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A randomised phase II trial of preoperative chemotherapy of cisplatin–docetaxel or docetaxel alone for clinical stage IB/II non-small-cell lung cancer: results of a Japan Clinical Oncology Group trial (JCOG 0204)

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Preoperative chemotherapy is a promising strategy in patients with early-stage resectable non-small-cell lung cancer (NSCLC); optimal chemotherapy remains unclear. Clinical (c-) stage IB/II NSCLC patients were randomised to receive either two cycles of docetaxel (D)–cisplatin (P) combination chemotherapy (D 60 mg m^{-2} and P 80 mg m^{-2} on day 1) every 3–4 weeks or three cycles of D monotherapy (70 mg m^{-2}) every 3weeks. Thoracotomy was performed 4–5 weeks (DP) or 3–4 weeks (D) after chemotherapy. The primary end point was 1-year disease-free survival (DFS). From October 2002 to November 2003, 80 patients were randomised. Chemotherapy toxicities were mainly haematologic and well tolerated. There were two early postoperative deaths with DP (one intraoperative bleeding and one empyema). Pathologic complete response was observed in two DP patients. Docetaxel–cisplatin was superior to D in terms of response rate (45 *vs* 15%) and complete resection rate (95 *vs* 87%). Both DFS and overall survival were better in DP. Disease-free survival at 1, 2 and 4 years were 78, 65 and 57% with DP, and were 62, 44 and 36% with D, respectively. Preoperative DP was associated with encouraging resection rate and DFS data, and phase III trials for c-stage IB/II NSCLC are warranted.

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Surgery is the standard of care for clinical (c-) stage IB/II non-small-cell lung cancer (NSCLC), but the treatment outcome remains poor, with 5-year survival rates of 50% or less (Mountain, 1997; Goya *et al*, 2005). The majority of post-surgical relapse occurs as distant metastases (Pisters and Le Chevalier, 2005); therefore, effective systemic therapy is necessary. Recently, a series of postoperative adjuvant chemotherapy trials reported modest but significant improvement in survival, mainly in patients with pathological stage II or IIIA NSCLC (Arriagada *et al*, 2004; Scagliotti, 2005; Winton *et al*, 2005; Douillard *et al*, 2006). Compliance to the chemotherapy remains a problem (Arriagada *et al*, 2004; Scagliotti, 2005; Winton *et al*, 2005; Douillard *et al*, 2006).

On the other hand, previous small phase III trials had reported that preoperative chemotherapy was better than surgery alone in stage III NSCLC (Rosell *et al*, 1994; Roth *et al*, 1994). Recent trials of preoperative platinum-based chemotherapy have reported promising results in c-stage IB/II NSCLC (Pisters *et al*, 2000; Depierre *et al*, 2002; Rosell *et al*, 2002). One advantage of the preoperative chemotherapy is better tolerability and compliance.

No data are available, however, as to the optimal preoperative therapy strategy for early-stage NSCLC. Although platinum-based 'standard' combination chemotherapy regimens have widely been used and reported to be generally safe, results of randomised trials

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reported nonsignificant but modest excess of post-surgical morbidity and mortality (Depierre *et al*, 2002; Pisters *et al*, 2007). Monotherapy with an active agent is associated with lower response rate, but less toxicity (Delbaldo *et al*, 2004); it might well be favourable for preoperative therapy in early stage, when surgery must not be compromised by adjuvant therapy.

Docetaxel (D) is a semisynthetic taxoid derived from the European yew *Taxus baccata*. It is active against NSCLC either in monotherapy (D)(Fossella *et al*, 1994; Francis *et al*, 1994; Kunitoh *et al*, 1996) or in combination with cisplatin (DP) (Zalcberg *et al*, 1998; Fossella *et al*, 2003). In advanced NSCLC, DP was reported to be better than P–vinca combination (Fossella *et al*, 2003; Kubota *et al*, 2004), one of the 'standard' adjuvant therapies. The DP combination was also reported to be active and promising as preoperative chemotherapy in c-stage III NSCLC (Betticher *et al*, 2006).

Docetaxel monotherapy, on the other hand, was reported to be not inferior to DP, with better tolerability in advanced NSCLC (Georgoulias *et al*, 2004). For stage III NSCLC, Mattson *et al* (2003) reported the results of D as preoperative chemotherapy; it was active, and did not compromise surgery.

On the basis of this rationale, we undertook a randomised phase II trial of DP *vs* D in resectable, c-stage IB/II NSCLC. The objectives of the study were to evaluate the safety and efficacy of the preoperative chemotherapy and to select promising one for future phase III trials. The primary end point was the disease-free survival (DFS) rate at 1 year.

PATIENTS AND METHODS

Patient eligibility criteria

Patients with untreated, histologically or cytologically documented NSCLC with clinical stage IB (c-T2N0M0), IIA (c-T1N1M0) or IIB (c-T2N1M0 or T3N0M0) were eligible for study entry. Each patient was required to meet the following criteria: 20–74 years of age, Eastern Cooperative Oncology Group (ECOG) performance status (PS) of 0 or 1; measurable disease; and adequate organ function (leukocyte count $\geqslant 4000/\mu l$ and $\leqslant 12\,000/\mu l$, neutrophil count $\geqslant 2000/\mu l$, platelet count $\geqslant 10^5/\mu l$, haemoglobin $\geqslant 10.0\,g\,dl^{-1}$, serum creatinine $\leqslant$ the upper limit of the institutional normal range (ULN), creatinine clearance calculated by the Cockcroft–Gault formula $\geqslant 60\,ml\,min^{-1}$, serum bilirubin $\leqslant$ ULN, serum ALT and AST $\leqslant 2 \times$ ULN and $PaO_2 \geqslant 70\,mm\,Hg$). Women who were pregnant or lactating were excluded from the study. Other exclusion criteria included patients with active infection, unstable angina or a history of myocardial infarction within 6 months, interstitial pneumonia or active lung fibrosis, uncontrolled diabetes or hypertension, systemic use of corticosteroid or active concomitant malignancy. Patients with tumour invading the first rib or more superior chest wall (Pancoast type) were also excluded. All mediastinal nodes measuring 1.0 cm or more in size on computed tomographic (CT) scans were required to be biopsied to be histologically benign before patient entry.

Patient eligibility was confirmed by the Japan Clinical Oncology Group Data Centre before registration. The study protocol was approved by the institutional review boards at each participating centre, and all patients provided written informed consent.

Treatment plan

This was an open-label, randomised trial. Patients were randomly assigned to one of two treatment arms. Dosages of the chemotherapy were based on the regulatory notes and clinical data in Japan (Kubota *et al*, 2004). In the DP combination arm, patients received D at $60\,mg\,m^{-2}$ as a 1-h intravenous infusion followed by P at $80\,mg\,m^{-2}$ as a 2-h infusion on day 1. Two cycles of the chemotherapy were repeated at an interval of 4 weeks. The interval was permitted to be shortened to 3 weeks, if the patient was judged to have adequately recovered enough from the first cycle. Surgery (lobectomy or pneumonectomy with systematic lymph node dissection) was performed 4–5 weeks after completion or early termination of the chemotherapy. Patients in the D monotherapy arm received D at $70\,mg\,m^{-2}$ as a 1-h intravenous infusion on day 1. Three cycles of the chemotherapy were repeated at 3 weeks intervals. Surgery in the D arm was performed 3–4 weeks after completion or early termination of chemotherapy. The preoperative periods were thus set at 8–10 weeks in each arm, which was designed to be easier to accept for the patients and the surgeons.

In each arm, when chemotherapy was judged to be ineffective with $\geqslant 10\%$ unidirectional tumour growth, or when the patient experienced unacceptable toxicity (such as, grade 3 neurotoxicity, grade 2 pulmonary toxicity, grade 3 cardiac toxicity or other grade 4 non-haematological toxicities), chemotherapy was discontinued and the patient was taken up for surgery as clinically indicated. With minor toxicities, such as uncomplicated grade 4 haematologic or grade 3 non-critical, non-haematological toxicities, dosages of subsequent chemotherapy courses were reduced (P by $20\,mg\,m^{-2}$ and D by $10\,mg\,m^{-2}$).

No protocol therapy was predetermined for those with unresectable tumours, either during chemotherapy or at operation, and those with microscopically or macroscopically incompletely resected tumours. Those who underwent curative resection were observed until recurrence without additional therapy.

Chemotherapy was supported with routine premedication for hypersensitivity and antiemetics. For the DP arm, ample hydration was ensured. Recombinant human granulocyte colony-stimulating factor was administered when grade 4 neutropaenia or neutropaenic fever occurred.

Patient evaluation and follow-up

Before study enrolment, a complete medical history and physical examination, blood cell count determinations, biochemistry testing, chest X-ray, ECG, CT scan of the chest and CT scan or ultrasound of upper abdomen were conducted for each patient. Whole-brain CT or magnetic resonance imaging (MRI) or isotope bone scanning was performed if clinically indicated. Positron emission tomography (PET) was not widely available in Japan at the time of the protocol activation and was not routinely used for staging. Blood cell counts, differential WBC counts and biochemistry testing were performed weekly during each course of chemotherapy.

Toxicity of the chemotherapy was evaluated with the National Cancer Institute Common Toxicity Criteria Tumour (NCI-CTC; version 2.0). Tumour responses were assessed radiographically according to the RECIST guideline (Therasse *et al*, 2000). Response confirmation at 4 weeks or longer intervals was not necessitated. Response was assessed by the attending physicians in each participating institution, and no central confirmation was performed. Chest X-ray was taken at each course, and when suggested for even minor tumour growth ($\geqslant 10\%$), confirmatory chest CT was performed to decide on the continuation of chemotherapy.

After curative resection, the patients were followed up with periodic reevaluation. This included chest CT every 6 months for the first 2 years and annually thereafter, until 5 years or tumour recurrence.

Statistical considerations

This trial was designed as a randomised phase II selection design. Therefore, formal statistical hypothesis testing of the differences between the arms, including the calculation of *P*-values, was not to be performed. The aim was to select the 'preferable' preoperative chemotherapy arm for a future definitive phase III trial, with the DFS rate at 1 year as primary end point. The DFS was calculated

from the date of enrolment by the Kaplan–Meier method, as was the overall survival (OS).The 'events' for the determination of the DFS included tumour relapse after curative surgery, death from any cause and non-curative operation. Those with non-curative operation include patients without surgery and those with incomplete resection, either microscopically or macroscopically. Non-curative operation was to be counted as an event on the date of registration, not on that of surgery. The sample size was determined to provide sufficient probability to choose the 'preferable' arm (Simon *et al*, 1985). Assuming DFS rates at 1 year of 70 and 80%, 40 patients per arm were required to correctly select the arm that is not inferior with the probability of 84.9%. The 'minimal' DFS rate of 70% was assumed with the prior report from North America, in which the 1- and 2-year survival rates were reported to be 85 and 56%, respectively (Pisters *et al*, 2000). The assumption was rough and might well be inaccurate, for no DFS data were available from the literature. The randomisation was carried out by the JCOG data centre using a minimisation method with c-stage (IB *vs* II) and institutions as balancing factors.

The secondary end points included the objective tumour response to chemotherapy, complete resection rate, intra- and post-surgical morbidity/mortality and the OS rate. Tumour responses in both arms were compared using Fisher's exact test.

During the accrual period, an interim analysis was planned after 40 patients were randomised and followed up for at least 4 months. All analyses were performed with the SAS software version 9.1 (SAS Institute, Cary, NC, USA).

RESULTS

Patient characteristics

From October 2002 to October 2003, 80 patients from 18 institutions were enroled and randomised. After 40 patients were randomised, an interim analysis was carried out. Following the JCOG Data and Safety Monitoring Committee's review, the study was continued. One patient in the D arm was found to be ineligible because of the wrong histology (sarcoma). All 80 patients were analysed for characteristics and chemotherapy toxicity, and the 79 eligible patients were analysed for the clinical and pathological response to chemotherapy, surgical results, DFS and OS.

Table 1 lists the characteristics of the patients, which were well balanced between the arms.

Chemotherapy delivery and toxicity

Table 2 summarises the chemotherapy delivery, and Table 3 summarises toxicity in the subject group. Only 60% in the D arm completed the planned chemotherapy courses, mainly arising from the clinical ineffectiveness of the therapy. On the other hand, compliance was very good in the DP arm, and the toxicity was not greater. Hyponatraemia, probably due to hydration with P administration, was an unexpected toxicity in the DP arm, but it was clinically silent and transient in all the cases. All patients recovered without any particular management, with no clinically relevant sequelae. Other toxicities were mainly haematologic, and both chemotherapy arms were generally well tolerated by the patients.

Clinical response and pathological results

Table 4 shows the clinical responses to the chemotherapy. The overall response rates, 45% in the DP arm and 15% in the D arm, were compatible with earlier reports for each of the chemotherapy regimen in patients with NSCLC.

Thoracotomy was performed in 39 of the 40 patients in the DP arm, and in 37 of the 39 patients in the D arm. The tumour was surgically resected in 39 (98%) patients in the DP arm, including pneumonectomy in 3 cases, bi-lobectomy in 2 cases and lobectomy in 34 cases. Tumour resection was performed in 35 (90%) patients of the D arm, including pneumonectomy in 1 case, bi-lobectomy in 4 cases and lobectomy in 30 cases. Five patients, including four in the DP arm and one in the D arm, suffered from massive ($\geqslant$1 l) intraoperative bleeding: due to severe adhesion in three cases (two in DP and one in D arm), to incomplete suture of the autostapler resulting in injury of pulmonary artery in one case (DP arm) and accidental injury to the aorta in one case (DP arm). None was judged to be related to preoperative therapy. The postoperative complications included one patient with empyema and another with pulmonary oedema, both in the DP arm. There were two surgical deaths, both in the DP arm; one died on postoperative day 59 because of empyema, and another on postoperative day 2 because of massive intraoperative bleeding resulting from surgical injury to the aorta.

Pathological complete resection (R0), without residual tumour found either macroscopically or microscopically, was achieved in 38 (95%) cases in the DP arm, and 34 (87%) cases in the D arm.

Table 1 Patient characteristics

Arm	Cisplatin–docetaxel	Docetaxel alone
N	40	40
Clinical stage		
IB	22	23
II	18	17
Clinical T stage		
T1	5	5
T2	31	29
T3	4	6
Clinical N stage		
N0	26	28
N1	14	12
ECOG performance status		
PS0	35	31
PS1	5	9
Histology		
Adenocarcinoma	30	24
Squamous cell carcinoma	10	11
Others	0	5
Body weight loss within 6 months		
None	24	22
$\leqslant$5 kg	13	14
>5 kg	1	2
Missing	2	2
Smoking		
Median smoking	40 pack-years	40 pack-years
Never-smoker	6	8

Table 2 Delivery of chemotherapy

Arm	Cisplatin–docetaxel	Docetaxel alone
N	40	40
Completed	38 (95%)	24 (60%)
Not completed	2	16
Ineffective[a]	1	11
Adverse event	1	3
Patient refusal	0	1
Found ineligible	0	1

[a]Ineffectiveness was judged upon $\geqslant$10% unidirectional increase in tumour size, and did not necessarily mean progressive disease by RECIST.

Table 3 Toxicity of chemotherapy

Arm	Cisplatin–docetaxel	Docetaxel alone
N	40	40
Grade	2/3/4 (% grade 3+4)	2/3/4 (% grade 3+4)
Haematological		
Leukopaenia	18/14/1 (38)	12/15/2 (43)
Neutropaenia	5/16/17 (83)	5/10/21 (78)
Anaemia	4/0/0 (0)	7/0/0 (0)
Thrombocytopenia	1/0/0 (0)	0/0/0 (0)
Nonhaematological		
Total bilirubin	4/0/0 (0)	0/0/0 (0)
Serum AST	0/0/0 (0)	3/1/0 (3)
Serum ALT	5/0/0 (0	5/1/0 (3)
Serum creatinine	3/0/0 (0)	0/0/0 (0)
Hypoxia	0/0/0 (0)	3/0/0 (0)
Hypercalcaemia	0/0/0 (0)	0/1/0 (3)
Hyponatraemia	–/6/0 (15)	–/1/0 (3)
Hypersensitivity	0/0/0 (0)	0/1/0 (3)
Fatigue	3/1/0 (3)	0/0/0 (0)
Constipation	4/1/0 (3)	5/0/0 (0)
Diarrhea	3/3/0 (8)	2/0/0 (0)
Nausea	9/7/– (18)	0/0/– (0)
Vomiting	5/1/0 (3)	0/0/0 (0)
Febrile neutropaenia	–/1/0 (3)	–/0/0 (0)
Infection with neutropaenia	–/2/0 (5)	–/3/0 (8)
Infection without neutropaenia	1/0/0 (0)	4/2/0 (5)
Neuropathy	0/0/0 (0)	1/0/0 (0)
Any grade 3/4 toxicity	35 (88%)	32 (80%)
Any grade 3/4		
Non-haematological toxicity	15 (38%)	9 (23%)

Table 4 Clinical response to chemotherapy based on RECIST

Arm	Cisplatin–docetaxel	Docetaxel alone
N	40	39
Completed chemotherapy	38 (95%)	24 (62%)
CR	1	0
PR	17	6
CR+PR	18	6
SD	18	23
PD	4	10
NE	0	0
ORR	45%	15%
(95% confidence interval)	(29–62%)	(6–31%)

CR = complete response; NE = not evaluable; ORR = overall response rate; PD = progressive disease; PR = partial response; RECIST = Response Evaluation Criteria in Solid Tumor; SD = stable disease.

On pathological examination, 23% of the 75 patients who underwent surgery were found to have N2 or N3 status. Pathologic CR was achieved in two patients, both in the DP arm. Clinical N-stage was poorly correlated to pathological nodal status (Table 5).

DFS and OS

The DFS and OS were updated in November 2007. The DFS rates at 1, 2 and 4 years were 78, 65 and 57% in the DP arm, and were 62, 44 and 36% in the D arm, respectively (Figure 1). Table 6 summarises the outcome at 1 year, the primary end point of the study. The DFS rate at 1 year was 78% (31 out of 40) in the DP arm, which was consistent with the study assumption that it would be 80% in the 'better' arm, whereas it was a disappointing 62% (24 out of 39) in the D arm. The 16% difference was more than presumed in the protocol.

Table 5 Pathological results

Arm	Cisplatin–docetaxel			Docetaxel alone		
c-N stage	N0	N1	Total	N0	N1	Total
Number of cases	26	14	40	27	12	39
p-N0	17	6	23	18	4	22
p-N1	3	5	8	3	1	4
p-N2	5	2	7	5	4	9
p-N3	1	0	1	0	0	0
Not assessable	0	1	1	1	3	4

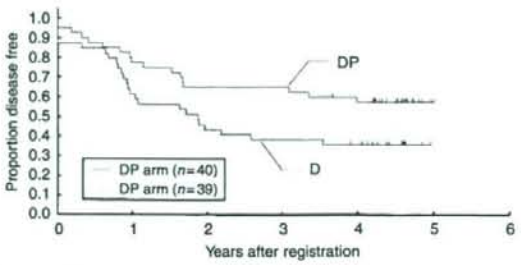

Figure 1 Disease-free survival.

Table 6 Outcome at 1 year

Arm	Cisplatin–docetaxel	Docetaxel alone	Total
Number of cases	40	39	79
Alive, disease-free	31	24	55
Alive with disease	4	11	15
Dead, due to cancer	3	2	5
Dead, treatment-related	2	0	2
Dead, other causes	0	2	2

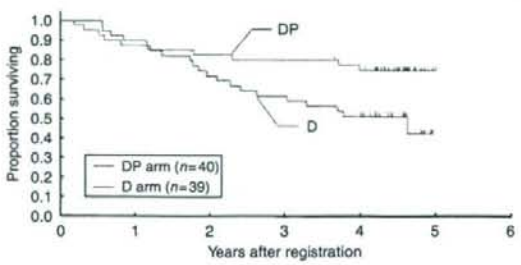

Figure 2 Overall survival.

The OS rates at 1, 2 and 4 years were 88, 83 and 75% in the DP arm, and were 87, 72 and 57% in the D arm, respectively (Figure 2). Both the DFS and the OS rates were better in the DP arm. The OS was better in the DP arm in both adenocarcinoma and non-adenocarcinoma histological subtypes.

DISCUSSION

As compared with post-surgical adjuvant therapy, preoperative chemotherapy has several practical as well as theoretical advan-

tages (Pisters *et al*, 2000; Pisters, 2003). The practical advantages include better patient tolerance and clinical visualisation of chemotherapy effect.

There are very few reports as to the optimal preoperative therapy strategy. The majority of trials have used 'standard' platinum-based doublets (Pisters, 2003). Although they are the 'standard' for advanced, stage IV NSCLC, a trade-off between the cytotoxic effect and toxicity of the chemotherapy, not only toxicity itself but also its influence on surgery and post-surgical morbidity and mortality (Depierre *et al*, 2002; Pisters *et al*, 2007), must be considered for preoperative therapy.

In this randomised phase II study, we evaluated DP combination chemotherapy and D monotherapy as preoperative treatment for early stage NSCLC. Although the DFS assumptions of the protocol, 70 *vs* 80% at 1 year, were rough and arbitrary due to lack of historical data, subsequent S9900 trial (Pisters *et al*, 2007) showed DFS rate of 68% in the surgery alone group and 69% in those with preoperative carboplatin–paclitaxel therapy, consistent with our assumption.

Our results showed that single-agent D was inadequate in this setting; an unexpectedly high progression rate led to an early chemotherapy termination rate of as high as 40%. The reason for the high PD rate is unknown. In addition, we tried to minimise the disadvantage of continuation of ineffective chemotherapy by defining the ineffectiveness as $\geqslant$10% tumour size increase instead of $\geqslant$20% in the RECIST guideline (Therasse *et al*, 2000). This subtle decision rule might require centralised confirmation. The DFS rate in the D arm was disappointing and was, in fact, very similar to that in the surgery-alone arm in the S9900 study in the United States (Pisters *et al*, 2007).

On the other hand, both the DFS and OS rates of the DP arm were promising. Disease-free survival at 1 year of 78% was fully consistent with the estimation in the study protocol. Although our data do not refute other platinum-based chemotherapy as candidates of preoperative treatment, it would be justified to conclude that DP was active and promising, regardless of disappointing data of D monotherapy. One might argue that DFS at 1 year was too premature as an end point. Because the DFS and OS curves of the DP arm seem to have reached to plateau at 2 years, DFS at 2 years might be a more appropriate end point.

The number of chemotherapy courses of the DP combination was two, whereas many previous studies used three courses. In the North American trials with carboplatin and paclitaxel, three preoperative courses appeared to have no advantage when compared with two courses (Pisters *et al*, 2000; Pisters, 2003). Although patients with 'two preoperative courses' were to have two additional courses after the operation, compliance to the post-surgical courses was very poor anyway (Pisters *et al*, 2000). But, as the majority of the patients appeared fit enough after two courses of DP and a major operation, we could consider the addition of a couple of postoperative chemotherapy cycles at least for responders.

One of the major disadvantages of preoperative therapy is the inaccuracy of the clinical staging, as reported by Depierre *et al* (2002). In our trial, 23% of the 74 patients who underwent thoracotomy were found to have p-N2/N3 disease. In Japan, mediastinoscopy for patients with mediastinal nodes measuring 1 cm or less in size on CT is not performed as a routine clinical practise, and nor was it in our study. Although the introduction of PET may improve the accuracy of the clinical staging, it would still be unlikely to be comparable to surgical staging (Lardinois *et al*, 2003; Cerfolio *et al*, 2004; Shim *et al*, 2005). This would inevitably lead to heterogeneity of the patient population, necessitating a sophisticated study design and large sample size for any future trial on preoperative therapy.

We conclude that the DP combination regimen is active and well tolerated as preoperative chemotherapy, with highly promising survival data. Future clinical trials are warranted based on our results.

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Conflict of interest

Hideo Kunitoh, Masahiro Tsuboi, Yukito Ichinose and Nagahiro Saijo have received honoraria from Sanofi-Aventis.

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Appendix

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Predominant Infiltration of Macrophages and CD8$^+$ T Cells in Cancer Nests Is a Significant Predictor of Survival in Stage IV Nonsmall Cell Lung Cancer

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BACKGROUND. The purpose of this study was to investigate whether tumor-infiltrating immune cells in biopsy specimens can be used to predict the clinical outcome of stage IV nonsmall cell lung cancer (NSCLC) patients.

METHOD. The authors performed an immunohistochemical study to identify and count the number of CD68$^+$ macrophages, c-kit$^+$ mast cells, and CD8$^+$ T cells in both cancer nests and cancer stroma in pretreatment biopsy specimens obtained from 199 patients with stage IV NSCLC treated by chemotherapy, and then analyzed for correlations between the number of immune cells and clinical outcome, including chemotherapy response and prognosis.

RESULTS. There was no correlation between the number of immune cells in either cancer nests or stroma and chemotherapy response. Patients with more tumor-infiltrating macrophages in cancer nests than in cancer stroma (macrophages, nests > stroma) had significantly better survival than nests < stroma cases median survival time (MST 440 days vs 199 days; $P < .0001$). Patients with more tumor-infiltrating CD8$^+$ T cells in cancer nests than in cancer stroma (CD8$^+$ T cells: nests > stroma) showed significantly better survival than in nests < stroma cases (MST 388 days vs 256 days; $P = .0070$). The proportion of tumor-infiltrating macrophages or CD8$^+$ T cells between cancer nests and stroma became independent prognostic factors in the multivariate analysis. Neither the number of mast cells in nests nor in stroma correlated with the clinical outcome.

CONCLUSIONS. Evaluation of the numbers of macrophages and CD8$^+$ T cells in cancer nests and stroma are useful biomarkers for predicting the prognosis of stage IV NSCLC patients treated with chemotherapy, but could fail to predict chemotherapy response. ***Cancer* 2008;113:1387–95.** © *2008 American Cancer Society.*

KEYWORDS: stage IV, nonsmall cell lung cancer, macrophage, CD8$^+$ T cell, mast cell.

Lung cancer is the leading cause of cancer deaths throughout the world, and nonsmall cell lung cancer (NSCLC) accounts for approximately 80% of lung cancer. The prognosis of NSCLC is poor, and patients with advanced NSCLC are candidates for systemic chemotherapy.[1] During the 1990s, 5 new drugs became available for the treatment of metastatic NSCLC: paclitaxel, docetaxel, vinorelbine, gemcitabine, and irinotecan. Each of them has since been evaluated in combination regimens with cisplatin or carboplatin, and the median survival time of patients with metastatic NSCLC treated with such regimens is approximately 8 to 10 months.[2,3] However, some patients with metastatic NSCLC exhibit long-term

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survival, and their tumors progress slowly after chemotherapy, or even in the absence of treatment.[4]

Tumor cells are surrounded by infiltrating inflammatory cells, such as lymphocytes, neutrophils, macrophages, and mast cells. Infiltration of $CD8^+$ T cells has been shown to be an important phenomenon for a specific immune response in several tumor systems, and $CD8^+$ T cells have been reported to play an important suppressive role in cancer progression, including ovarian cancer, esophageal cancer, pancreatic cancer, bile duct cancer, gallbladder cancer, and colorectal cancer.[5-12] Immunologists have long considered the presence of tumor-infiltrating macrophages as evidence of a host response against the growing tumor, and the presence of tumor-infiltrating macrophages has been reported to be associated with anticancer immunomechanisms of the tumor-bearing host and a favorable prognosis. However, recently, tumor-associated macrophage infiltration has been found to be correlated with angiogenesis and an unfavorable prognosis in several kinds of cancer, including gastric cancer, endometrial cancer, and breast cancer.[13-15] Furthermore, it has been reported that mast cells produce many angiogenic factors and a variety of cytokines, including transforming growth factor-beta, tumor necrosis factor-α (TNF-α), interleukin-8 (IL-8), fibroblast growth factor-2, and vascular endothelial growth factor, which are implicated in both normal and tumor-associated neoangiogenesis.[16] In fact, mast cell density has been reported to be highly correlated with the extent of both normal and pathologic angiogenesis, such as the angiogenesis observed in chronic inflammatory diseases and tumors, including gastric cancer and endometrial cancer.[17,18]

Tumor-infiltrating immune cells are thought to play important roles in disease progression and therapeutic efficacy. The effect of chemoradiotherapy has been found to be correlated with the presence of $CD8^+$ T cells in esophageal cancer,[11] and cervical cancer patients with T-cell infiltration showed improved local response to radiation therapy.[19]

In the current study, we evaluated the status of tumor-infiltrating immune cells in tumor biopsy specimens obtained from stage IV NSCLC patient and analyzed the numbers of immune cells and clinical outcome, including chemotherapy response and prognosis, for correlations.

MATERIALS AND METHODS

Patients and Tissue Specimens

The tumor specimens analyzed in this study were obtained from a total of 199 patients with stage IV NSCLC who received platinum-based combination chemotherapy (cisplatin plus paclitaxel, docetaxel, gemcitabine, vinorelbine, or irinotecan, or carboplatin plus paclitaxel), which is considered to be the standard regimen[20,21] at the National Cancer Center Hospital East in Kashiwa, Chiba, Japan, between January 1996 and December 2004, with performance status (PS) 0 or 1 on the Eastern Cooperative Oncology Group scale. Of the 199 patients, 184 had died by the time of the analysis. All patients had adequate tumor biopsy specimens obtainable before chemotherapy and were analyzed in this study. The tumor specimens were obtained by bronchoscopy in 152 patients, and by percutaneous needle biopsy in 47 patients. The histological classification was based on a World Health Organization report. Clinical staging was based on an initial evaluation consisting of a clinical assessment, chest radiography, computed tomography of the chest and abdomen, computed tomography or magnetic resonance imaging of the brain, and bone scintigraphy. The current International Staging System was used to stage clinical disease.[22] All target lesions were evaluated for response by computed tomography or magnetic resonance imaging after completion of the first-line chemotherapy, and all patients underwent tumor biopsy and chemotherapy, after obtaining informed consent in accordance with institutional guidelines.

Immunohistochemistry and Cell Counts

All specimens were fixed in 10% formalin and paraffin embedded. Four-micrometer-thick sections were mounted on silanized slides and deparaffinized with xylene and ethanol. To retrieve the antigen for macrophages, sections were pretreated in 0.05% trypsin and incubated for 20 minutes at 37°C in a humidity chamber. Endogenous peroxidase was blocked with 0.3% H_2O_2 in methanol for 15 minutes. We used mouse antihuman CD68 antibody (Dako, Kyoto, Japan) to detect macrophages, mouse antihuman CD8 antibody (Novocastra, Newcastle, UK) to detect T cells, and mouse antihuman c-kit antibody (Dako) to detect mast cells; immunostaining was performed with Envision (Dako). To retrieve the antigen for CD8 and c-kit, sections were immersed in 10 mM citric buffer solution (pH 6.0) and heated to 95°C by exposure to microwave irradiation for 20 minutes.

We performed an immunohistochemical study to identify and count the number of $CD68^+$ macrophages, $c\text{-}kit^+$ mast cells, and $CD8^+$ T cells and confirmed that cancer cells and mesenchymal cells such as endothelial cells were not immunostained with these antibodies.

Immunostained cells counts were blinded to the patients' clinical data. Macrophages, $CD8^+$ T cells,

and mast cells in the specimen were counted in 2 locations: in the "cancer nests" and in the "cancer stroma." Cancer nests were defined as "cancer nests without fibroblasts and vasculatures" and cancer stroma as "connective tissues surrounding cancer nests without any cancer cells." Every biopsy specimen had both cancer nest and stroma, and it was possible to distinguish these lesions. We counted $CD68^+$ round cells as macrophages, c-kit^+ round cells as mast cells, and $CD8^+$ round cells as T cells. By using a high-power microscopic field (×400; 0.0625 mm^2), we separately counted the number of macrophages, $CD8^+$ T cells, and mast cells in each 2 most intensive areas. Two pathologists (O.K. and G.I) reviewed all slides and counted the cells.

Statistical Analysis

Statistical analysis was performed using the Scientific Package for Social Sciences (SPSS, Chicago, Ill) software. We used median values to calculate category correlations between macrophages, $CD8^+$ T cells, mast cells, and survival rate by the Kaplan-Meier method, and performed univariate analyses by means of log-rank test. The chi-square test was used to test for relationships between categorical variables. Multivariate analysis was performed by means of the Cox proportional hazards model. Student t test was used to test for correlation between macrophage counts, $CD8^+$ T cell counts, mast cell counts and response to first-line chemotherapy. We evaluated test results as significant if the P value was $P < .05$.

RESULTS

Patient Characteristics

The clinicopathological characteristics of all patients are listed in Table 1. Their median age at the time of diagnosis was 62 years (range, 39 years-79 years), and 139 of the 199 patients were men. There were 134 patients with adenocarcinoma, 41 patients with squamous cell carcinoma, and 24 patients with NSCLC that could not be specified by biopsy specimen.

Macrophages, Mast Cells, and $CD8^+$ T Cells, in Cancer Nests and Cancer Stroma

Macrophages were observed in cancer nests (Fig. 2A) in 194 of the 199 tumors, and the mean number was 18.0 ± 2.4 (median, 13; range, 0-76). Macrophages were also observed in cancer stroma (Fig. 2B) in 195 of the 199 tumors, and the mean number was 15.3 ± 1.9 (median, 12; range, 0-105). Mast cells were observed in cancer nests (Fig. 2C) in 149 tumors and in the cancer stroma (Fig. 2D) in 158 tumors, and the mean number was 4.5 ± 0.8 (median, 2; range, 0-52), and 5.4 ± 0.8 (median, 3; range, 0-28), respectively. $CD8^+$ T cells were observed in cancer nests (Fig. 2E) in 197 tumors, and the mean number was 16.9 ± 2.2 (median, 12; range, 0-89). $CD8^+$ T cells were observed in the cancer stroma (Fig. 2F) in 198 tumors, and the mean number was 15.7 ± 1.8 (median, 13; range, 0-88).

TABLE 1
Patient Characteristics and Response to First-Line Chemotherapy

	Patients (N = 199)	
	No.	%
Age		
Median, y (range)	62 (39-79)	
<70 y	158	79.3
≥70 y	41	20.6
Sex		
Women	60	30.1
Men	139	69.8
Histological diagnosis		
Adenocarcinoma	134	67.3
Squamous cell carcinoma	41	20.6
NSCLC	24	12
ECOG performance status		
0	44	22.1
1	155	77.8
Smoking history		
<20 pack years	73	36.6
≥20 pack years	126	63.3
Median survival time, d (range)	317 (19-1969)	
Response to first-line chemotherapy		
PR	53	26.6
SD	95	47.7
PD	51	25.6

NSCLC indicates nonsmall cell lung cancer; ECOG, Eastern Cooperative Oncology Group; PR, partial response; SD, stable disease; PD, progressive disease.

Relationships between the number of infiltrating macrophages, mast cells, $CD8^+$ T cells, and clinicopathological variables

The numbers of infiltrating macrophages, mast cells, and $CD8^+$ T cells were divided into 2 groups at the median value. The relationships between these groups in cancer nests or stroma and the individual clinicopathological characteristics (sex, age, smoking history, PS, histological type) were examined by the chi-square test. More macrophages were present in cancer nests in the nonadenocarcinomas than in the adenocarcinomas (data not shown).

Correlations between the numbers of macrophages, $CD8^+$ T cells, mast cells, and first-line chemotherapy response

We analyzed the number of macrophages, mast cells, and $CD8^+$ T cells in cancer nests and stroma and

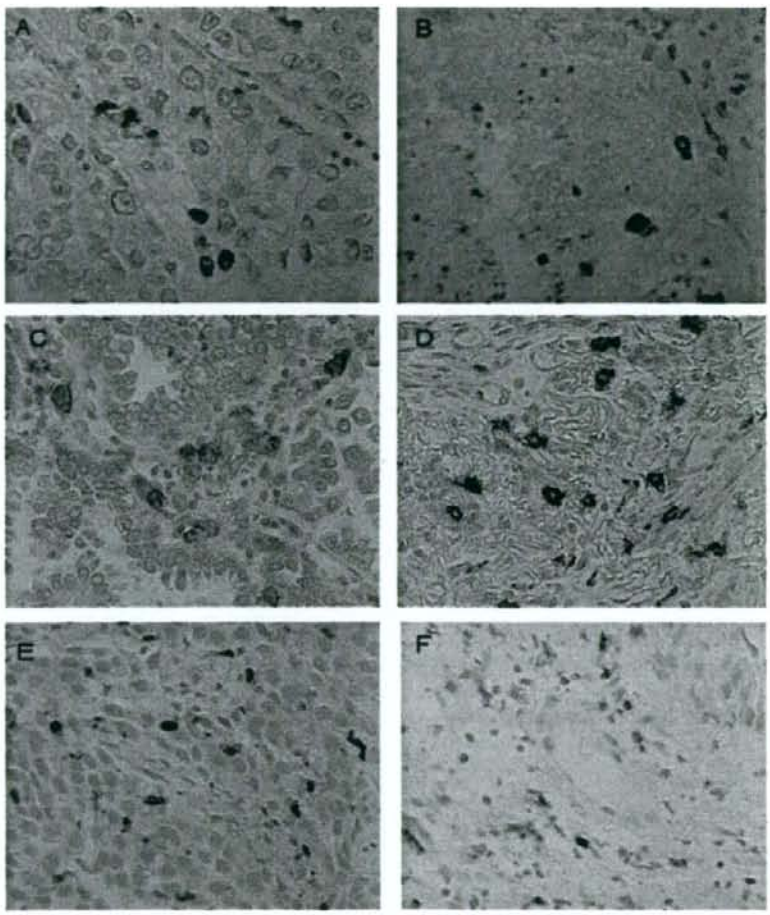

FIGURE 1. Typical photographs are shown of the results of immunostaining for the presence of $CD68^+$ macrophages in (A) cancer nests and in (B) stroma, C-kit^+ mast cells in (C) cancer nests and in (D) stroma, and $CD8^+$ T cells in (E) cancer nests and in (F) stroma.

first-line chemotherapy response for correlations by Student *t* test (Table 2), but the results showed no correlations between numbers of any of the infiltrating immune cells and response to first-line chemotherapy.

Correlations between the numbers of tumor-infiltrating macrophages, mast cells, and $CD8^+$ T cells and patient survival

Kaplan-Meier survival analyses and the log-rank test were performed to compare survival with the number of infiltrating cells (Fig. 2, Table 3). Patients with more macrophages in cancer nests than the median value had the same survival rate as patients with fewer macrophages. By contrast, patients with more macrophages in the cancer stroma had significantly poorer survival than those with fewer macrophages ($P = .0001$). The median survival time was 243 days in the group with higher numbers of macrophages in the stroma, versus 391 days in the group with fewer macrophages in the stroma (1-year survival rate, 33.9% and 55.2%, respectively). Patients were divided into 2 groups, according to the distribution of infiltrating macrophages; a High Nests Macrophage (HNM) group, in which the number of macrophages in the cancer nests was higher than in the cancer stroma (macro-

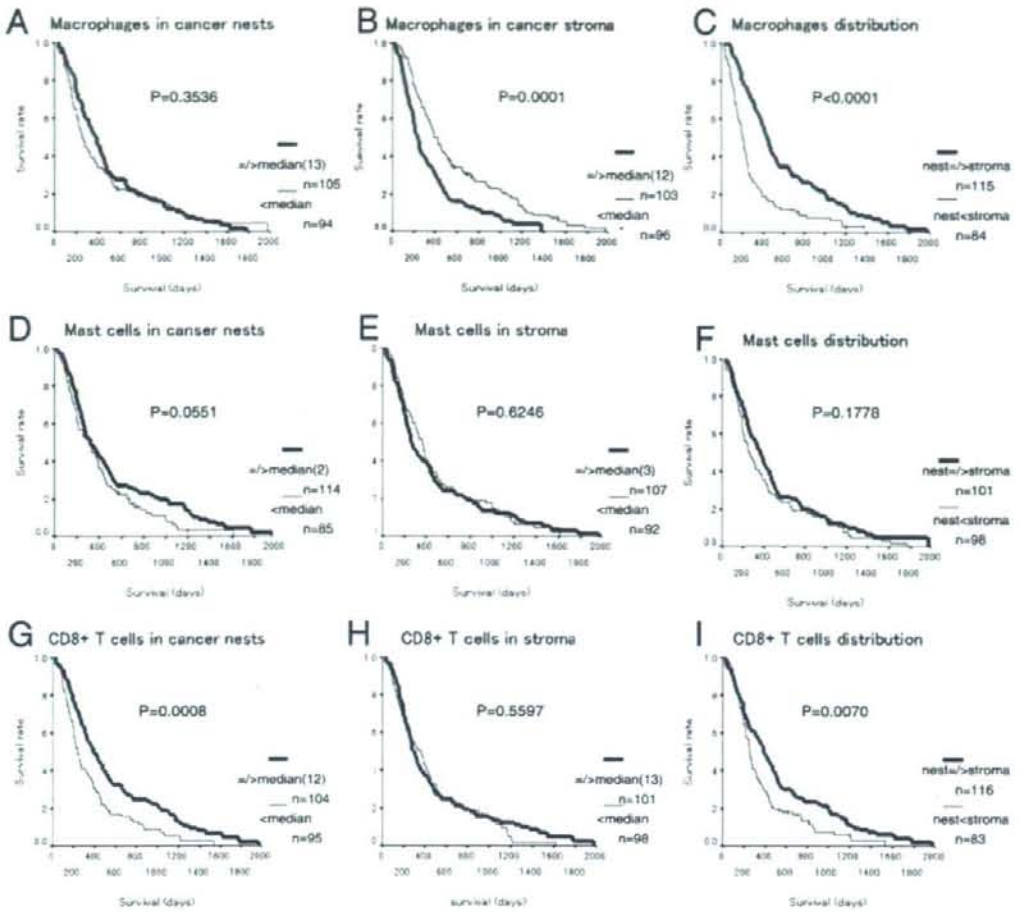

FIGURE 2. Kaplan-Meier analysis of overall survival is shown according to the level of infiltration by macrophages, mast cells, and $CD8^+$ T cells in cancer nests (A), (D), (G) and stroma (B), (E), (H) and their distribution (C), (F), (I). Data were dichotomized at the median value for each parameter (A), (B), (D), (E), (G), (H) and the distribution of infiltrating macrophages, mast cells, and $CD8^+$ T cells (C), (H), (I).

phages, nests > stroma) and a Low Nests Macrophage (LNM) group (nests < stroma). The HNM group had significantly better survival than the LNM group ($P < .0001$) (Fig. 2C). Median survival time was 440 days in the HNM group versus only 199 days in the LNM group, and the 1-year survival rate was 60.8% and 21.4%, respectively. Although mast cells in the cancer nests have been reported to contribute to a favorable outcome,[23] there was no significant relationship with patient survival in this study (Fig. 2D-F). Figure 2G-I shows the relation between the number of $CD8^+$ T cells and patient survival; there was a positive association between survival and the number of $CD8^+$ T cells in cancer nests (Fig. 2G, $P = .0008$). Median survival was 388 days in the group with the higher number of $CD8^+$ T cells in the cancer nests, versus 242 days in the group with the lower number (1-year survival rate, 52.8% and 34.3%, respectively). According to the distribution of infiltrating $CD8^+$ T cells, patients in the High Nests $CD8^+$ T cell (HNT) group, in which the number of tumor-infiltrating $CD8^+$ T cells was higher in the cancer nests than in the cancer stroma (nests > stroma), had significantly better survival than those in the Low Nests $CD8^+$ T cell (LNT) (nests < stroma) group ($P = .0070$) (Fig. 2I). Median survival time was 440 days in the HNT group, versus

TABLE 2
Correlations Between Immune Cells and Response to First-Line Chemotherapy

Parameter	t	95% CI	P*
Macrophages in cancer nests	−0.577	−7.173-3.946	.556
Macrophages in cancer stroma	0.119	−4.094-4.617	.905
Mast cells in cancer nests	−0.413	−2.310-1.512	.680
Mast cells in cancer stroma	1.476	−0.427-2.929	.143
$CD8^+$ T cells in cancer nests	−1.045	−7.114-2.201	.298
$CD8^+$ T cells in cancer stroma	−0.586	−5.162-2.807	.559

CI indicates confidence interval.
* Student *t* test.

only 199 days in the LNT group, and 1-year survival rate was 53.4% and 31.3%, respectively.

We then classified the patients into 4 groups according to macrophage and $CD8^+$ T cell distribution: 1) the HNM and HNT group (macrophages, nests > stroma; $CD8^+$ T cells, nests > stroma), 2) the HNM and LNT group (macrophages, nests > stroma; $CD8^+$ T cells, nests < stroma), 3) the LNM and HNT group (macrophages, nests < stroma; $CD8^+$ T cells, nests > stroma), and 4) the LNM and LNT group (macrophages, nests < stroma; $CD8^+$ T cells, nests < stroma). Median survival time was 495 days in the HNM and HNT group, versus only 196 days in the LNM and LNT group, and the 1-year survival rate was 68.4% and 12.5%, respectively. Median survival time was 342 days, and 1-year survival rate was 45.0% in the HNM and LNT group; median survival time was 221 days, and the 1-year survival rate was 27.2% in the LNM and HNT group. Patients in the HNM and HNT group had significantly better survival than patients in the other groups (Fig. 3, Table 3)

Multivariate Regression Analysis of Survival in NSCLC Patients

As immune cells in cancer nests and cancer stroma would have different biological activity in regard to tumor progression, it would be meaningful to evaluate immune cell distribution. Considering that the distributions of macrophages in cancer nests and cancer stroma may impact clinical outcome, multivariate analysis of macrophage and $CD8^+$ T cell distribution and clinicopathological predictors of survival was performed by means of the Cox proportional hazards model (Table 4), and both macrophage distribution ($P < .001$) and $CD8^+$ T cell distribution ($P = .040$) emerged as independent favorable prognostic indicators. Smoking status also emerged as an independent prognostic indicator ($P = .033$).

TABLE 3
Overall Survival

Groups	No.	Survival, d Median	95% CI	P
Macrophages in cancer nests				.3536
<Median	94	248	192-304	
≥Median	105	376	299-453	
Macrophages in stroma				.0001
<Median	96	391	307-475	
≥Median	103	243	206-280	
Macrophage distribution				<.0001
Nests < stroma	84	199	178-220	
Nests > stroma	115	440	370-505	
Mast cells in cancer nests				.0551
<Median	85	307	201-413	
≥Median	114	317	230-404	
Mast cells in stroma				.6246
<Median	92	366	301-431	
>Median	107	259	200-318	
Mast cell distribution				.1778
Nests < stroma	98	250	188-324	
Nests ≥ stroma	101	370	304-436	
$CD8^+$ T cells in cancer nests				.0008
<Median	95	242	199-285	
≥Median	104	388	290-486	
$CD8^+$ T cells in stroma				.5597
<Median	98	358	237-479	
≥Median	101	297	247-347	
$CD8^+$ T cell distribution				.0070
Nests < stroma	83	256	224-288	
Nests ≥ stroma	116	388	316-460	

CI indicates confidence interval.
*Log-rank test.

DISCUSSION

This is the first study to investigate the relationship between the number of tumor-infiltrating macrophages, mast cells, and $CD8^+$ T cells in tumor biopsy specimens and the clinical outcome of patients with stage IV NSCLC. Patients with higher numbers of tumor-infiltrating macrophages and $CD8^+$ T cells in cancer nests than in cancer stroma had significantly better survival. These factors were also independent prognostic factors in multivariate analysis.

Immunologists have long considered the presence of tumor-infiltrating immune cells as evidence of a host response against the growing tumor. However, recently reports have shown that macrophages and mast cells in cancer stroma secrete several growth factors and proteases involved in angiogenesis, thereby promoting cancer progression. An experimental study has demonstrated that interaction between lung cancer cells and macrophages promotes the invasiveness and matrix-degrading activity of cancer cells,[24] and infiltration by these cells has been reported to be

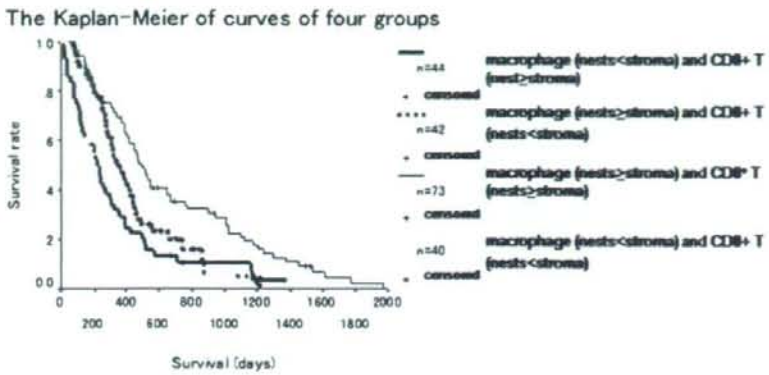

FIGURE 3. Kaplan-Meier analysis of overall survival is shown according to distribution in 4 groups of macrophages and $CD8^+$ T cells. Patients whose tumors contained macrophages in the nest and more $CD8^+$ T cells in the nest had significantly better survival (macrophages, nest > stroma; $CD8^+$ T cells, nest > stroma) than patients with macrophages nest > stroma and $CD8^+$ T cells nest < stroma ($P = .0070$), patients with macrophages nest < stroma and $CD8^+$ T cells nest > stroma ($P = .0010$), and patients with macrophages nest < stroma and $CD8^+$ T cells: nest < stroma ($P < .0001$).

TABLE 4
Multivariate Cox Proportional Hazards Analysis of Overall Survival

Parameter	Hazard Ratio	95% CI	P
Age (<70 y vs ≥70 y)	1.093	0.740-1.613	.655
Sex (men vs women)	1.166	0.772-1.760	.897
PS (0 vs 1)	1.41	0.971-2.049	.071
Smoking (< pack years vs > pack years)	1.561	1.037-2.348	.033
Histology (adeno vs nonadeno)	1.031	0.742-1.432	.856
Macrophage distribution (nests < stroma vs nests > stroma)	0.439	0.320-0.602	<.001
$CD8^+$ T cells distribution (nests < stroma vs nests > stroma)	0.723	0.530-0.985	.040

CI indicates confidence interval; PS, performance status; adeno, adenocarcinoma.

associated with an unfavorable outcome in several kinds of cancers.[25-27] Conversely, macrophages in cancer nests produce cytotoxic cytokines, such as IL-1α, IL-1β, IL-6, and TNF-α, which may protect against tumor progression.[28] Considering the results of this study showing that the distributions of macrophages in cancer nests and cancer stroma impacted outcome of stage IV NSCLC, the macrophages in cancer nests and cancer stroma may have different biological activity in regard to tumor progression. Welsh et al demonstrated that higher numbers of macrophages in cancer stroma and lower numbers of macrophages in cancer nests were unfavorable prognosis factors in surgically resected NSCLC,[23] and their findings are in part consistent with the results of our study. No relationship between the numbers of macrophages in cancer nests and patient survival was found in our study. This can be explained by the difference between the specimens from operable cases of NSCLC (stage I-III) and stage IV cases.

$CD8^+$ T cells with cytotoxic activity play an important role in antitumor immunity. $CD8^+$ T cells can circumvent many of the barriers inherent in cancer-induced stroma, while optimizing T-cell specificity, activation, homing, and antitumor function.[29] The presence of tumor-infiltrating $CD8^+$ T cells has previously been reported to be associated with a favorable outcome, the same as in our own study.[5-12,30] Patients in the HNM and HNT group had significantly better survival (median survival was 495 days; 1-year survival rate was 68.5%) than patients in the HNM group (median survival, 440 days; 1-year survival rate, 60.8%; Fig. 2C) and patients in the HNT group (median survival, 388 days; 1-year survival rate, 53.4%; Fig. 2I). There were also many long-term survivors in the HNM and HNT group, which notably had a 3-year survival rate of 19.1%. Because aggregation of tumor-infiltrating macrophages in cancer nests has been reported to have a beneficial effect by activating cytotoxic T cells,[31] the macrophages and $CD8^+$ T cells in cancer nests should exert synergistic antitumor effects. Infiltration of $CD8^+$ T cells in gastric carcinoma is actually directly correlated with macrophage infiltration, suggesting that macrophages play an important part in the activation of T cells and subsequent tumor cell destruction.[31]

Whether there is any correlation between the presence of tumor-infiltrating mast cells and cancer progression is a matter of controversy. In previous studies, mast cells were found to have antitumor

functions, including serving as natural cytotoxic effectors[32,33] and antitumor compounds,[34] and to be a favorable prognostic factor in surgically resected NSCLC, breast cancer, and colorectal cancer.[35-37] Although mast cells produce histamine, basic fibroblast growth factor, heparin, chymase, and tryptase, which have been shown to promote cancer progression, including in surgically resected NSCLC, gastric cancer, and endometrial cancer,[18,38] no significant relation to survival was found in this study.

Accumulation of immune cells in tumor tissue either before or during chemoimmunotherapy has been reported to be associated with a better clinical response and improved survival.[39-41] The effect of chemoradiotherapy in esophageal cancer is correlated with the number of $CD8^+$ T cells in the tumor of each patient, and the patterns of gene expressions for T cell activation and for tumor vessel formation may become good markers for identifying potential long-term survivors.[11] However, in the present study, no correlations between numbers of macrophages, $CD8^+$ T cells, or mast cells and response to first-line chemotherapy were found (Table 2). The results of our study suggested that patients with a favorable or unfavorable prognosis could be identified by the status of tumor-infiltrating macrophages and $CD8^+$ T cells in tumor biopsy specimens before receiving chemotherapy regardless of chemotherapy response. Cancer patients can mount cellular immune responses against their own tumor cells, and hosts can respond to a large compendium of tumor-associated antigens and epitopes. The natural immune system within the cancer microenvironment may affect its ability to control malignant disease beyond the response to chemotherapy. The only treatment currently available for metastatic NSCLC is chemotherapy, but patients with a poor prognosis, and patients with a predominant distribution of macrophages and $CD8^+$ T cells in the cancer stroma, require some additional therapy to prolong life. For example, elimination of macrophages from the cancer stroma or transfer of $CD8^+$ T cells to cancer nests might be beneficial in prolonging the life of stage IV NSCLC patients in these unfavorable groups.

In conclusion, we found that predominant distribution of macrophages and/or $CD8^+$ T cells in cancer nests as opposed to cancer stroma was correlated with a favorable prognosis in stage IV NSCLC patients. Patients with advanced NSCLC require additional therapy, because the response rate to chemotherapy has been poor (only 30%-40%), and the median survival time of patients with metastatic NSCLC is approximately 8 months to 10 months.[20,21] The results of our study indicate the possibility of using macrophages and $CD8^+$ T cells to treat advanced NSCLC in the future.[42] Decreasing the number of tumor-associated macrophages in the tumor stroma in an animal model of breast cancer effectively altered the tumor microenvironment involved in tumor angiogenesis and progression and markedly suppressed tumor growth and metastasis.[43]

Thus, a more accurate insight into the role of macrophages and $CD8^+$ T cells in tumors and consideration of the local microenvironment in regulating the functions of these cells is needed and has important implications for the design of future clinical trials of adjuvant therapy, as well as for our understanding of the immunopathobiology of stage IV NSCLC.

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ORIGINAL ARTICLE

Impact of *CYP3A4* haplotypes on irinotecan pharmacokinetics in Japanese cancer patients

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Abstract
Background and purpose Cytochrome P450 3A4 (CYP3A4) converts an anticancer prodrug, irinotecan, to inactive metabolites such as APC. However, the contribution of *CYP3A4* genetic polymorphisms to irinotecan pharmacokinetics (PK) and pharmacodynamics (PD) is not fully elucidated. In paclitaxel-administered cancer patients, an association of *CYP3A4*16B* harboring the low activity allele **16* [554C > G (Thr185Ser)] has been shown with altered metabolite/paclitaxel area under the plasma concentration–time curve (AUC) ratios, suggesting a possible impact of **16B* on the PK of other drugs. In this study, the effects of *CYP3A4* haplotypes including **16B* on irinotecan PK/PD were investigated in irinotecan-administered patients.
Methods The *CYP3A4* genotypes for 177 Japanese cancer patients who received irinotecan were defined in terms of

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4 major haplotypes, i.e., **1A* (wild type), **1G* (IVS10 + 12G > A), **16B* [554C > G (Thr185Ser) and IVS10 + 12G > A], and **18B* [878T > C (Leu293Pro) and IVS10 + 12G > A]. Associations of *CYP3A4* genotypes with irinotecan PK and severe toxicities (grade 3 diarrhea and grade 3 or 4 neutropenia) were investigated.

Results Area under the concentration–time curve ratios of APC/irinotecan, an in vivo parameter for CYP3A4 activity, were significantly higher in females than in males. The male patients with **16B* showed significantly decreased AUC ratios (APC/irinotecan) with 50% of the median value of the *non-*16B* male patients (no **16B*-bearing female patients in this study), whereas no significant alteration in the AUC ratios was observed in the patients with **18B*. A slight trend toward increasing AUC ratios (20%) was detected in both male and female patients bearing **1G*. Multivariate analysis confirmed contributions of *CYP3A4*16B* (coefficient ± SE = −0.18 ± 0.077, $P = 0.021$) and **1G* (0.047 ± 0.021, $P = 0.029$) to the AUC ratio. However, no significant association was observed between the *CYP3A4* genotypes and total clearance of irinotecan or toxicities (severe diarrhea and neutropenia).

Conclusion This study suggested that *CYP3A4*16B* was associated with decreased metabolism of irinotecan to APC. However, the clinical impact of *CYP3A4* genotypes on total clearance and irinotecan toxicities was not significant.

Keywords CYP3A4 · Haplotype · Irinotecan · Pharmacogenetics

Introduction

Human cytochrome P450 3A4 (CYP3A4) is a major CYP enzyme, abundant in the liver and intestine, and is involved in the metabolism of endogenous substances, including steroid hormones, and a variety of exogenous compounds such as environmental chemicals and pharmaceuticals. Large inter-individual differences in liver and intestinal CYP3A4 expression levels are known and thought to be caused by multiple factors including genetic variations, disease status, and modulation by exogenous stimuli, such as smoking, diet, and drugs [5, 18, 31]. The tissue-specific CYP3A4 expression is regulated by constitutive and inducible mechanisms via activation of the nuclear receptors, pregnane X receptor (PXR), constitutive androstane receptor (CAR), and vitamin D receptor (VDR) [5, 18]. Since approximately half of clinical drugs currently in use are metabolized by CYP3A4 [5, 33], it is important to find suitable biomarkers, including genetic polymorphisms, which can reflect in vivo CYP3A4 activity and predict individual responses to CYP3A4-metabolized drugs. Recent progress in pharmacogenetic research has led to the accumulation of knowledge about *CYP3A4* genetic variations responsible for altered expression or function. To date, more than 30 *CYP3A4* variations have been identified (http://www.cypalleles.ki.se/cyp3a4.htm), and large ethnic differences in their frequencies have been recognized. *CYP3A4*1B* (−392A > G), a single nucleotide polymorphism (SNP) in the 5′-flanking region, is found in Caucasians (2–9.6%) and African–Americans (35–67%), but not in Asians [16]. As relatively frequent coding SNPs, **2* [664T > C (Ser222Pro)] (2.7%) and **17* [566T > C (Phe189Ser)] (2%) were detected in Caucasians; **10* [520G > C (Asp174His)] in Caucasians (0.24–2%) and Mexicans (5%); **15* [485G > A (Arg162 Gln)] (2–4%) in African–Americans; **16* [554C > G (Thr185Ser)] in East Asians (1.4–5%) and Mexicans (5%); **18* [878T > C (Leu293Pro)] (2.3–10%) in East Asians [2, 4, 17, 24]. We previously identified 25 *CYP3A4* haplotypes in a Japanese population [4]. The haplotypes **6* [including 830_831insA (Glu277fsX8)] (0.1%), **11* [including 1088C > T (Thr363Met)] (0.2%), **16B* [including 554C > G (Thr185Ser)] (1.4%), and **18B* [including 878T > C (Leu293Pro)] (2.8%) were identified, but **1B* (−392A > G) was not found. These findings indicate that ethnic-specific *CYP3A4* haplotypes must be taken into consideration in pharmacogenetic studies.

Irinotecan, an anticancer prodrug, is used for treatment of various cancers including lung and colon, and metabolized by CYP3A4 to produce inactive compounds such as APC (a major CYP3A4-mediated product) and NPC (a minor product) [6, 7]. An active metabolite SN-38 (a topoisomerase I inhibitor) is produced from the parent compound by carboxylesterases (CES) [28] and subsequently glucuronidated by UDP-glucuronosyltransferase 1As (UGT1As) to form inactive compound SN-38G [12] (Fig. 1). The parent compound and its metabolites are mainly excreted into the bile [29], where several ABC transporters, such as P-glycoprotein (P-gp), breast cancer resistance protein (BCRP), and multidrug resistance-associated protein 2 (MRP2) are involved in excretion [30]. The dose-limiting toxicities of irinotecan are severe diarrhea and neutropenia, and high plasma concentrations of SN-38 and/or its accumulation in tissues are thought to cause these toxicities [3, 30]. Recent extensive pharmacogenetic studies on irinotecan, mostly focusing on the *UGT1A1* genotypes, have revealed important roles for *UGT1A1*28* and **6* in reduced in vivo UGT activity and enhanced toxicities [1, 8, 9, 11, 13, 22, 26]. On the other hand, CYP3A4 can modulate irinotecan pharmacokinetics (PK). Co-administration of ketoconazole, a CYP3A4 inhibitor and also a potent UGT1A1 inhibitor [34], with irinotecan resulted in a decreased value of the area under the concentration–time curve (AUC) for APC and also increased AUC for SN-38 [14]; and vice versa, co-administration of St. John's Wort,

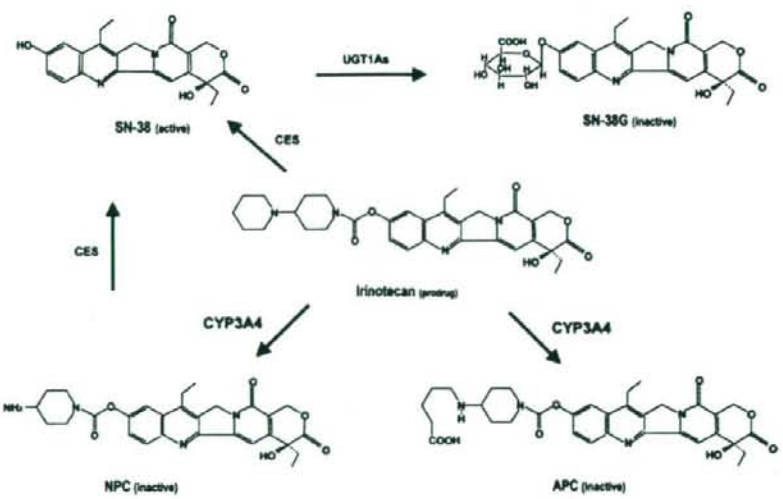

Fig. 1 Irintoecan metabolism in human liver. CYP3A4 mediates oxidation of irinotecan to produce inactive compounds, such as APC (a major CYP3A4-mediated product) and NPC (a minor product)

a CYP3A4 inducer, decreased the AUC of SN-38 [19]. A close association was also reported between in vivo CYP3A4 phenotypes and irinotecan clearance [21]. To date, however, no clinical impact by *CYP3A4* polymorphisms, such as **1B* (−392A > G) and **3* [1334T > C (Met445Thr)], has been demonstrated on irinotecan PK in Caucasians [20]. We previously found that **16* [554C > G (Thr185Ser)] caused decreased in vitro CYP3A4 activities [23]. Furthermore, a significant association of **16B* [harboring 554C > G (Thr185Ser)] was demonstrated with decreased AUC ratios of metabolite/paclitaxel, an in vivo parameter of CYP3A4 activity, in paclitaxel-administered Japanese patients [24].

In this study, to determine the clinical impact of the *CYP3A4* polymorphisms on irinotecan therapy, we identified the *CYP3A4* diplotypes of 177 Japanese cancer patients who received irinotecan and analyzed associations of the *CYP3A4* genotypes with irinotecan PK and toxicities.

Materials and methods

Patients and irinotecan treatment

One hundred seventy-seven patients with cancers who started irinotecan-containing therapy from 2002 to 2004 at two National Cancer Center Hospitals (Tokyo and Kashiwa, Japan) were enrolled for this pharmacogenetic study on irinotecan. This study was approved by the ethics committees of the National Cancer Center and the National Institute of Health Sciences, and written informed consent was obtained from all participants. No participant received irinotecan previously, and other eligibility criteria included: bilirubin < 2 mg/dl, aspartate aminotransferase (GOT) < 105 IU/l, alanine aminotransferase (GPT) < 120 IU/l, creatinine < 1.5 mg/dl, white blood cell count > 3000/μl, performance status of 0–2, and an interval of at least 4 weeks after the last session of chemotherapy (2 weeks after the last session of radiotherapy). Exclusion criteria were diarrhea, active infection, intestinal paralysis or obstruction, and interstitial pneumonitis. Irinotecan was administered as a single agent or in combination chemotherapy at the discretion of attending physicians. Doses and schedules were applied according to the approved treatment recommendations in Japan: intravenous 90-min infusion at a dose of 100 mg/m^2 weekly or 150 mg/m^2 biweekly for irinotecan-monotherapy, and 60 mg/m^2 weekly for combination therapy with cisplatin. Profiles of the patients and irinotecan regimens are summarized in Table 1.

Genotyping of *UGT1A1* and *CYP3A4*

DNA was extracted from pretreatment whole-blood samples taken from 177 patients who received irinotecan. Data on *UGT1A1* genetic polymorphisms obtained from the same set of DNA samples have been published elsewhere [22]. The *CYP3A4* genotypes for 88 patients were previously determined [4]. Additional *CYP3A4* genotyping for the remaining 89 patients was conducted using the pyrosequencing method described previously [24], and the *CYP3A4* diplotypes/haplotypes [4] were inferred using an expectation-maximization-based program, LDSUPPORT [15].

Pharmacokinetics and toxicities

Pharmacokinetic analysis for irinotecan in 176 patients (data on one patient was unavailable) was performed as

Table 1 Profiles of Japanese cancer patients in this study

			No. of patients
Patients for genotyping			177
(Male/female)			(135/42)
Age			
Mean/range	60.5/26–78		
Performance status	0/1/2		84/89/4
Combination therapy, tumor type and initial dose of irinotecan[a]			
Irinotecan monotherapy	Lung	100 (60–100)/w	21
	Colon	150 (120–150)/2w	28
	Others	100 (100–150)/w	7
With platinum-containing drug[b]	Lung	60 (50–90)/w	58
	Stomach	70/2w	9
	Others	60/w	5
With 5-fluorouracil (5-FU)/leucovorin (LV)[c] or tegafur/gimeracil/oteracil potassium[d]	Colon	100 (90–180)/w or 150/2w	34
	Others	90/w or 100/w	2
With mitomycin C (MMC)[e]	Stomach	150/2w	10
	Colon	150/2w	1
With amrubicin[f]	Lung	60/w	2

[a] The median value and range in the parentheses are shown. "/w" and "/2w" represent weekly and biweekly, respectively

[b] Mostly, cisplatin (60 or 80 mg/m^2) was administered after irinotecan treatment

[c] LV (10 mg/m^2) was administered right after irinotecan treatment and then followed by 5-FU treatment. (500 mg/m^2 injection); or LV (200 mg/m^2) was administered simultaneously with irinotecan and followed by 5-FU treatment (400 mg/m^2 bolus injection and 2.0–2.4 g/m^2 infusion)

[d] Tegafur (80 mg/m^2 per day)/gimeracil/oteracil potassium was administered twice (before irinotecan treatment and on the next day)

[e] MMC (5 mg/m^2) was administered just before irinotecan treatment

[f] Amrubicin (30 or 35 mg/m^2) was administered 24 h after irinotecan treatment

previously described [26]. Briefly, heparinized blood was collected before administration of irinotecan, and 0, 0.3, 1, 2, 4, 8, and 24 h after termination of the first infusion of irinotecan. Plasma concentrations of irinotecan and APC were determined by HPLC [25], and AUC_{inf} and other PK parameters were calculated using the trapezoidal method of the 202 non-compartmental model for a constant infusion in WinNonlin ver. 4.01 (Pharsight Corporation, Mountain View, CA, USA). As for the co-administered anti-cancer and other drugs which were administered within 1 week before irinotecan-treatment, no drugs significantly affected the PK parameters related to CYP3A4 activity. Information on foods and drinks taken by the patients which might induce or inhibit CYP3A4 activity was not available.

A complete medical history and data on physical examinations were recorded prior to irinotecan therapy. Complete blood cell counts with differentials and platelet counts, as well as blood chemistry, were measured once a week during the first 2 months of irinotecan treatment. Toxicities were graded according to the Common Toxicity Criteria of National Cancer Institute version 2. Association of genetic factors with irinotecan toxicities was analyzed primarily in patients who received irinotecan as a single agent.

Statistical analysis

Statistical analysis on the differences in PK parameters between sexes and among *CYP3A4* genotypes was performed using the Mann–Whitney test or Kruskal–Wallis test, and associations of *CYP3A4* genotypes with the irinotecan toxicities were assessed by the Chi-square test, using Prism version 4.0 (GraphPad Prism Software Inc. San Diego, CA, USA). $P = 0.05$ (two-tailed) was set as a significant level of difference. Multivariate analysis for the log-transformed AUC ratio (APC/irinotecan) was performed using age, sex, body surface area, dosage of irinotecan, history of smoking or drinking, performance status, co-administered drugs, serum biochemistry parameters at baseline, and genetic factors (including *CYP3A4* haplotypes and the *UGT1A1*6 or *28* haplotype obtained in our previous study [22]) as independent variables. Multivariate analysis on toxicities (grade 3 diarrhea or nadir of absolute neutrophil counts) was conducted for the patients who received irinotecan monotherapy, where the variables included dosing interval and the absolute neutrophil count at baseline, in addition to the other patient background and genetic factors described above. The variables in the final

models for both AUC ratio and toxicities were chosen by the forward and backward stepwise procedure at the significance level of 0.1 using JMP version 6.0.0 software (SAS Institute, Inc., Cary, NC, USA).

Results

Sex difference in PK parameters

Since hepatic CYP3A4 levels were reported to be significantly higher in females than in males [24, 32], we first analyzed the sex differences in the major PK parameters for irinotecan and APC, a major CYP3A4 metabolite (Table 2). As for irinotecan, lower total clearance and MRT, and higher AUC/dose were observed in females, but the differences (3, 5 and 3%, respectively) were not significant. A small but significant increase in C_{max}/dose for irinotecan was observed in females. This is attributable to the smaller distribution volume of females. On the other hand, the median values of AUC/dose and C_{max}/dose for APC of the females were significantly higher than those of the males (1.29- and 1.33-fold, respectively). The AUC ratio (APC/irinotecan), a parameter of in vivo CYP3A4 activity, was significantly higher (1.28-fold) in females than in males. These findings suggest that these differences may reflect the higher CYP3A4 activity in the females.

CYP3A4 genotypes

CYP3A4 diplotypes/haplotypes in 177 Japanese cancer patients were determined according to the previous definition [4]. The *CYP3A4* haplotypes found in this population were **1A* (wild type), **1G* (IVS10 + 12G > A alone), **16B* [554C > G (Thr185Ser) and IVS10 + 12G > A], and **18B* [878T > C (Leu293Pro) and IVS10 + 12G > A]. In the current study, neither **6* [830_831insA (Glu277fsX8)] nor **11* [1088C > T (Thr363Met)] were found. The frequencies of **1G*, **16B*, and **18B* were 0.215, 0.014, and 0.020 (Table 3), and they were comparable to those obtained in previous reports [4, 24]. Note that the haplotypes **16B* and **18B* were detected only in male patients.

Associations of *CYP3A4* genotypes with PK parameters

Considering the significant sex difference in APC levels, associations between the *CYP3A4* genotypes and PK parameters were analyzed for each sex separately. In male patients, no significant differences among the *CYP3A4* genotypes were observed for total clearance and MRT of irinotecan (Fig. 2a, b). In females, a slightly but significantly lower (10%) median value for MRT of irinotecan was observed in patients bearing **1G* compared with those carrying the wild type (**1A*/**1A*) ($P = 0.022$, Mann–Whitney test) (Fig. 2b), whereas no significant **1G*-dependency was observed for total clearance (Fig. 2a). No significant

Table 3 Frequencies of *CYP3A4* haplotypes (A) and diplotypes (B) for Japanese cancer patients in this study

(A) Haplotype group[a]	No. of chromosomes ($N = 354$)	Frequency
**1A*	266	0.751
**1G*	76	0.215
**16B*	5	0.014
**18B*	7	0.020
(B) Diplotype	No. of patients ($N = 177$)	Frequency
1A*/1A*	100	0.565
1G*/1A*	55	0.311
1G*/1G*	10	0.056
16B*/1A*	4	0.023
16B*/1G*	1	0.006
18B*/1A*	7	0.040

[a] Groups based on tagging SNPs of major haplotypes previously defined [4]; **1A* wild type, **1G* IVS10 + 12G > A; **16B* 554C > G (Thr185Ser) and IVS10 + 12G > A; **18B* 878T > C (Leu293Pro) and IVS10 + 12G > A

Table 2 Pharmacokinetic parameters for irinotecan-administered Japanese patients and sex differences

Parameters	Male ($N = 134$) Median (25–75%ile)	Female ($N = 42$) Median (25–75%ile)	P value[a]
Irinotecan			
Total CL (l/h per m^2)	22.6 (18.5–26.9)	21.8 (17.8–25.1)	0.242
AUC/dose (10^{-3} h m^2 per l)	44.4 (37.3–54.1)	45.8 (39.8–55.8)	0.242
C_{max}/dose (10^{-3} m^2 per l)	10.0 (8.96–11.3)	11.4 (10.4–12.4)	0.0003
MRT (h)	6.61 (6.01–7.40)	6.29 (5.78–7.12)	0.202
APC			
AUC/dose (10 h m^2 per l)	6.72 (5.23–9.49)	8.66 (6.57–13.1)	0.0071
C_{max}/dose (10^{-3} m^2 per l)	0.560 (0.430–0.805)	0.745 (0.610–1.14)	0.0007
AUC ratio (APC/irinotecan)	0.151 (0.114–0.210)	0.194 (0.132–0.266)	0.0179

CL clearance; *MRT* mean residence time

[a] Mann–Whitney test