice in the first week of chemotherapy, followed by weekly monitoring on the same day of and prior to the next chemotherapeutic administration. Chest radiogram was performed every week primarily for evaluation of pulmonary toxicity. Chest CT and other radiographic modalities necessary for evaluating target lesions by RECIST were repeated every month until evidence of disease progression.

2.5. Skip, dose modification and termination criteria of chemotherapy

Drug administration was skipped when encountering any of the following criteria on the same day and before the administration: grade 1 or more of fever (axillary) or diarrhoea, grade 2 or more of leukocytopenia, elevated creatinine, total bilirubin, peripheral neuropathy, myalgia/arthralgia, pulmonary fibrosis or cardiac events, grade 3 or more of nausea/vomiting/appetite loss, or thrombocytopenia of less than 1.0×10^5 mm^{-3}. If the condition recovered, the treatment was resumed the following week after skipping. The dose of irinotecan was reduced to 45 mg/m^2 when encountering any of the following criteria during the previous cycle: grade 3 or more of thrombocytopenia, diarrhoea, or fever, or grade 4 of leukocytopenia or neutropenia. Cisplatin dose was also reduced to 20 mg/m^2 when encountering grade 2 or more of elevated creatinine, peripheral neuropathy, or myalgia/arthralgia during the previous cycle. The treatment was totally terminated when encountering any of the following conditions: disease progression, successive three-time administration skipping, meeting with any of the dose reduction criteria after one dose reduction experience, other serious adverse events, or patient's refusal.

2.6. Response and toxicity criteria

Tumour response to therapy was evaluated according to RECIST, and was classified into four categories: complete response (CR), partial response (PR), stable disease (SD) and progressive disease (PD). All target lesions were re-evaluated with the same imaging studies as used in the pretreatment evaluation. A minimum of 6-week-interval from the start of therapy was required for establishing SD. All of the evaluation was confirmed or corrected by external reviews of the CT scans. Toxicity during the entire course of the therapy was evaluated, and the worst event was scored according to the National Cancer Institute Common Toxicity Criteria (NCI-CTC) version 2.0.

2.7. Statistical and ethical considerations

The primary endpoint of the study was to assess the overall response rate (ORR), and the secondary ones were to evaluate toxicity and survival data consisting of median survival time (MST), median progression free survival (PFS) and 1-year survival rate. Calculated minimum sample size was 43 based on Simon's two-stage optimal design with target and threshold response rate of 25 and 10%, respectively, with α and β errors of 0.05 and 0.20, respectively, and a final sample size of 48 was determined. The first stage required at least 2 or more patients of 18 to have confirmed CR or PR before proceeding to the second stage. In the second stage, if a total of 7 or more among 43 assessable patients achieved CR or PR, then the primary endpoint would have been met. Survival time was measured from enrollment into the study, and was analysed by Kaplan–Meier method. After completing the study, some unplanned sub-set analyses on response rate and survival were performed. The study was approved by our institutional review board. It was registered to the clinical trial registration system of UMIN-CTR with the identification number of C000000084 on 29 August 2005.

3. Results

3.1. Patient characteristics

From February 2002 to November 2005, 48 patients were enrolled, and their characteristics are listed in Table 1. The majority of the patients (81%) were male, and 60% had adenocarcinoma. Prior chemotherapies were platinum-based in 41 patients (86%) and non-platinum in 7 patients. Thirteen patients (27%) were treated with thoracic irradiation of curative or palliative intent. Twenty-four patients (50%) achieved tumour response including one CR and 23 PR, and the other 24 patients showed no response to the front-line treatment. As one patient died due to tumour progression after enrollment and before starting chemotherapy, 47 patients were eligible for evaluation of response rate and toxicity, whereas all 48 were analysed for survival.

3.2. Treatment delivery and dose reduction

Chemotherapy was administered for a median of six cycles (ranging from 0 to 15), and median duration of chemotherapy was 49 days (ranging from 0 to 168 days). Median dose intensity was 49 (ranging from 24 to 65) mg/(m^2 week) for irinotecan, and 20 (ranging from 10 to 25) mg/(m^2 week) for cisplatin.

Dose reduction of irinotecan was done in three patients because of grade 3 diarrhoea in 1 patient at the third cycle (three cycles were administered and discontinued because of recurrence of grade 3 diarrhoea in this patient), grade 3 diarrhoea plus grade 4 neutropenia in one patient at the third cycle (four cycles were administered and discontinued because of recurrent grade 3 diarrhoea in this patient), and grade 4 thrombocytopenia in one patient at the fourth cycle (six cycles were administered and discontinued because of recurrent grade 3 thrombocytopenia in this patient). Cisplatin was not reduced in dose in any of the cases. No patient required delivery omission due to unresolved toxicity.

Table 1 Patients characteristics

Characteristics	No. of patient (n=48)	Year	%
Sex			
Male	39		81
Female	9		19
Age			
Median		62	
Range		36–73	
PS			
0	29		60
1	18		38
2	1		2
Histology			
Adeno	29		60
Squamous	14		29
Large	3		6
NSCLC[a]	2		4
Prior chemotherapy			
Cisplatin-containing	20		42
Carboplatin-containing	21		44
Non-platinum	7		15
Prior radiotherapy			
Thoracic, curative intent	12		25
Thoracic, palliative	1		2
Other than thoracic	7		15

[a] NSCLC not further specified.

3.3. Antitumour activity

ORR was 26% (12/47) with a 95% confidence interval (CI) of 13–38%, with no CR, 12 PR, 30 SD and 5 PD. Unplanned subset analyses demonstrated that response rates of the second-line treatment were comparable between responders and non-responders to the front-line therapy (29% or 7/24 versus 22% or 5/23, p=0.74 by χ^2 test), whereas PD by the second-line therapy was seen significantly more often in the non-responders (22% or 5/23) than the responders (0/24), with a p value of 0.02. Similarly, patients with a longer interval (3 or more than 3 months) from the last

administration of the first-line chemotherapy to the study enrollment and ones with a shorter interval (less than 3 months) had comparable ORRs (28% or 9/32 versus 27% or 4/15, $p=0.92$ by χ^2 test). Patients treated with non-platinum-based front-line therapy showed a trend of better response rate (57% or 4/7) than patients with platinum-based front-line therapy (20% or 8/40) without statistical significance ($p=0.06$ by χ^2 test).

3.4. Toxicity

Toxicities affecting patients with grade 3 or 4 are listed in Table 2, together with the grading of elevated serum creatinine. The most frequent grade 3 or 4 toxicities were neutropenia, anaemia, and nausea. Grade 3 or 4 diarrhoea was seen in 11% of the 47 assessable patients. Elevated serum creatinine was observed in 15 patients, 11 of them with grade 1 and 4 with grade 2. Out of 42 patients with a pre-treatment serum creatinine level of grade 0, elevated creatinine was seen in 10 of them, 8 (19%) with grade 1 and 2 (4%) with grade 2. In contrast, 2 out of 5 patients with a pre-treatment creatinine level of grade 1 upgraded to 2 (Table 2). There was no treatment-related death.

3.5. Third-line therapy and survival

Thirty-six out of the 47 patients underwent third-line therapy. Fifteen of them were again treated with a platinum-containing regimen, and 20 were treated with a non-platinum regimen alone; gefitinib was administered at least once for 20 patients, and one patient was treated by thoracic radiotherapy alone. At a median follow-up interval of 11 months, five cases were still alive and censored (survival times of these five patients exceeded MST); median PFS and MST were 3 (95% CI: 3–4) and 11 (95% CI: 8–13) months, respectively, and 1- and 2-year survival rates were 46% and 15%, respectively. Fig. 1 shows the Kaplan–Meier curves of overall survival (OS) and PFS. Unplanned analyses demonstrated that responders and non-responders to the front-line chemotherapy had comparable MST (11 months in both groups, $p=0.50$ by Log-rank test) and comparable median PFS (4 months versus 2 months, respectively, $p=0.12$) by the present second-line chemotherapy. Similarly, patients with a longer interval (3 or more than 3 months) from the last administration of the first-line chemotherapy to the study enrollment and ones with a shorter interval (less than 3 months) had comparable MST (11 months in both groups, $p=0.70$) and comparable median PFS (4 months versus 2 months, respectively, $p=0.09$) by the present second-line chemotherapy.

Table 2 Toxicity and its frequency ($n=47$)

Toxicity	Grade[a]				
	1	2	3	4	% (3 and 4)
Haematological					
Leucocytopenia	4	14	5	3	17
Neutropenia	7	11	9	5	30
Neutropenic fever	—	—	6	0	13
Hypohaemoglobinemia	1	13	11	3	30
Thrombocytopenia	0	0	2	1	6
Non-haematological[b]					
Nausea	13	7	10	0	21
Vomitting	4	4	4	0	9
Fatigue	6	8	0	1	2
Diarrhoea	13	8	5	0	11
Colitis	0	0	2	0	4
Infection w/o colitis	0	0	1	0	2
Increased serum creatinine					
Total	11	4	0	0	0
Pretreatment Cr level					
Grade 0 ($n=42$)	8	2	0	0	0
Grade 1 ($n=5$)	3	2	0	0	0

[a] The worst grade during entire chemotherapeutic courses in each patient.
[b] Only non-haematological toxicities including grade 3 and/or 4 are listed.

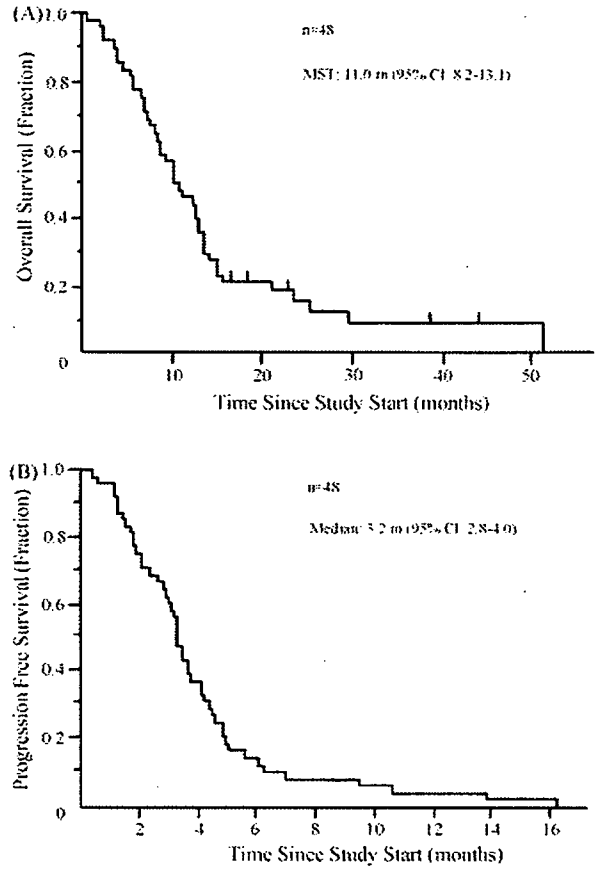

Fig. 1 Kaplan–Meier survival curves of the 48 patients treated with second-line chemotherapy consisting of weekly irinotecan and cisplatin for OS (A) and PFS (B) times starting from study enrollment.

4. Discussion

Most patients with advanced NSCLC treated with chemotherapy suffer from recurrence, and survival benefit of the

Table 3 Summary of reported phase II studies with irinotecan as a second-line therapy for NSCLC

First author	Year	Pretreatment		Irinotecan		Combined agent, its dose and schedule	Interval (week)	Pts No.	ORR		PFS/TTP (month)	MST (month)	One-year survival (%)
		% Platinum	Non-platinum	Dose (mg/m^2)	Schedule				(%)	95%CI			
Nakanishi [19]	1999	100%	–	60	Days 1, 8 and 15	CDDP (30 mg/m^2, days 1, 8 and 15)	4	16[a]	31	6–57	ND	ND	ND
Kakalyris [20]	2001	23%	DTX based	100, 110	Days 1 and 8	CDDP (80 mg/m^2, day 8)	3	44	22	9–35	8	8	30
Georgoulias et al. [21]	2005	0%	Taxane+Gem	100, 110	Days 1 and 8	CDDP (80 mg/m^2, day 8)	3	74	23	13–32	ND	8	34[b]
Negoro [22]	1991	ND	ND	100	Day 1	No	1	26	0	-	ND	ND	ND
Nakai [23]	1991	ND	ND	200	Day 1	No	3–4	22	14	0–29	ND	ND	ND
Pectasides [24]	2002	100%	–	150	Day 1	VNR (25 mg/m^2, days 1 and 8)	4	41	15	6–29	5	8	37
Gonzalez Cao [25]	2002	100%	–	300	Day 1	VNR (30 mg/m^2, days 1 and 14)	4	33	9	2–24	10	25	23
Font [26]	2003	96%	Misc	70	Days 1, 8 and 15	DTX (25 mg/m^2, days 1, 8 and 15)	4	51	6	0–13	3	8	30
Pectasides [27]	2003	100%	–	150	Days 1 and 15	Gem (1800 mg/m^2, days 1 and 8)	4	50	16	7–29	6	8	36
Han [28]	2003	ND	Misc	100 → 90	Days 1 and 8	Cape (2000 mg/m^2, bid, days 1–14)	3	37	11	0–21	ND	7	12
Georgoulias [29]	2004	100%	–	300	Day 8	Gem (1000 mg/m^2, days 1 and 8)	3	76	18	10–27	8	9	25[c]
				300	Day 1	No	3	71	4	0–9	5	7	29
Wachters [30]	2005	75%	Misc	200	Day 1	DTX (60 mg/m^2, day 1)	3	52	10	2–18	4	7	30[d]
Pectasides [31]	2005	100%	–	60	Days 1 and 8	DTX (30 mg/m^2, days 1 and 8)	3	65	20	10–29	6	7	37[e]

Abbreviations—ND: not described; misc: miscellaneous; CDDP: cisplatin; VNR: vinorelbine; DTX: docetaxel; Gem: gemcitabine; Cape: capecitabine; ORR: objective response rate; CI: confidence interval; PFS: progression free survival; TTP: time to progression; MST: median survival time.

[a] Date extracted from a patient population including small-cell lung cancer.

[b] No survival benefit over CDDP alone (randomized phase II).

[c] No survival benefit over irinotecan alone (randomized phase II).

[d] No response and survival benefit over DTX alone (randomized phase II).

[e] No survival benefit over DTX alone (randomized phase II).

standard second-line chemotherapy is limited. Therefore, more active regimens for second-line chemotherapy are eagerly awaited.

The present study was designed to address the clinical relevance of a new regimen as a second-line chemotherapy for patients with NSCLC. The combination of weekly irinotecan and cisplatin yielded a high response rate of 26% (12/47) with a 95% confidence interval of 13–38%. It should be emphasized that the response rates of the present second-line treatment were comparable between responders (29% or 7/24) and non-responders (22% or 5/23) to the initial chemotherapy, whereas PD was seen exclusively in non-responders to the initial treatment. Survival data was also promising, with median PFS of 3 months, MST of 11 months, and 1-year survival rate of 46%, when calculated from enrollment to the second-line study. Again, there were no statistically significant differences in MST and PFS between responders and non-responders to the first-line chemotherapy. In addition, ORR, MST and PFS by the present second-line chemotherapy were comparable between patients with a longer (≥3 months) interval from the last administration of the front-line chemotherapy to the study enrollment and patients with a shorter (<3months) interval, suggesting that the present second-line treatment might be effective independent of the outcome of the first-line chemotherapy.

Although toxicities were not insignificant, consisting of neutropenia, neutropenic fever, anaemia, nausea and diarrhoea, they were manageable without hospitalization in most cases, and median cycles administered were 6 in a median treatment period of 49 days with relatively high dose-intensity. This treatment delivery may be equivalent to a dose intensity of two cycles of standard combination consisting of irinotecan (60 mg/m^2, days 1, 8 and 15) and cisplatin (80 mg/m^2, day 1). In addition, it is noteworthy that there was not even a single case of grade 3 or 4 serum creatinine. That is to say, weekly cisplatin administration at a dose of 25 mg/m^2 required only 1000 ml hydration each week, ensuring a convenience for outpatient treatment.

Thirteen phase II studies [19–31] regarding irinotecan-containing second-line chemotherapy for patients with NCSLC were found, and they are summarized in Table 3. Among them, three studies employed regimens consisting of irinotecan and cisplatin (the top three lines of Table 3), and reported promising response rates and survival data similarly to ours. In addition, they disclosed better response rates than other studies employing non-platinum regimens in Table 3. It should be pointed out that patients in the studies by Nakanishi et al. [19] and Kakolyris et al. [20] included patients who had been pretreated with cisplatin. Although a direct comparison of different phase II studies cannot be justified in terms of drawing any definitive conclusions, this observation may suggest a relevance of including platinum in second-line treatment even when the patients had been pretreated with platinum. Definitive conclusions need to be reached on the basis of carefully designed phase III studies. The most important finding in the series of phase II studies listed in Table 3 is that chemotherapeutic regimens including irinotecan yielded a reproducibly high response rate and promising survival data in the clinical setting of second-line chemotherapy for NSCLC. The present regimen consisting of convenient weekly irinotecan plus cisplatin showed a good

response rate, MST and 1-year survival rate, and they were equally observed regardless of tumour response to pretreatment.

In conclusion, the present phase II trial with weekly irinotecan and cisplatin as a second-line chemotherapy for patients with pretreated NSCLC provided encouraging response rate, survival data, and tolerable safety profile, in concordance with the reproducible excellent tumour response and survival data in the previous studies. Unplanned subset analyses demonstrated possible non-cross resistance to the front-line chemotherapy. Given this promising information regarding the regimen, further studies in previously treated patients with NSCLC, especially for patients refractory to previous therapy, are warranted.

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References

[1] Socinski MA, Morris DE, Masters GA, Lilenbaum R. Chemotherapeutic management of stage IV non-small cell lung cancer. Chest 2003;123:226S–43S.

[2] Shepherd FA, Rodrigues Pereira J, Ciuleanu T, Tan EH, Hirsh V, Thongprasert S, et al. Erlotinib in previously treated non-small-cell lung cancer. New Engl J Med 2005;353:123–32.

[3] Herbst RS, Johnson DH, Mininberg E, Carbone DP, Henderson T, Kim ES, et al. Phase I/II trial evaluating the anti-vascular endothelial growth factor monoclonal antibody bevacizumab in combination with the HER-1/epidermal growth factor receptor tyrosine kinase inhibitor erlotinib for patients with recurrent non-small-cell lung cancer. J Clin Oncol 2005;23:2544–55.

[4] Georgoulias VA. Second-line chemotherapy in relapsing or refractory patients with non-small cell lung cancer. Lung Cancer (Amsterdam, Netherlands) 2002;38(Suppl 3):S61–6.

[5] Huisman C, Smit EF, Giaccone G, Postmus PE. Second-line chemotherapy in relapsing or refractory non-small-cell lung cancer: a review. J Clin Oncol 2000;18:3722–30.

[6] Shepherd FA, Dancey J, Ramlau R, Mattson K, Gralla R, O'Rourke M, et al. Prospective randomized trial of docetaxel versus best supportive care in patients with non-small-cell lung cancer previously treated with platinum-based chemotherapy. J Clin Oncol 2000;18:2095–103.

[7] Hanna N, Shepherd FA, Fossella FV, Pereira JR, De Marinis F, von Pawel J, et al. Randomized phase III trial of pemetrexed versus docetaxel in patients with non-small-cell lung cancer previously treated with chemotherapy. J Clin Oncol 2004;22:1589–97.

[8] Fukuoka M, Niitani H, Suzuki A, Motomiya M, Hasegawa K, Nishiwaki Y, et al. A phase II study of CPT-11, a new derivative of camptothecin, for previously untreated non-small-cell lung cancer. J Clin Oncol 1992;10:16–20.

[9] Fukuoka M, Masuda N. Clinical studies of irinotecan alone and in combination with cisplatin. Cancer Chemother Pharmacol 1994;34:S105–11.

[10] Rothenberg ML. CPT-11: an original spectrum of clinical activity. Semin Oncol 1996;23:21–6.

[11] Devore III R, Johnson D, Crawford J, Dimery I, Eckardt J, Eckhardt SG. Irinotecan plus cisplatin in patients with advanced non-small-cell lung cancer. Oncology 1998;12:79–83.

[12] Masuda N, Fukuoka M, Fujita A, Kurita Y, Tsuchiya S, Nagao K, et al. A phase II trial of combination of CPT-11 and cisplatin for advanced non-small-cell lung cancer. CPT-11 Lung Cancer Study Group. Br J Cancer 1998;78:251–6.

[13] Adjei AA, Argiris A, Murren JR. Docetaxel and irinotecan alone and in combination, in the treatment of non-small cell lung cancer. Semin Oncol 1999;26:32–40.

[14] DeVore RF, Johnson DH, Crawford J, Garst J, Dimery IW, Eckardt J, et al. Phase II study of irinotecan plus cisplatin in patients with advanced non-small-cell lung cancer. J Clin Oncol 1999;17:2710–20.

[15] Chemotherapy in non-small cell lung cancer: a meta-analysis using updated data on individual patients from 52 randomised clinical trials. Non-small Cell Lung Cancer Collaborative Group. BMJ 1995;311:899–909.

[16] Ferrigno D, Buccheri G. Weekly chemotherapy with cisplatin and paclitaxel in inoperable non-small cell lung cancer: an extended phase II study. Anticancer Res 2005;25:4685–92.

[17] Berardi R, Porfiri E, Scartozzi M, Lippe P, Silva RR, Nacciarriti D, et al. Elderly patients with advanced non-small cell lung cancer. A phase II study with weekly cisplatin and gemcitabine. Oncology 2003;65:198–203.

[18] Kim S-W, Suh C, Lee SD, Kim WS, Kim DS, Kim WD, et al. Weekly low dose paclitaxel and cisplatin as first-line chemotherapy for advanced non-small cell lung cancer. Lung Cancer (Amsterdam, Netherlands) 2003;41:221–6.

[19] Nakanishi Y, Takayama K, Takano K, Inoue K, Osaki S, Wataya H, et al. Second-line chemotherapy with weekly cisplatin and irinotecan in patients with refractory lung cancer. Am J Clin Oncol 1999;22:399–402.

[20] Kakolyris S, Kouroussis C, Souglakos J, Agelaki S, Kalbakis K, Vardakis N, et al. Cisplatin and irinotecan (CPT-11) as second-line treatment in patients with advanced non-small cell lung cancer. Lung Cancer (Amsterdam, Netherlands) 2001;34(Suppl 4):S71–6.

[21] Georgoulias V, Agelidou A, Syrigos K, Rapti A, Agelidou M, Nikolakopoulos J, et al. Second-line treatment with irinotecan plus cisplatin vs cisplatin of patients with advanced non-small-cell lung cancer pretreated with taxanes and gemcitabine: a multicenter randomised phase II study. Br J Cancer 2005;93:763–9.

[22] Negoro S, Fukuoka M, Niitani H, Suzuki A, Nakabayashi T, Kimura M, et al. A phase II study of CPT-11, a camptothecin derivative, in patients with primary lung cancer. CPT-11 Cooperative Study Group (in Japanese, with English abstract). Gan To Kagaku Ryoho 1991;18:1013–9.

[23] Nakai H, Fukuoka M, Furuse K, Nakao I, Yoshimori K, Ogura T, et al. An early phase II study of CPT-11 in primary lung cancer (in Japanese, with English abstract). Gan To Kagaku Ryoho 1991;18:607–12.

[24] Pectasides D, Fountzilas G, Rigopoulos A, Bountouroglou NG, Koutras A, Glotsos J, et al. Irinotecan and vinorelbine in patients with non-small cell lung cancer previously treated with platinum-based chemotherapy. A phase II study of the Hellenic Cooperative Oncology Group. Anticancer Res 2002;22:3501–6.

[25] Gonzalez Cao M, Aramendia JM, Salgado E, Aristu J, Martinez Monje R, Algarra SM, et al. Second-line chemotherapy with irinotecan and vinorelbine in stage IIIB and IV non-small-cell lung cancer: a phase II study. Am J Clin Oncol 2002;25:480–4.

[26] Font A, Sanchez JM, Taron M, Martinez-Balibrea E, Sanchez JJ, Manzano JL, et al. Weekly regimen of irinotecan/docetaxel in previously treated non-small cell lung cancer patients and

correlation with uridine diphosphate glucuronosyltransferase 1A1 (UGT1A1) polymorphism. Invest New Drugs 2003;21:435–43.

[27] Pectasides D, Mylonakis N, Farmakis D, Nikolaou M, Koumpou M, Katselis I, et al. Irinotecan and gemcitabine in patients with advanced non-small cell lung cancer, previously treated with cisplatin-based chemotherapy. A phase II study. Anticancer Res 2003;23:4205–11.

[28] Han J-Y, Lee DH, Kim HY, Kim E-A, Lee JJ, Ju SY, et al. A Phase II study of weekly irinotecan and capecitabine in patients with previously treated non-small cell lung cancer. Clin Cancer Res 2003;9:5909–14.

[29] Georgoulias V, Kouroussis C, Agelidou A, Boukovinas I, Palamidas P, Stavrinidis E, et al. Irinotecan plus gemcitabine vs irinotecan for the second-line treatment of patients with advanced non-small-cell lung cancer pretreated with docetaxel and cisplatin: a multicentre, randomised, phase II study. Br J Cancer 2004;91:482–8.

[30] Wachters FM, Groen HJM, Biesma B, Schramel FMNH, Postmus PE, Stigt JA, et al. A randomised phase II trial of docetaxel vs docetaxel and irinotecan in patients with stage IIIb-IV non-small-cell lung cancer who failed first-line treatment. Br J Cancer 2005;92:15–20.

[31] Pectasides D, Pectasides M, Farmakis D, Kostopoulou V, Nikolaou M, Gaglia A, et al. Comparison of docetaxel and docetaxel-irinotecan combination as second-line chemotherapy in advanced non-small-cell lung cancer: a randomized phase II trial. Ann Oncol 2005;16:294–9.

Prediction of breast cancer prognosis by gene expression profile of *TP53* status

Shin Takahashi,[1] Takuya Moriya,[2] Takanori Ishida,[3] Hiroyuki Shibata,[1] Hironobu Sasano,[4] Noriaki Ohuchi,[3] and Chikashi Ishioka[1,5]

[1]Department of Clinical Oncology, Institute of Development, Aging and Cancer, Tohoku University, 4-1, Seiryo-machi, Aobaku, Sendai, 980-8575; [2]Department of Pathology, Tohoku University Hospital, 1-1, Seiryo-machi, Aobaku, Sendai, 980-8574; and [3]Division of Surgical Oncology, Tohoku University School of Medicine, 1-1, Seiryo-machi, Aobaku, Sendai, 980-854; and [4]Division of Anatomical Pathology, Tohoku University School of Medicine, 1-1, Seiryo-machi, Aobaku, Sendai, 980-8574, Japan

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***TP53* mutations are a poor prognostic factor in breast cancers. The present study sets out to identify the gene set that determines the expression signature of the *TP53* status (*TP53* signature) and to correlate it with clinical outcome. Using comprehensive expression analysis and DNA sequencing of the *TP53* gene in 38 Japanese breast cancer patients, a gene set from differentially expressed genes was isolated, depending on the *TP53* status. As independent external datasets, two published datasets were introduced for validation of *TP53* status predictions (251 Swedish samples) and survival analysis (both the Swedish and 295 Dutch samples). Thirty-three gene sets were identified from microarray analysis. Predictive accuracy of the *TP53* status by gene expression profiling was 83.3% in the test set ($n = 12$). *TP53* signature has the ability to predict recurrence-free survival (RFS) of 29 stage I and stage II Japanese breast cancers (log rank, $P = 0.032$), and RFS, overall survival of two independently published datasets (log rank, both $P < 0.0001$). Multivariate analysis has shown that the *TP53* signature an independent and significant prognostic factor with a hazard ratio (HR) for recurrence and survival in two external datasets ($P < 0.0001$). The *TP53* signature is also a strong prognostic factor in the subgroups: estrogen-receptor positive, lymph node positive and negative, intermediate/high risk in St. Gallen criteria, and high risk in National Cancer Institute (NCI) criteria (log rank, $P < 0.0001$). *TP53* signature is a reliable and independent predictor of the outcome of disease in operated breast cancer. (*Cancer Sci* 2008; 99: 324–332)**

Breast cancer is one of the common malignant tumors affecting women with an increasing incidence rate in many countries. The prognosis of breast cancer is improving because of multidisciplinary adjuvant treatments including chemotherapy, hormone therapy, and radiotherapy.[1] To assist patient management, breast cancers are stratified into hormone receptor-positive and -negative groups, lymph node (LN)-positive or -negative groups or through prognostic criteria, such as St. Gallen and National Institute of Health (NIH).[1,2] The current prognostic criteria, however, are inadequate for predicting patients who have a high mortality, especially in the early stages of the condition and in patients who are most likely to benefit from adjuvant therapy.

Several studies have investigated the predictive value of *TP53* mutation status for tumor response to treatment and patient outcome in various cancers with different clinical and methodological settings. In the majority of breast cancer studies that screened *TP53* mutations by gene sequencing, the presence of a mutation correlates with a shorter survival or a poor response to treatment.[3] Although the *TP53* mutations identified by sequencing the exons 5–8 as independent prognostic factor in a large number (1790) of breast cancer patients,[4] *TP53* is an unpredictable tool for individual risk evaluation, metastases, and overall survival. Because it is not easy to correctly evaluate *TP53* status by standard DNA sequencing analysis,[5] an efficient biomarker for *TP53* status may potentially improve its predictive value.

Microarray technologies have contributed to the characterization of molecular pathways on tumorigenesis, drug sensitivity, and prognosis of several cancers. For breast cancer, gene expression profiles that determine the status of estrogen receptor (ER), human epidermal growth factor receptor (HER2), distant metastasis, and prognosis by microarray-based transcriptome analysis may predict patient outcome even in the early stages of cancer.[6–17] These profiles therefore seem to be beneficial for decision-making in adjuvant treatment.

In our study, a gene set capable of determining the *TP53* mutation status has been identified. Furthermore, the expression signature for the *TP53* status has been shown to be an independent prognostic marker and a significantly better biomarker for predicting recurrence and patient survival than the currently used clinical and pathological markers and hormonal markers in the two independent datasets, 251 Swedish and 295 Dutch breast cancer cases.[11,18]

Materials and Methods

Patients. As internal dataset, 40 Japanese patients with invasive ductal carcinoma of the breast, operated at the Tohoku University Hospital between March 2000 and November 2002 were collected. An informed consent was obtained from the patients prior to their participation in the study, which had been approved by the ethical and legal committee of the Tohoku University School of Medicine. Clinicopathological information was obtained from patient records. Among the 40 patients, two samples with heterozygous *TP53* mutation were excluded for further analysis and the remaining 38 samples were divided into two groups: learning sample set ($n = 26$) for *TP53* status predictor gene set selection, and test sample set ($n = 12$) for validation of the gene set. No overlapping sample was included in each sample set.

As two independent external datasets, microarray and clinicopathological data of 251 Swedish breast cancer samples (including *TP53* status),[18] and 295 Dutch breast cancer samples (not including *TP53* status),[11] were obtained from Gene Expression Omnibus (GEO) and the Rosetta Inpharmatics, respectively. These datasets were used for validation of *TP53* status prediction (Swedish samples) and survival analysis (both Swedish and Dutch samples).

Microdissection and nucleic acid extraction. The frozen tumor materials were embedded and sliced into 20-μm thin sections by a cryotome (Thermo Electron Corp., Waltham, MA, USA). Frozen

[5]To whom correspondence should be addressed.
E-mail: chikashi@idac.tohoku.ac.jp
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sections were made according to the manufacturer's instructions and refrozen in −80°C until microdissection. From tumor tissue sections, only tumorous cells were microdissected using a Leica AS LMD system (Leica, Wetzlar, Germany). For RNA extraction, tumor cells were collected in 50-μL RLT buffer (Qiagen, Valencia, CA, USA), containing 1% 2-mercaptoethanol and 100–450 ng of total RNA was extracted by using RNeasy micro kit (Qiagen). The quality of RNA was confirmed by checking the 28S and 18S ribosomal RNA ratios using a Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). For DNA extraction, tumor cells were collected in 50 μL Buffer ATL (Qiagen) and 20–200 ng genomic DNA was obtained using a DNeasy tissue kit (Qiagen).

_TP53_ mutation analysis. *TP53* gene fragments covering an entire open reading frame were amplified from 2 to 10 ng genomic DNA by polymerase chain reaction (PCR) and sequenced using an automated CEQ2000XL DNA analysis system (Beckman Coulter, Fullerton, CA, USA) under specific cycle and temperature conditions as listed in Suppl. Table S1 and using oligonucleotides primers listed in Suppl. Table S2.

Immunohistochemistry. Immunohistochemistry (IHC) analyses were carried out at the pathology department of the Tohoku University Hospital by using the following antibodies: monoclonal antibody estrogen receptor (ER) (Immunotech, Marseilles, France) and mouse antiprogesterone receptor (PgR) monoclonal antibody (Chemicon, Temecula, CA, USA) until December 2002; DAKO monoclonal antihuman PgR, clone 636 (Dako, Carpinteria, CA, USA) and polyclonal rabbit antihuman c-erbB2 oncoprotein (DAKO) until June 2001; followed by Hercep test and monoclonal mouse antihuman p53 protein (Biomedia, Foster City, CA, USA).

Microarray analysis. Reference RNA pool was prepared by mixing 50 ng RNA from each tumor RNA sample. RNA from each sample was amplified and labeled using a low input RNA fluorescent linear amplification kit (Agilent). Microarray hybridizations (Agilent: whole human genome oligo microarray; 41 k unique probe) were carried out with 1 μg Cy3 labeled cRNA and 1 μg Cy5 labeled cRNA, both prepared from each sample and reference pool, respectively, according to manufacturer's instruction. The gene expression data were analyzed by GeneSpring 6.2 (Silicon Genetics, Redwood City, CA, USA).

Microarray data analysis. To exclude lower confident genes, during analysis, a cut-off value of 200 was defined, which was higher than the mean (104.4) plus twice the standard deviation (24.4) of the reference (Cy5) background values. Following the Lowess normalization, 19 206 genes, which had a signal intensity value exceeding 200 in more than 32 samples (80% of samples), were selected from 41 000 genes and were used for statistical analysis.

Predictor gene set for _TP53_ status. The gene set that predicts *TP53* mutation status correctly ('the predictor gene set for *TP53* status') was extracted using the following process. First, Wilcoxon rank sum test with or without Benjamini and Hochberg false discovery rate (FDR),[19] was used to select genes that were differentially expressed between mutant *TP53* and wild type *TP53* in the learning sample set ($n = 26$, see Materials and Methods). Second, selected genes were cross-validated by the K-nearest neighbor method with the leave-one-out method in the same learning set. Finally, to confirm the prediction accuracy of the gene set, the test sample set, which consists of independent samples from the learning sample set ($n = 12$), was used for validation.

_TP53_ status prediction. The mutant p53 expression signature (mPES) was defined as the average of the expression level of each predictor gene for the samples with *TP53* mutation in the learning sample set. The wild type p53 expression signature (wPES) was defined as the average of the expression level of each predictor gene for the samples with wild type *TP53* in the learning sample set. The prediction was based on correlations of the expression profile of each sample with mPES and the wPES, respectively. In the simple prediction, if a sample showed better correlation with wPES than mPES, it was considered as a wild type. In the wild-type specific prediction, if a sample showed positive correlation with wPES and negative correlation with mPES with $P < 0.05$, respectively, it was assigned to wild type. The other samples were assigned to non-wild type.

Survival analyses. Kaplan-Meier survival curves for recurrence-free survival (RFS), overall survival (OS), and survival after recurrence were compared using the Cox-Mantel log rank test. Multivariate analyses were carried out using the Cox-proportional hazards model and statistical analyses were carried out using SPSS14.0 (SPSS Inc., Chicago, IL, USA).

Results

Mutation analysis of _TP53_. *TP53* mutations were detected in 20 out of 40 tumors (50%). There were 13 missense mutations, five protein-truncating mutations (three nonsense and two frameshift mutations), and two splicing-site mutations (Suppl. Table S3). All of the mutations were plotted in the DNA binding domain of p53. To validate whether mutations were functionally inactivating p53 protein, the 13 missense mutations were confirmed as loss-of-function mutations by our database for transactivation activity of p53 mutants (Suppl Fig. S1).[20] In two samples of 13 missense mutations, heterozygous *TP53* missense mutations were detected. The five protein-truncating mutations and the two splicing-site mutations were assigned to loss-of-function mutations, because these mutations should eliminate functionally important domains and the carboxyl-terminal tetramerization domain and/or part of the DNA binding domain. Furthermore, by immunohistochemical staining of the p53 protein (p53 IHC), nuclear accumulation of the p53 protein was confirmed in all of the missense mutation cases except for the seven non-missense mutation cases (Suppl. Table S4). The characteristics of 40 Japanese patients with invasive ductal carcinoma of the breast by *TP53* status (sequence based), are shown in Suppl. Table S5. Consistent with previous studies, significant differences were noted in nuclear pleomorphism, mitotic counts, histological grade, ER, progesteron receptor, and p53 IHC ($P < 0.05$), but not in other clinical parameters (age, tumor size, LN status, and stage) among the *TP53* wild type and mutant status in the cohort.

Predictor gene set for _TP53_ status. From the result of mutation analysis, the two samples with heterozygous *TP53* missense mutations were excluded because the tumor cells may retain normal p53 function. The remaining 38 samples were divided into two groups: learning sample set (26 samples consisting of 12 samples with *TP53* mutation and 14 samples with wild type *TP53*) for predictor gene selection and test sample set (12 samples consisting of six samples with *TP53* mutation and six samples with wild type *TP53*) for validation of the gene set. Wilcoxon rank sum test with or without FDR was used to select genes that were differentially expressed between mutant *TP53* (12 samples) and wild type *TP53* (14 samples) in the learning sample set. The four predictor gene sets were identified depending on the distinct selection conditions. These consisted of 497 genes, 70 genes, 33 genes, and 10 genes for four distinct conditions: $P < 0.05$ without FDR, $P < 0.1$ with FDR, $P < 0.05$ with FDR, and $P < 0.01$ with FDR, respectively (Suppl. Table S6). Selected genes were cross-validated by the leave-one-out method in the learning sample set. Finally, the gene sets were validated by using the test sample set that consisted of six samples with mutant *TP53* and six samples with wild type *TP53*. The prediction accuracy of the 33 genes was the best predictor gene set among the four selection conditions and was 100% in the learning set (leave-one-out cross-validation) and 83.3% in the test set (overall 94.7%) (Suppl. Table S6). The 33 genes consisted of 24 upregulated genes and nine downregulated genes in mutant *TP53* samples (Table 1).

Table 1. The 33 genes identified as a predictor gene set for *TP53* status

GenBank	Common	Map	Description	31 gene set	26 gene set
Upregulated gene in mutant TP53					
NM_012325	MAPRE1	20q11.1–11.23	Microtubule-associated protein, RP/EB family, member 1	*	*
NM_018131	C10orf3	10q23.33	Chromosome 10 open reading frame 3	*	*
NM_012222	MUTYH	1p34.3-p32.1	mutY homolog (E. coli)	*	*
NM_152259	MGC45866	15q26.1	Hypothetical protein MGC45866	*	
NM_003981	PRC1	15q26.1	Protein regulator of cytokinesis 1	*	*
NM_006845	KIF2C	1p34.1	Kinesin family member 2C	*	*
AK001581	FLJ10719	15q25-q26	Hypothetical protein FLJ10719	*	
NM_004701	CCNB2	15q21.3	Cyclin B2	*	*
NM_001813	CENPE	4q24-q25	Centromere protein E, 312 kDa	*	*
NM_004219	PTTG1	5q35.1	Pituitary tumor-transforming 1 (securin)	*	*
NM_005563	STMN1	1p36.1-p35	Stathmin 1/oncoprotein 18	*	*
NM_018101	CDCA8	1p34.2	Cell division cycle associated 8	*	*
NM_005030	PLK	16p12.3	Polo-like kinase (Drosophila)	*	*
NM_138555	ANAPC7	12q13.12	Anaphase-promoting complex subunit 7	*	*
NM_018136	ASPM	1q31	Asp (abnormal spindle)-like, microcephaly associated (Drosophila)	*	*
NM_181803	UBE2C	20q13.12	Ubiquitin-conjugating enzyme E2C	*	*
BC000784	BIRC5	17q25	Baculoviral IAP repeat-containing 5 (survivin)	*	*
NM_014176	HSPC150	1q32.1	HSPC150 protein similar to ubiquitin-conjugating enzyme	*	*
NM_016343	CENPF	1q32-q41	Centromere protein F, 350/400 ka (mitosin)	*	*
NM_182687	PKMYT1	16p13.3	Membrane-associated tyrosine- and threonine-specific cdc2-inhibitory kinase	*	*
NM_003504	CDC45L	22q11.21	CDC45 cell division cycle 45-like (S. cerevisiae)		*
NM_138553	BCL11A	2p16.1	B-cell CLL/lymphoma 11 A (zinc finger protein)	*	*
NM_173083	TGS	1q42.13	TUDOR gene similar	*	
BF930764	BF930764		Homo sapiens transcribed sequence with strong similarity to protein sp.P05215 (H.sapiens) TBA4_HUMAN Tubulin alpha-4 chain (Alpha-tubulin 4)		
Down-regurated gene in mutant TP53					
NM_017572	MKNK2	19p13.3	MAP kinase-interacting serine/threonine kinase 2	*	*
NM_015920	RPS27L	15q22.1	Ribosomal protein S27-like	*	*
NM_080392	PTP4A2	1p35	Protein tyrosine phosphatase type IVA, member 2	*	*
NM_016210	LOC51161	3p21.3	G20 protein	*	*
NM_018837	SULF2	20q12-q13.2	Sulfatase 2	*	
AK023624	HIS1	17q21.31	HMBA-inducible	*	*
NM_018379	FLJ11280	1q21.3	Hypothetical protein FLJ11280	*	*
NM_145058	MGC7036	12q24.31	Hypothetical protein MGC7036	*	
NM_032780	FLJ14399	11q23.3	Hypothetical protein FLJ14399	*	

HMBA, hexamethylene bis-acetamide; MAP, mitogen activating protein.

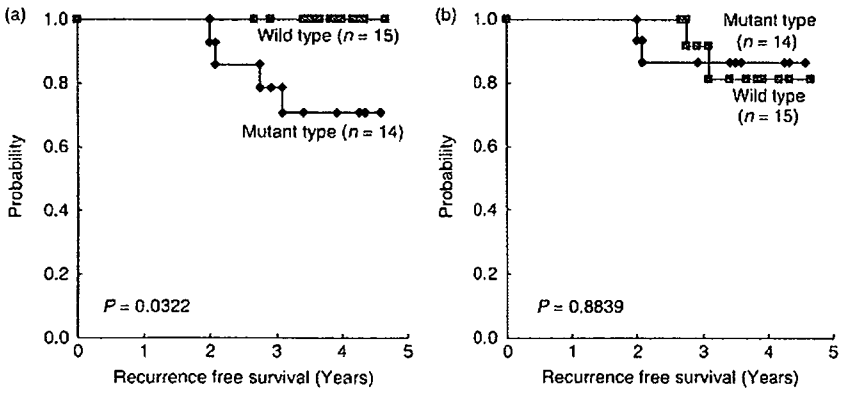

Fig. 1. Recurrence free survival analysis stratified by a wild type and mutant type signature (a) or the result of immunohistochemistry of p53 (b) in 29 Japanese patients with stage I and II breast cancer. *P*-value of log rank test is indicated.

The gene expression patterns of the learning sample set and the test sample set are shown in Suppl. Fig. S2.

Survival analysis by predicted *TP53* status in patients with stage I and II tumors. To determine whether the best predictor gene set (33 genes) for *TP53* status has also the ability to predict prognosis in stage I and II breast cancer, survival analysis was carried out. To exclude advanced disease samples, eight stage III and IV cases, as well as one stage IIA case that died because of gallbladder cancer, were excluded from the 38 samples. Then the remaining 29 samples (containing 14 and 15 cases predicted

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as mutant and wild type *TP53*, respectively) were analyzed for RFS (Fig. 1). Because the number of patients in the test set for validation was too small for survival analysis, we used both the learning set and the test set in survival analysis. Although the observation period was short (mean observation period: 44 months) and the sample size small, RFS showed significant difference between *TP53* mutation and *TP53* wild type cases ($P = 0.0322$, log rank test) (Fig. 1a). All four recurrent cases were only from the *TP53* mutant cases, whereas no recurrent case was observed in 15 *TP53* wild type cases. However, there was no difference between IHC positive and negative cases (Fig. 1b).

TP53 status prediction in external Swedish dataset. To confirm the accuracy of *TP53* status prediction in a large external sample set, 251 Swedish breast cancer samples,[18] were used. Among the predictor gene set for *TP53* status (33 genes), 31 genes were found in the provided Swedish dataset (Table 1). The predictive accuracy of the 31 genes (100% in learning set and 83.3% in test set) was equal to that of the 33 genes (Suppl. Table S6). *TP53* status prediction was carried out by the simple prediction (wild type or mutant type) and the wild-type specific prediction (wild type or non-wild type) (see Materials and Methods) using 31 genes (Suppl. Table S7). The predictive accuracy was 78.5% (197/251) and 62.5% (157/251) in the simple prediction and the wild-type specific prediction, respectively. Although the predictive accuracy of the wild-type specific prediction was lower than that of the simple prediction, specificity of predicting wild type was higher in the wild-type specific prediction (102 of 105, 97.1%) than in the simple prediction (154 of 169, 91.1%).

Survival analysis using TP53 status prediction in two external datasets. To examine the predictive value of our gene set on patient survival, two external datasets were used. In the Swedish dataset, 15 cases with no survival data were excluded and the remaining 236 samples were analyzed by using the 31 genes. In the simple prediction (158 wild type and 78 mutant type), OS was significantly ($P < 0.005$, log rank test) better in cases predicted as wild type than that of mutants (Fig. 2a). Five/10-year survival rates of wild type or mutant type were 87.0/81.0% or 76.0/62.8%, respectively. In the wild-type specific prediction (96 wild type and 140 non-wild type), the difference between the survival rate of wild type and that of non-wild type was also significant ($P < 0.0001$, log rank test) (Fig. 2b). Five/10-year survival rates of wild type or non-wild type were 94.7/89.8% or 73.3/65.7%, respectively. Obviously, predictive value of OS in the wild-type specific prediction was better than that in the simple prediction, although the predictive accuracy of *TP53* mutation in the former was worse than that in the latter.

As a second external dataset, the 295 Dutch breast cancer cases[11] were analyzed. Among the predictor gene set for *TP53* status (33 genes), 26 genes were found in the provided Dutch dataset (Table 1). The predictive accuracy of the 26 genes (100% in learning set and 83.3% in the test set) was equal to that of the 33 genes (Suppl. Table S6). *TP53* status of the 295 cases was predicted using the 26 genes by the simple prediction (176 wild type and 119 mutant type) and the wild-type specific prediction (104 wild type, 191 non-wild type). Sequence-based *TP53* status in the 295 cases was not available and therefore the predictive accuracy of the *TP53* status was not verified in the cohort. Based on the *TP53* status prediction results, the RFS (Fig. 2c,d) and OS (Fig. 2e,f) were analyzed. All the results showed significant difference in both RFS and OS between wild type and the other type (mutant type or non-wild type) ($P < 0.0001$, log rank test). In the simple prediction, five/10-year survival rates of wild type or mutant type were 93.4/82.2% or 68.1/52.3%, respectively. In the wild-type specific prediction, five/10-year survival rates of wild type or non-wild type were 98.0/90.2% or 75.4/59.6%, respectively. These results were very similar to the 251 Swedish breast cancer dataset and showed that the wild-type specific prediction was superior in predicting the wild type subgroup to the simple prediction. The expression profile of the *TP53* status could also predict survival periods after recurrence (Suppl. Fig. S3). There were significant differences between the wild type and the mutant type by the simple prediction ($P < 0.0001$, log rank test) and the non-wild type in the wild specific prediction ($P < 0.005$, log rank test). Predictive value of the survival period after recurrence by the simple prediction seemed to be better than that by the wild-type specific prediction.

RFS and OS analysis in a stratified group of patients. Predictive value of the expression profile of the 31 genes (251 Swedish dataset, Fig. 3a and b) and 26 genes (295 Dutch dataset, Fig. 3c–f) were examined in a stratified subgroup on ER status and LN status. We chose the wild-type specific prediction because it was more predictable than the simple prediction in unstratified samples. Clearly, there were significant differences between the wild type and non-wild type for survival in ER-positive, LN-positive and LN-negative subgroups in terms of both RFS and OS. In contrast, there was no significant difference between ER-positive and ER-negative or between LN-positive and LN-negative subgroups in both wild type and non-wild type.

Comparison with St. Gallen and NIH criteria. By the two published consensus criteria, the 2001 St. Gallen criteria,[21] and the 2001 NIH criteria,[1] only 22 (7.5%) and 11 (3.7%) of the 295 Dutch patients were classified into low risk subgroup, respectively.[11] Therefore the majority of the remaining patients were recommended to receive adjuvant systemic therapy. In contrast, the wild-type specific prediction classified 295 patients into 104 wild type (35.3%) and 191 non-wild types (64.7%) for the *TP53* status (Fig. 4). As expected, there were significant differences between wild type and non-wild type in *TP53* status for both RFS and OS in St. Gallen high-risk group and NIH high-risk group ($P < 0.0001$, log rank test). These results suggest that the gene set used in this study has the ability to identify low-risk patients who were not identified by the St. Gallen (Fig. 4a) or NIH criteria (Fig. 4b). They would not necessarily receive adjuvant systemic therapy. In contrast, there were no differences between the St. Gallen low risk group and the St. Gallen high risk group in wild type and in non-wild type cases.

Multivariable analysis. Table 2 shows the results of multivariable analysis of RFS and OS by the Cox proportional-hazard model. Hazard ratio (HR) of the wild-type specific prediction for OS was 3.08 (95% confidence interval [CI] [1.52–6.24], $P = 0.002$) in the Swedish dataset of 251 and 4.11 (95% CI [1.89–8.95], $P < 0.001$) in the Dutch dataset of 295. The HR for RFS was 2.63 (95% CI [1.49–4.35], $P < 0.001$) in the Dutch dataset. These results indicate that the wild-type specific prediction for *TP53* status was independent and a significant prognostic factor in the two external datasets.

Discussion

Techniques used to determine the *TP53* mutation should be highly specific and sensitive in order to isolate the accurate predictor gene set for *TP53* status. The sensitivity of mutation detection was maximized by microdissecting only the tumor cells in order to avoid contamination of normal cells and by sequencing the entire coding region of the *TP53* including the mutation outside of the DNA-binding domain.[4,22] Maximum specificity was achieved by evaluating pathogenicity of the detected missense mutations using the comprehensive *TP53* database for transactivation function,[20] thereby avoiding rare polymorphism or junk sequences caused by PCR error,[5] and by confirming nuclear accumulation of the expressed mutant p53 using IHC in the case of missense mutations. Although the characteristics (distribution and mutation types) of detected *TP53* mutations were consistent with previous reports, the detection rate (50%) exceeded the average prevalence of the *TP53* mutation of breast cancers (20.0–25.6%).[23] The technique used

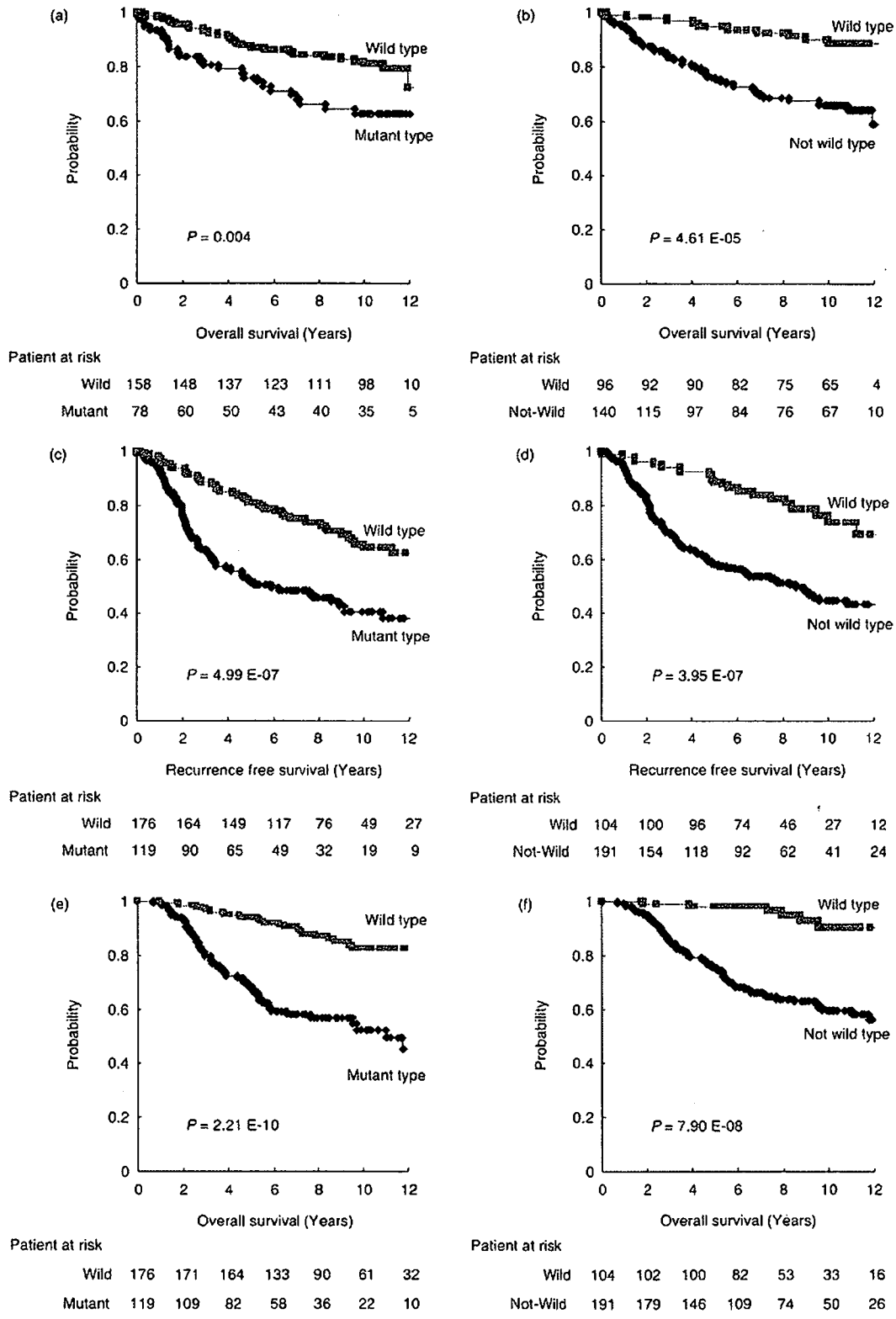

Fig. 2. Overall survival (OS) (a, b, e and f) and recurrence free survival (RFS) (c and d) analysis stratified by the simple prediction (left panel; a, c and e) and the wild-type specific prediction (right panel; b, d and f) in Swedish dataset (a and b) and Dutch dataset (c–f). Patients at risk and *P*-value of log rank test are indicated.

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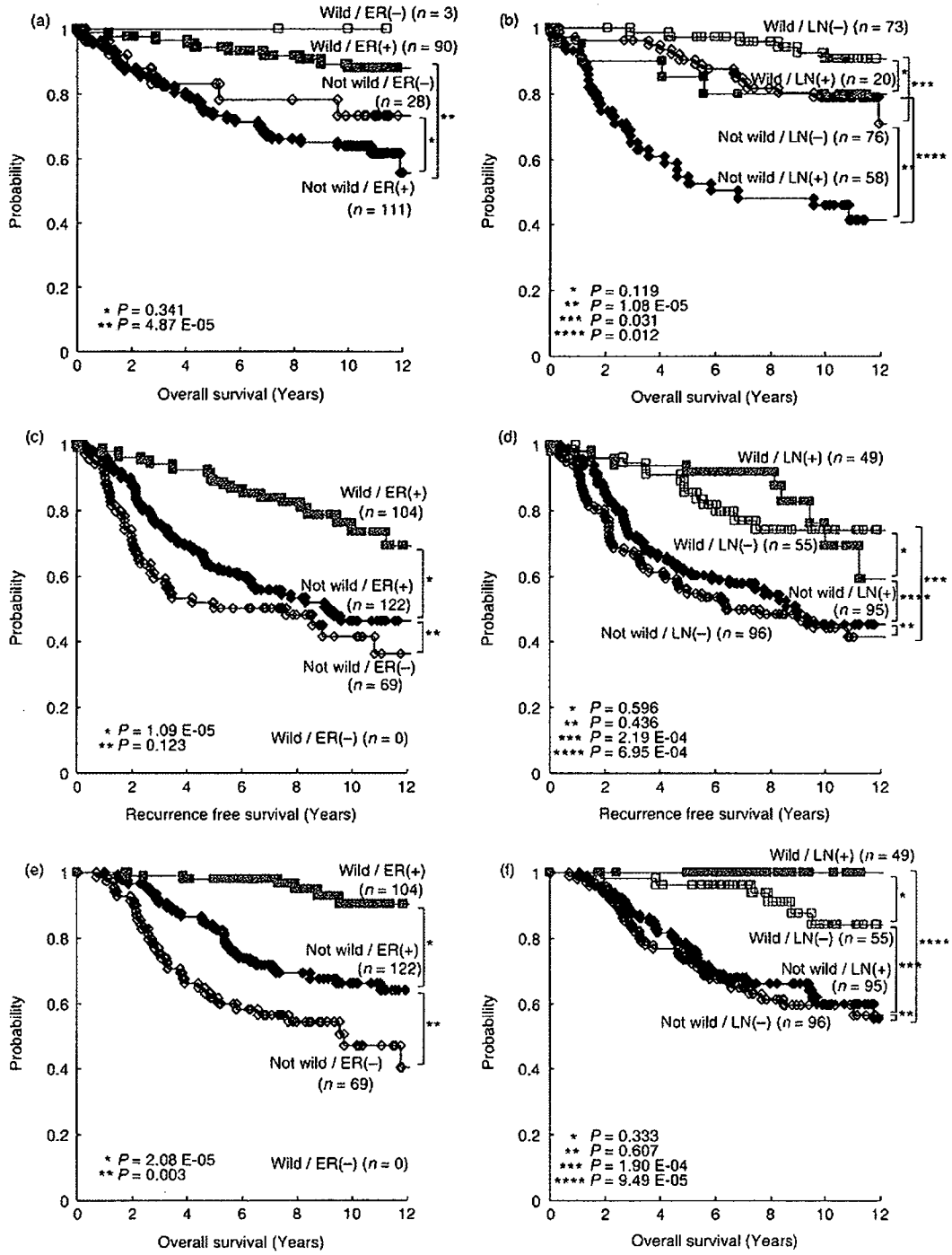

Fig. 3. Recurrence free survival (RFS) (c and d) and overall survival (OS) (a, b, e and f) analyses stratified by estrogen-receptor (ER) expression (a, c and e) and lymph node-metastasis status (b, d and f) in Swedish dataset (a and b) and in Dutch dataset (c–f). *P*-values of log rank test are indicated.

achieved maximal detection of *TP53* mutation and satisfactorily isolated the predictor 33 genes for the *TP53* status. Recently, Miller *et al.* reported that the expression signature of 32 genes for the *TP53* status has the ability to predict overall survival of 251 Swedish patients.[18] Surprisingly, there was no overlapped gene between the 32 genes and our 33 genes. The predictive accuracy for *TP53* status by the 33 genes in the Swedish dataset (78.5%) seems to be slightly lower than that in the test set of Japanese patients (83.3%). The interpretation of the predictive accuracy is difficult because there are large differences in the mutation prevalence between Japanese samples (50%) and Swedish samples (23%). We speculate that the difference may be due to the different sensitivity of mutation detection (with or without microdissection and genomic or cDNA sequencing) and the different specificity of the detected mutations (with or without functional analysis).

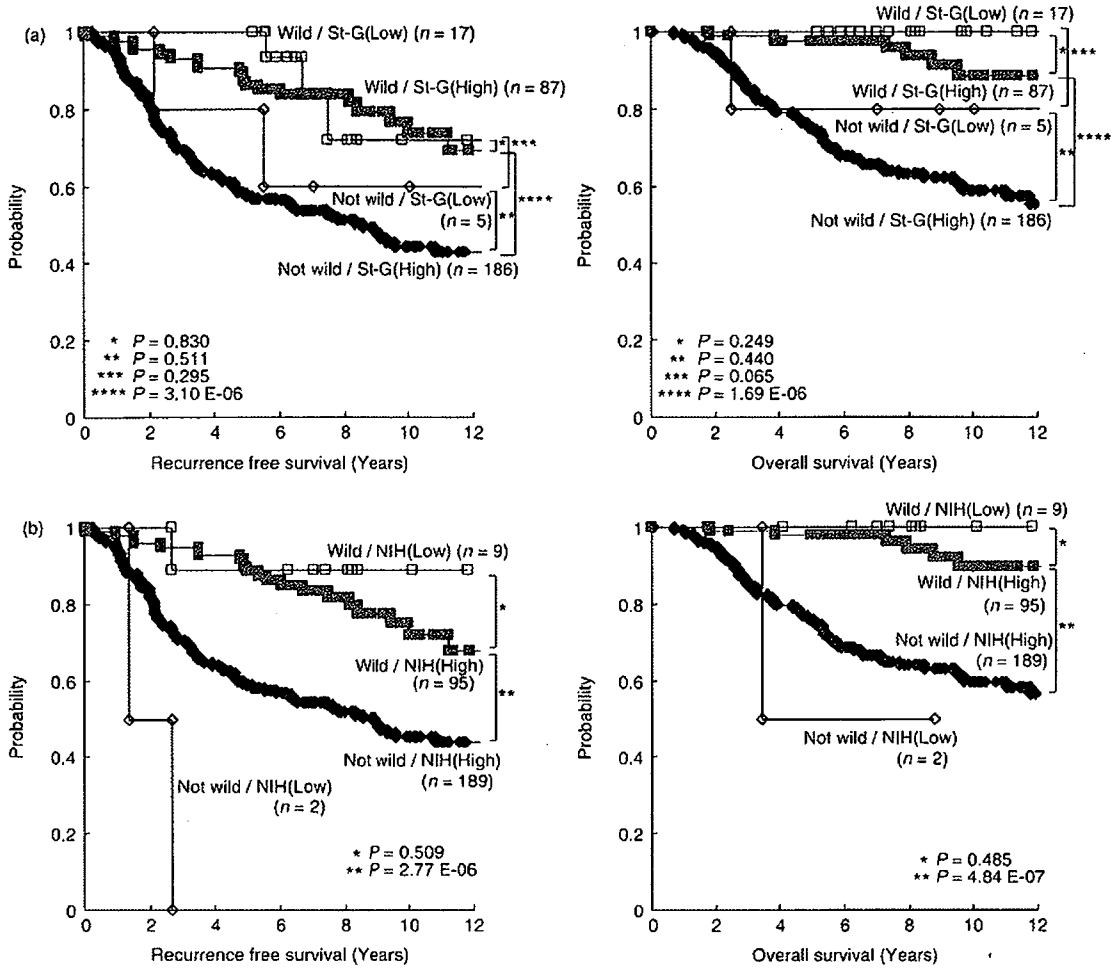

Fig. 4. Recurrence free survival (RFS) (left panel) and overall survival (OS) (right panel) analyses stratified by St. Gallen criteria (a) and National Institute of Health (NIH) criteria (b) in the Dutch dataset. *P*-values of log rank test are indicated.

Table 2. Multivariate analyses by Cox-proportional hazards regression model

Vriable	251 Swedish dataset			295 Dutch dataset					
	Overall survival			Recurrence free survival			Overall survival		
	HR[4]	95% CI	*P*	HR	95% CI	*P*	HR	95% CI	*P*
ER[1]	1.79	0.73–4.37	0.20	1.17	0.84–1.63	0.36	1.13	0.77–1.67	0.54
HER2[4]	1.32	0.99–1.76	0.06	1.35	0.96–1.89	0.08	1.83	1.24–2.73	0.00264
Lymphnode (versus negative node)	3.49	2.01–6.06	8.56E-06	0.87	0.61–1.25	0.45	0.89	0.57–1.40	0.62
TP53 status (versus wild type)[5]	3.08	1.52–6.24	1.79E-03	2.63	1.58–4.35	0.000179	4.11	1.89–8.95	0.000368

[1]Biochemical assay data (*versus* positive) was used in the Swedish dataset and the log ratio of ESR1 expression was used as a continuous variable in the Dutch dataset. [4]The log ratio of HER2 expression was used as a continuous variable. [5]The wild-type specific prediction by expression profile. CI, confidence interval; HR, hazard ratio.

The predictor gene set for *TP53* status (33 genes) consists of 24 upregulated genes and nine downregulated genes in samples with *TP53* mutations. Of the 24 upregulated genes, 14 genes have molecular functions related to cell cycle and/or cell division. The high frequency of cell cycle/division-related genes is also observed when analysis is extended to 757 genes, depending on *TP53* status (identified by Wilcoxon rank sum test) and analyzed for gene (or pathway) ontology (data not shown). It is speculated that cell cycle control was directly or indirectly disrupted by *TP53* mutation in tumor cells and that proliferation of tumor cells with *TP53* mutation increased rapidly compared with cells without *TP53* mutation. In fact, mitotic counts of samples with *TP53* mutation were higher compared with the wild type used in this study (Suppl. Table S1, $P < 0.001$). Recently, Dai *et al.* examined a gene expression signature related to a poor outcome in breast cancer and they identified 50 genes as a prognostic gene set.[12] Because many of the genes that were highly expressed in the tumors of poor prognosis were cell cycle-associated genes, they speculated that the cell proliferation signature was a marker of poor outcome in ER/age in high-risk

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breast cancer patients. Interestingly, seven genes (UBE2C or UBCH10, CCNB2, KIF2C or KNSL6, PRC1, CDC45L, PKMYT1, and BRIC5) in the *TP53*-status gene set (33 genes), all of which have cell cycle or cell division associated molecular function, were also found in their prognostic gene set (50 genes). Although the two studies used different samples and different technologies, the similar results confirmed that cell proliferation in breast cancer was greatly influenced by *TP53* status and that the *TP53* status was one of the important factors that define malignant potential of cancer cells and poor outcome of patients. The 70 gene set for prognosis prediction was reported by van't Veer *et al.*[9] There was only one overlapping gene (PRC1) between the 70 gene set and our *TP53* predictor gene set. It is conceivable that most of the breast cancers with good signature in the 70 genes should retain wild type p53, even though information on *TP53* mutation status for the 295 Dutch cases was not available. This observation again underlines the importance of the biological function of p53 in clinical behavior of breast cancer.

Based on expression signature, the prognosis for the predicted *TP53* status by both the simple prediction and the wild-type specific prediction significantly correlated to RFS in 29 Japanese stage I and II breast cancer patients. Moreover, it was shown that predicted *TP53* status provided a highly significant correlation to OS in 251 Swedish breast cancers and to RFS, OS, and survival after recurrence in the 295 Dutch breast cancers. Although both prediction methods were significantly useful, the wild-type specific prediction (wild type, or non-wild type) had the greater ability to predict prognosis than the simple prediction. For example, in Dutch dataset, 6.3% (11 of 176) of wild type and 31.3% (37 of 119) of mutant type by the simple prediction died within 5 years. In contrast, 1.9% (2 of 104) of wild type and 38.7% (46 of 119) of non-wild type by the wild-type specific prediction died within 5 years. From the point of clinical practice, a better prognostic stratification is important because the application of adjuvant chemotherapy after surgery may be reduced because patients are expected to have less chance of recurrence. The current St. Gallen criteria recommend that low risk patients should be treated by only adjuvant endocrine therapy (not by chemotherapy).[2] Unfortunately, only a small fraction of patients were stratified as low risk by St. Gallen criteria (21 of 295, 7%) and NIH criteria (11 of 295, 4%). Therefore, many patients with a potentially good prognosis have possibly been over-treated under the current decision-making criteria. In contrast, the wild-type specific prediction has the ability to stratify 96 of 236 Swedish patients (40.7%) and 104 of 295 Dutch patients (35.3%) into the wild type category in which the clinical outcome for both RFS and OS was comparable to that of the St. Gallen and the NIH low risk group (Figs 2b,d,f; 4a,b). Therefore, the microarray-based method should improve the selection of patients who may benefit from adjuvant systemic therapy as described by the other groups.[9,11,14,24] Beyond the microarray analysis, expression profile of a limited number of genes is clearly simpler than the sequence analysis and once the gene set is identified, the technique will soon be automated and the cost will be minimized. Furthermore, the statistical analysis needed for prediction was only correlation coefficient: once the system is established, full-powered statistical analyses are no longer required.

This study has shown that the expression signature of the *TP53* status was useful for stratification of good or poor risk groups of breast cancer patients. The data also indicates that the metastatic potential of breast cancer is acquired at a relatively early stage of the condition (initiation model, rather than progression model),[25] and that p53 seems to be involved in the early stages of carcinogenesis of the mammary gland. Because *TP53* mutation status is a prognostic factor in various cancers, including lung cancer, colonic cancer, and urinary bladder cancer, and because the biological role of p53 should be no different from breast cancer and other cancers, the *TP53* signature is potentially applicable to cancers other than breast cancer.

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References

1 Eifel P, Axelson JA, Costa J *et al.* National institutes of health consensus development conference statement: adjuvant therapy for breast cancer, November 1–3, 2000. *J Natl Cancer Inst* 2001; **93**: 979–89.
2 Goldhirsch A, Glick JH, Gelber RD, Coates AS, Thurlimann B, Senn HJ. Meeting highlights: international expert consensus on the primary therapy of early breast cancer 2005. *Ann Oncol* 2005; **16** (10): 1569–83.
3 International Agency for Research on Cancer (IARC). IARC *Tp53* mutation database. *Database Downlords: Data set of Somatic Mutation.* [Cited 28 July 2005] Available from URL: http://www-p53.iarc.fr/Somatic.html.
4 Olivier M, Langerod A, Carrieri P *et al.* The clinical value of somatic *TP53* gene mutations in 1794 patients with breast cancer. *Clin Cancer Res* 2006; **12** (4): 1157–67.
5 Soussi T, Asselain B, Hamroun D *et al.* Meta-analysis of the p53 mutation database for mutant p53 biological activity reveals a methodologic bias in mutation detection. *Clin Cancer Res* 2006; **12** (1): 62–9.
6 Gruvberger S, Ringner M, Chen Y *et al.* Estrogen receptor status in breast cancer is associated with remarkably distinct gene expression patterns. *Cancer Res* 2001; **61** (16): 5979–84.
7 Oh DS, Troester MA, Usary J *et al.* Estrogen-regulated genes predict survival in hormone receptor-positive breast cancers. *J Clin Oncol* 2006; **24** (11): 1656–64.
8 West M, Blanchette C, Dressman H *et al.* Predicting the clinical status of human breast cancer by using gene expression profiles. *Proc Natl Acad Sci USA* 2001; **98** (20): 11 462–7.
9 van 't Veer LJ, Dai H, van de Vijver MJ *et al.* Gene expression profiling predicts clinical outcome of breast cancer. *Nature* 2002; **415** (6871): 530–6.
10 Bertucci F, Borie N, Ginestier C *et al.* Identification and validation of an ERBB2 gene expression signature in breast cancers. *Oncogene*, 2004; **23** (14): 2564–75.
11 van de Vijver MJ, He YD, van't Veer LJ *et al.* A gene-expression signature as a predictor of survival in breast cancer. *N Engl J Med* 2002; **347** (25): 1999–2009.
12 Dai H, van't Veer L, Lamb J *et al.* A cell proliferation signature is a marker of extremely poor outcome in a subpopulation of breast cancer patients. *Cancer Res* 2005; **65** (10): 4059–66.
13 Chang HY, Nuyten DS, Sneddon JB *et al.* Robustness, scalability, and integration of a wound-response gene expression signature in predicting breast cancer survival. *Proc Natl Acad Sci USA* 2005; **102** (10): 3738–43.
14 Wang Y, Klijn JG, Zhang Y *et al.* Gene-expression profiles to predict distant metastasis of lymph-node-negative primary breast cancer. *Lancet* 2005; **365** (9460): 671–9.
15 Sorlie T, Perou CM, Tibshirani R *et al.* Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc Natl Acad Sci USA* 2001; **98** (19): 10 869–74.
16 Foekens JA, Atkins D, Zhang Y *et al.* Multicenter validation of a gene expression-based prognostic signature in lymph node-negative primary breast cancer. *J Clin Oncol* 2006; **24** (11): 1665–71.
17 Huang E, Cheng SH, Dressman H *et al.* Gene expression predictors of breast cancer outcomes. *Lancet* 2003; **361** (9369): 1590–6.
18 Miller LD, Smeds J, George J *et al.* An expression signature for p53 status in human breast cancer predicts mutation status, transcriptional effects, and patient survival. *Proc Natl Acad Sci USA* 2005; **102** (38): 13 550–5.
19 Benjamini, Y and Hochberg, Y. A practical and powerful approach to multiple testing. *Jornal Royal Statist Soc* 1995; **57**: 289–300.
20 Kato S, Han SY, Liu W *et al.* Understanding the function-structure and function-mutation relationships of p53 tumor suppressor protein by high-resolution missense mutation analysis. *Proc Natl Acad Sci USA* 2003; **100** (14): 8424–9.

21 Aapro MS. Adjuvant therapy of primary breast cancer. A review of key findings from the 7th international conference, St. Gallen, February 2001. *Oncologist* 2001; **6** (4): 376–85.
22 Borresen-Dale AL. *TP53* and breast cancer. *Hum Mutat* 2003; **21** (3): 292–300.
23 International Agency for Research on Cancer (IARC). *IARC* Tp53 *Mutation Database: Database Statistics: R10* July 2005 [Cited 28 July 2005] Available from URL: http://www-p53.iarc.fr/Somatic.html.
24 Glinsky GV, Higashiyama T, Glinskii AB. Classification of human breast cancer using gene expression profiling as a component of the survival predictor algorithm. *Clin Cancer Res* 2004; **10** (7): 2272–83.
25 Bernards R, Weinberg RA. A progression puzzle. *Nature* 2002; **418** (6900): 823.

Supplementaly Material

The following supplementary material is available for this article:

Fig. S1. Functional validation of 12 missense mutations detected in this study. Transcriptional activities of missense mutations and wild type p53 through p53-binding sequences derived from eight indicated genes were shown as a color gradiation based on standardized values among 2314 reported mutations (ref). Red, transcriptionally active; blue, transcriptionally inactive.

Fig. S2. Expression profile of 33 genes predictable for TP53 status and correlation coefficient. (A) The mutant (mPES) and wild type p53 expression signatures (wPES) were defined as the average of the expression level of the each predictor gene for the 12 samples with TP53 mutation and the 14 samples with wild type TP53, respectively, in the learning sample set (upper panel). Prediction was made based on the correlations of the expression profile of each sample with the mPES and the wPES (lower panel). (B) Validation of prediction accuracy in the test set. Two samples were incorrectly predicted (triangles). The order of the samples and the 33 genes depends on the correlation with mPES and the average expression value of each gene in the learning set, respectively.

Fig. S3. Survival after recurrence analyzed by the simple prediction (left panel) or the wild-type specific prediction (right panel) analysis.

Table S1. Polymerase chain reactionprimers
Table S2. Polymerase chain reaction conditions
Table S3. Summary of *TP53* mutation detected in this study
Table S4. Relation between *TP53* mutation and immunohistochemistry
Table S5. Patients and tumor characteristics
Table S6. Predictor Gene Set for *TP53* Status
Table S7. Validation of *TP53* status prediction by expression profile by external dataset.

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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

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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/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)

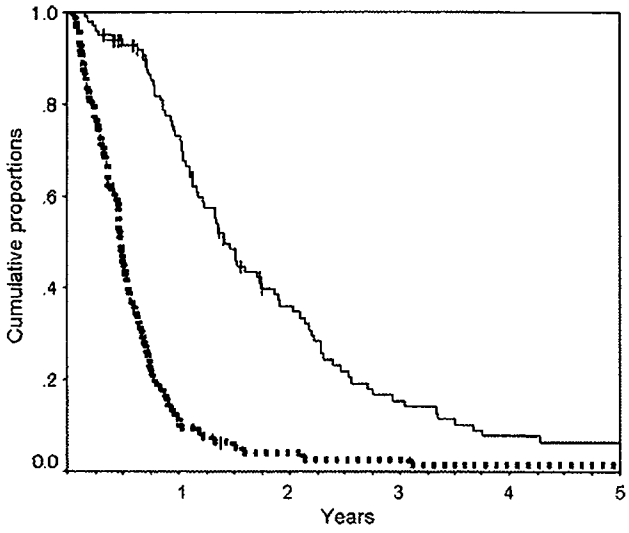

Fig. 1 Kaplan–Meier analysis of time to progression (*dotted line*) and overall survival (*solid line*). *Vertical bars* indicate censored cases

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