Table 1 Patient characteristics

Arm	A (drainage alone)	B (ipc BLM)
N	42	38
Gender		
Male	27	24
Female	15	14
Median age (range)	60.5 (39–75)	60 (42–73)
Histology		
Small cell	3	2
Non-small cell	39	36
Prior chemotherapy		
Yes	29	24
No	13	14
Prior thoracic radiotherapy		
Yes	11	9
No	31	29
Drainage methods		
Surgical	19	17
Others	23	21
Median drainage volume in ml (range)	550 (250–1750)	600 (130–1930)
Effusion cytology		
Negative	6	11
Indeterminate	1	0
Positive	33	25
Not examined	2	2

ipc BLM = intrapericardial bleomycin instillation.

cytology-positive effusions in arm A. Cytology of the effusion was positive in 58 cases out of the 76 examined (76%).

In arm B, all 38 patients received at least one ipc BLM instillation and a total of 74 administrations: seven patients received four administrations (total BLM dose: 45 mg), five received three administrations (total BLM: 35 mg), five received two administrations (total BLM: 25 mg) and the remaining 21 received a single administration (total BLM: 15 mg). There was no apparent relationship between total dose and efficacy end points such as EFFS, except that those required four administrations had a worse primary control of the MPE.

A total of 24 patients (14 in arm A and 10 in arm B) received systemic chemotherapy after drainage tube removal. Nine patients (five in arm A and four in arm B) received gefitinib. Cytotoxic chemotherapy was administered to 21 patients (11 in arm A and 10 in arm B).

Morbidity and early deaths

Table 2 summarises the morbidity of the protocol therapy. Although 30 (38%) of the patients experienced some pain, no significant difference in the incidence and severity of pain was observed between the arms. Bleeding and infections were rare and generally controllable. Two patients in arm B developed transient fever of moderate degree (38–38.7°C). One case with constrictive pericarditis at 4 months and another with late cardiac dysfunction at 12 months after the registry, both reported to be grade 2, were observed in arm B.

As anticipated, there were as many as nine early deaths within 30 days of randomisation; five in arm A and four in arm B. Although the death was ascribed to disease progression in the majority, two patients in arm A died of massive bleeding during surgical attempts at re-drainage for recurrent MPE, possibly due to crack formation in the ventricular wall upon dissection of the adherent pericardium. Another patient in arm B died suddenly on day 12 of the protocol without a clear cause.

Table 2 Morbidity of the protocol therapy

Arm	A (drainage alone)	B (ipc BLM)
N	42	38
Pain		
None	25	25
Medication not required	4	4
Controlled with non-opioid analgesics	9	7
Controlled with opioid analgesics	4	2
Uncontrollable	0	0
Infection		
None	39	35
Controllable	3	3
Uncontrollable	0	0
Bleeding		
None	42	36
Controllable	0	1
Severe	0	1
Late complications		
None	42	36
Pulmonary	0	0
Cardiac function	0	1 (grade 2)
Constrictive pericarditis	0	1 (grade 2)

ipc BLM = intrapericardial bleomycin instillation.

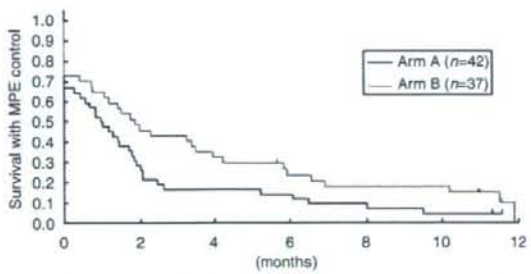

Figure 1 Effusion failure-free survival (EFFS). The median EFFS was 30 days in arm A and 57 days in arm B, with a hazard ratio of 0.64 (95% confidence interval: 0.40–1.03), with arm B significantly favouring this parameter (one-sided $P = 0.030$ by log-rank test).

Efficacy end points

Primary control of the MPE with successful tube removal within 7 days of randomisation was achieved in 28 of the 42 cases (67%) in arm A and 27 of the 37 eligible cases (73%) in arm B, the difference between the two groups not being statistically significant. The median time to tube removal was 7 days in each arm. Arm B favoured EFFS (Figure 1), with a hazard ratio of 0.64 (95% confidence interval: 0.40–1.03, and one-sided $P = 0.030$ by log-rank test).

The EFFS at 1, 2, 4, 6 and 12 months was 50, 29, 17, 14 and 5%, respectively, for arm A, and 65, 46, 32, 24 and 10%, respectively, for arm B. Although arm B also favoured the primary end point, EFFS at 2 months (46 *vs* 29%), the difference between the two

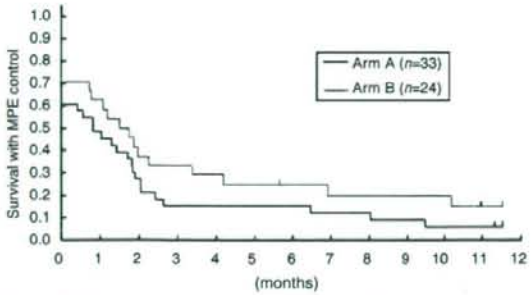

Figure 2 Effusion failure-free survival (EFFS) in effusion cytology-positive patients. In the effusion cytology-positive patient subset, arm B favoured EFFS. The hazard ratio was 0.69 (95% confidence interval: 0.39–1.21).

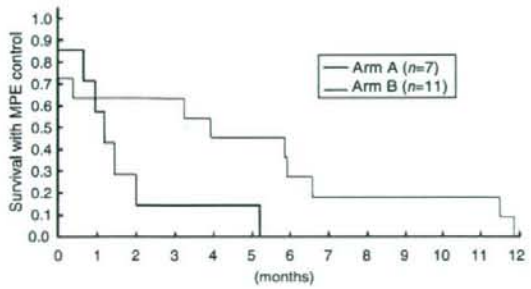

Figure 3 Effusion failure-free survival (EFFS) in effusion cytology-negative or -indeterminate patients. In the effusion cytology-negative or -indeterminate patient subset, arm B favoured EFFS. The hazard ratio was 0.39 (95% confidence interval: 0.12–1.21).

groups was not statistically significant (one-sided $P=0.086$ by Fisher's exact test).

The median OS was not significantly different between the two arms: 79 days in arm A and 119 days in arm B. The OS rates at 6 months were 27 and 31% in arm A and arm B, respectively.

Subgroup analysis

As more patients in arm A had cytology-positive effusion, which has been reported to be associated with a poor prognosis (Gornik *et al*, 2005), subset analysis was performed according to the effusion cytology status (Figures 2 and 3). In both cytology-positive patients (Figure 2) and cytology-negative or -indeterminate patients (Figure 3), arm B favoured EFFS.

Thirty-six patients had undergone surgical (subxiphoid pericardiostomy) and 43 had undergone non-surgical (percutaneous tube pericardiostomy) drainage before randomisation. Patients with surgical drainage tended to have a longer EFFS (Figure 4). The effect of ipc BLM was observed irrespective of the drainage method employed; arm B tended to favour EFFS both in patients with surgical drainage (hazard ratio 0.62, 95% confidence interval: 0.30–1.29) and in those with non-surgical drainage (hazard ratio 0.56, 95% confidence interval: 0.29–1.05).

Symptom palliation

The baseline symptom scores were taken for all of the 79 eligible patients, at enrolment (after drainage). At the 1-month follow-up,

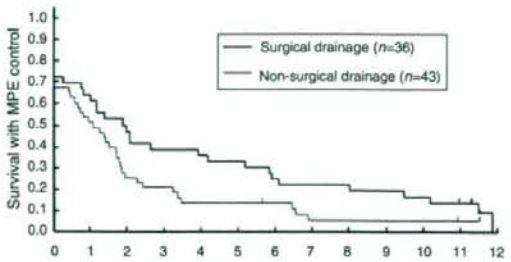

Figure 4 Effusion failure-free survival (EFFS) and drainage method. Patients with surgical drainage tended to have longer EFFS (median EFFS: 2.0 *vs* 1.1 month).

Table 3 Symptom palliation

Arm	A (drainage alone)	B (ipc BLM)
N eligible	42	37
% of those with improved or stable scores[a]		
Cough	60%	57%
Pain	50%	62%
Anorexia	55%	62%
Dyspnoea	62%	46%
Total	55%	51%

ipc BLM = intrapericardial bleomycin instillation. [a]The scores at 1 month were compared with those at enrolment.

approximately half of the patients (55% in arm A and 51% in arm B) had stable or improved overall scores. There were no significant differences between the arms for any of the symptom scores (Table 3).

DISCUSSION

Malignant pericardial effusion is a potentially life-threatening complication of malignancy that usually manifests itself at an advanced or terminal stage of the disease. It brings great agony to the patient once it becomes symptomatic, with dyspnoea, orthopnoea, chest pain and cough. Although the prognosis of the patients with MPE is very poor, especially in those with chemotherapy-resistant tumours such as non-small-cell lung cancer (Press and Livingston, 1987; Okamoto *et al*, 1993; Gornik *et al*, 2005; Yonemori *et al*, 2007), optimal management is very important for palliation.

Pericardial sclerosis following drainage has been widely performed. However, data are available mainly from phase II trials or case series. In fact, historical comparison has failed to demonstrate the efficacy of pericardial sclerosis over drainage alone (Okamoto *et al*, 1993; Vaitkus *et al*, 1994). It has also been suggested that sclerosis may be effective in preventing re-accumulation of MPE after percutaneous tube pericardiostomy, but not after subxiphoid pericardiotomy, because the surgical intervention alone was considered to be sufficient to prevent recurrent MPE (Press and Livingston, 1987; Park *et al*, 1991; McDonald *et al*, 2003).

In addition, there are some potential morbidities associated with pericardial sclerosis; most of the agents used as sclerosants produce unpleasant adverse effects, such as fever and pain (Liu *et al*, 1996). There is also concern about the complications of the procedure, both in the short term, such as bleeding and infection,

and in the long term, such as constrictive pericarditis, as the inflammatory response causes adhesion of the visceral and parietal pericardium (Shepherd, 1997).

We undertook a randomised trial to evaluate the efficacy of pericardial sclerosis following drainage as compared with drainage alone. We chose BLM as the sclerosant agent for ipc instillation, because of its low toxicity as compared with doxycycline, reported from an earlier randomised trial (Liu *et al*, 1996). We included only patients with non-small-cell lung cancer or chemotherapy-treated small cell cancer to minimise the influence of systemic chemotherapy after the protocol study (Vaitkus *et al*, 1994). We randomised the patients after the pericardial drainage, as we judged that obtaining informed consent before it, that is when the patients suffer from symptoms of MPE, would be very difficult. Therefore, we did not specify the indication for drainage and enrolled cases after both emergent and elective drainage. We thus focused on the prevention of MPE recurrence. We could not find any comparable phase III trial on this participant, and no such trial is registered in ClinicalTrial gov.

We found that ipc BLM instillation seemed to be effective at preventing the recurrence of MPE. However, the benefit in the primary end point, that is, EFFS at 2 months, was not significantly different, which is a major drawback to make a definitive conclusion. The therapeutic benefit, which could not be demonstrated with our modestly sample-sized trial, therefore, might be only a modest one. On the other hand, the benefit of ipc BLM seemed to be unrelated to the drainage method. As expected, the OS was poor in both arms and not significantly different.

Our study has several limitations. One is that without significant survival prolongation and difference of symptom scores, modest improvement of the EFFS might not represent true patient benefit. We believe, however, that conductance of our trial itself would be fully justified; given the severe symptoms of uncontrolled MPE and the inconvenience of the drainage tube, survival without MPE would be a worthwhile treatment goal.

The second limitation was that we limited the participants to lung cancer patients, which makes it difficult to evaluate late complications due to short OS. In patients with more chemotherapy-sensitive tumours such as breast cancer or lymphoma, many more patients may be expected to live for up to at least 1 year longer. There would be greater concern about late pericardial or cardiac complications, which we did observe in two of our own cases. Even for lung cancer patients, advances in systemic therapy may be expected to improve the outcome of those with even far-advanced disease in the future, which would evidently modify the risk/benefit of ipc BLM.

The third limitation of our study was that we did not control for the method of primary pericardial drainage, and each institution chose it in accordance with its daily practice. We do not believe that our results were much biased by the drainage methods, as each participating institution basically adhered to one method of its choice, and the ipc BLM arm tended to favour EFFS in both subgroups with surgical and non-surgical drainage. However, control for the drainage method or indication (emergent *vs* elective) for drainage might be necessary in future trials, as they might well affect the patient outcomes. In fact, we did observe that, although not a randomised comparison and thus it should be interpreted with caution, patients who underwent surgical drainage tended to have a better MPE control.

Recently, less invasive techniques for surgical treatment of MPE have been described, such as percutaneous balloon pericardiotomy (Ziskind *et al*, 1993; Wang *et al*, 2002), which create a pleuro-pericardial communication and allow fluid drainage into pleural space. It was reported to be effective and safe, and may potentially obviate the need for surgical intervention. However, it has yet to be compared with other drainage methods and its role has not been established. No patient underwent this procedure in our study.

One ancillary finding of our study was that two patients died of major bleeding during surgical attempts at re-drainage for recurrent MPE. Although it has rarely been reported in the literature, partial adhesions could have led to injury to the cardiac wall during the surgical procedure.

In this trial, we evaluated the safety and efficacy of pericardial sclerosis with a 'classic' sclerosant agent of BLM. Future trial designs would include one to compare BLM with another agent with a different mode of action, such as intrapericardial instillation of a platinum compound as 'local chemotherapy'.

In conclusion, we found that pericardial sclerosis with ipc BLM after drainage appears to be safe and effective, overall, in the management of MPE in patients with lung cancer and should be a valid therapeutic option in these patients. We could not, however, demonstrate a statistical significance in the primary end point with the modest sample size of 80. The therapeutic advantage might not be large enough, and more trials are warranted.

ACKNOWLEDGEMENTS

We thank the members of the JCOG data center and operations office for their support in preparing the paper and oversight of the study management (Drs Isamu Saito and Haruhiko Fukuda), statistical analysis (Mr Takashi Asakawa and Dr Naoki Ishizuka) and data management (Mr Hidenobu Yamada).

Conflict of interest

The authors have no conflicts of interest to declare.

Registered in www.clinicaltrials.gov, ClinicalTrials.gov number, NCT00132613 and in UMIN-CTR[www.umin.ac.jp/ctr/], identification number, C000000030.

REFERENCES

Abraham KP, Reddy V, Gattuso P (1990) Neoplasms metastatic to the heart: review of 3314 consecutive autopsies. *Am J Cardiovasc Pathol* **3:** 195–198

Colleoni M, Martinelli G, Beretta F, Marone C, Gallino A, Fontana M, Graffeo R, Zampino G, De Pas T, Cipolla G, Martinoni C, Goldhirsch A (1998) Intracavitary chemotherapy with thiotepa in malignant pericardial effusions: an active and well-tolerated regimen. *J Clin Oncol* **16:** 2371–2376

Dempke W, Firusian N (1999) Treatment of malignant pericardial effusion with 32P-colloid. *Br J Cancer* **80:** 1955–1957

Gornik HL, Gerhard-Herman M, Beckman JA (2005) Abnormal cytology predicts poor prognosis in cancer patients with pericardial effusion. *J Clin Oncol* **23:** 5211–5216

Imamura T, Tamura K, Takenaga M, Nagatomo Y, Ishikawa T, Nakagawa S (1991) Intrapericardial OK-432 instillation for the management of malignant pericardial effusion. *Cancer* **68:** 259–263

Kaira K, Takise A, Kobayashi G, Utsugi M, Horie T, Mori T, Imai H, Inazawa M, Mori M (2005) Management of malignant pericardial effusion with instillation of mitomycin C in non-small cell lung cancer. *Jpn J Clin Oncol* **35:** 57–60

Kawashima O, Kurihara T, Kamiyoshihara M, Sakata S, Ishikawa S, Morishita Y (1999) Management of malignant pericardial effusion resulting from recurrent cancer with local instillation of aclarubicin hydrochloride. *Am J Clin Oncol* **22:** 396–398

Klatt EC, Heitz DR (1990) Cardiac metastases. *Cancer* **65:** 1456–1459

Lerner-Tung MB, Chang AY, Ong LS, Kreiser D (1997) Pharmacokinetics of intrapericardial administration of 5-fluorouracil. *Cancer Chemother Pharmacol* **40:** 318–320

Liu G, Crump M, Goss PE, Dancey J, Shepherd FA (1996) Prospective comparison of the sclerosing agents doxycycline and bleomycin for the primary management of malignant pericardial effusion and cardiac tamponade. *J Clin Oncol* **14:** 3141–3147

Maher EA, Shepherd FA, Todd TJ (1996) Pericardial sclerosis as the primary management of malignant pericardial effusion and cardiac tamponade. *J Thorac Cardiovasc Surg* **112:** 637–643

Martinoni A, Cipolla CM, Cardinale D, Civelli M, Lamantia G, Colleoni M, Fiorentini C (2004) Long-term results of intrapericardial chemotherapeutic treatment of malignant pericardial effusions with thiotepa. *Chest* **126:** 1412–1416

Maruyama R, Yokoyama H, Seto T, Nagashima S, Kashiwabara K, Araki J, Semba H, Ichinose Y (2007) Catheter drainage followed by the instillation of bleomycin to manage malignant pericardial effusion in non-small cell lung cancer: a multi-institutional phase II trial. *J Thorac Oncol* **2:** 65–68

McDonald JM, Meyers BF, Guthrie TJ, Battafarano RJ, Cooper JD, Patterson GA (2003) Comparison of open subxiphoid pericardial drainage with percutaneous catheter drainage for symptomatic pericardial effusion. *Ann Thorac Surg* **76:** 811–815; discussion 816

Moriya T, Takiguchi Y, Tabeta H, Watanabe R, Kimura H, Nagao K, Kuriyama T (2000) Controlling malignant pericardial effusion by intrapericardial carboplatin administration in patients with primary non-small-cell lung cancer. *Br J Cancer* **83:** 858–862

Norum J, Lunde P, Aasebo U, Himmelmann A (1998) Mitoxantrone in malignant pericardial effusion. *J Chemother* **10:** 399–404

Okamoto H, Shinkai T, Yamakido M, Saijo N (1993) Cardiac tamponade caused by primary lung cancer and the management of pericardial effusion. *Cancer* **71:** 93–98

Park JS, Rentschler R, Wilbur D (1991) Surgical management of pericardial effusion in patients with malignancies. Comparison of subxiphoid window *vs* pericardiectomy. *Cancer* **67:** 76–80

Press OW, Livingston R (1987) Management of malignant pericardial effusion and tamponade. *JAMA* **257:** 1088–1092

Primrose WR, Clee MD, Johnston RN (1983) Malignant pericardial effusion managed with Vinblastine. *Clin Oncol* **9:** 67–70

Shepherd FA (1997) Malignant pericardial effusion. *Curr Opin Oncol* **9:** 170–174

Shepherd FA, Morgan C, Evans WK, Ginsberg JF, Watt D, Murphy K (1987) Medical management of malignant pericardial effusion by tetracycline sclerosis. *Am J Cardiol* **60:** 1161–1166

Theologides A (1978) Neoplastic cardiac tamponade. *Semin Oncol* **5:** 181–192

Tobinai K, Kohno A, Shimada Y, Watanabe T, Tamura T, Takeyama K, Narabayashi M, Fukutomi T, Kondo H, Shimoyama M, Suemasu K (1993) Toxicity grading criteria of the Japan Clinical Oncology Group. The Clinical Trial Review Committee of the Japan Clinical Oncology Group. *Jpn J Clin Oncol* **23:** 250–257

Tomkowski WZ, Wisniewska J, Szturmowicz M, Kuca P, Burakowski J, Kober J, Fijalkowska A (2004) Evaluation of intrapericardial cisplatin administration in cases with recurrent malignant pericardial effusion and cardiac tamponade. *Support Care Cancer* **12:** 53–57

Vaitkus PT, Herrmann HC, LeWinter MM (1994) Treatment of malignant pericardial effusion. *JAMA* **272:** 59–64

Wang HJ, Hsu KL, Chiang FT, Tseng CD, Tseng YZ, Liau CS (2002) Technical and prognostic outcomes of double-balloon pericardiotomy for large malignancy-related pericardial effusions. *Chest* **122:** 893–899

Wilkes JD, Fidias P, Vaickus L, Perez RP (1995) Malignancy-related pericardial effusion. 127 cases from the Roswell Park Cancer Institute. *Cancer* **76:** 1377–1387

Yonemori K, Kunitoh H, Tsuta K, Tamura T, Arai Y, Shimada Y, Fujiwara Y, Sasajima Y, Asamura H, Tamura T (2007) Prognostic factors for malignant pericardial effusion treated by pericardial drainage in solid-malignancy patients. *Med Oncol* **24:** 425–430

Ziskind AA, Pearce AC, Lemmon CC, Burstein S, Gimple LW, Herrmann HC, McKay R, Block PC, Waldman H, Palacios IF (1993) Percutaneous balloon pericardiotomy for the treatment of cardiac tamponade and large pericardial effusions: description of technique and report of the first 50 cases. *J Am Coll Cardiol* **21:** 1–5

Appendix

Supported by the Grant-in-Aid for Cancer Research from the Ministry of Health, Labour and Welfare of Japan (11S-2, 11S-4, 14S-2, 14S-4, 17S-2, 17S-5).

Presented in part at the 43rd Annual Meeting of the American Society of Clinical Oncology, June 1–5, 2007, Chicago IL.

Study participants: The following institutions and investigators participated in the trial:

National Hospital Organization Dohoku Hospital (Yuka Fujita and Satoru Fujiuchi), Tochigi Cancer Center (Kiyoshi Mori and Yukari Kamiyama), National Cancer Center Hospital East (Kaoru Kubota, Yutaka Nishiwaki and Nagahiro Saijo), National Cancer Center Hospital (Noboru Yamamoto, Tomohide Tamura and Hideo Kunitoh), International Medical Center (Koichiro Kudo and Yuichiro Takeda), Cancer Institute Hospital (Takeshi Horai and Makoto Nishio), Kanagawa Cancer Center (Kazumasa Noda and Fumihiro Oshita), Yokohama Municipal Citizen's Hospital (Koshiro Watanabe and Hiroaki Okamoto), Niigata Cancer Center Hospital (Akira Yokoyama and Yuko Tsukada), Gifu City Hospital (Yoshiyuki Sawa and Takashi Ishiguro), Aichi Cancer Center Hospital (Toyoaki Hida), National Hospital Organization Nagoya Medical Center (Hideo Saka), Kinki University Hospital (Kazuhiko Nakagawa and Isamu Okamoto) and Kyushu University Hospital (Yoichi Nakanishi and Koichi Takayama).

0090-9556/09/3702-272–276$20.00
DRUG METABOLISM AND DISPOSITION
Copyright © 2009 by The American Society for Pharmacology and Experimental Therapeutics
DMD 37:272–276, 2009

Vol. 37, No. 2
24208/3426314
Printed in U.S.A.

Short Communication

Close Association of *UGT1A9* IVS1+399C>T with *UGT1A1*28*, **6*, or **60* Haplotype and Its Apparent Influence on 7-Ethyl-10-hydroxycamptothecin (SN-38) Glucuronidation in Japanese

Received August 26, 2008; accepted October 30, 2008

ABSTRACT:

The anticancer prodrug, irinotecan, is converted to its active form 7-ethyl-10-hydroxycamptothecin (SN-38) by carboxylesterases, and SN-38 is inactivated by UDP-glucuronosyltransferase (UGT)1A1-mediated glucuronidation. UGT1A9 also mediates this reaction. In a recent study, it was reported that the *UGT1A9* IVS1+399 (I399)C>T polymorphism is associated with increased SN-38 glucuronidation both in vitro and in vivo. However, its role in UGT1A9 expression levels and activity is controversial. Thus, we evaluated the role of I399C>T in SN-38 glucuronidation using 177 Japanese cancer patients administered irinotecan. I399C>T was detected at a 0.636 allele frequency. This polymorphism was in strong linkage disequilibrium (LD) with *UGT1A9*1b* (−126_−118T_9>T_{10}, |D′| = 0.99) and *UGT1A1*6* (211G>A, 0.86), in moderate LD with *UGT1A1*60* (−3279T>G, 0.55), but weakly associated with *UGT1A1*28* (−54_−39A(TA)$_6$TAA>A(TA)$_7$TAA, 0.25). Haplotype analysis showed that 98% of the I399C alleles were linked with low-activity haplotypes, either *UGT1A1*6*, **28*, or **60*. On the other hand, 85% of the T alleles were linked with the *UGT1A1* wild-type haplotype **1*. Although I399T-dependent increases in SN-38 glucuronide/SN-38 area under concentration-time curve (AUC) ratio (an in vivo marker for UGT1A activity) and decreases in SN-38 AUC/dose were apparent ($P < 0.0001$), these effects were no longer observed after stratified patients by *UGT1A1*6*, **28*, or **60* haplotype. Thus, at least in Japanese populations, influence of I399C>T on SN-38 glucuronidation is attributable to its close association with either *UGT1A1*6*, **28*, or **60*.

Irinotecan is an important drug for treatment of various tumors including lung, colon, and gastric (Smith et al., 2006). The infused drug is metabolized to its active form 7-ethyl-10-hydroxycamptothecin (SN-38) by carboxylesterases, and SN-38 is inactivated by glucuronidation. At least four UDP-glucuronosyltransferase (UGT) isoforms, namely UGT1A1, UGT1A7, UGT1A9, and UGT1A10, are known to glucuronidate SN-38 (Gagné et al., 2002; Saito et al., 2007).

The UGT1A gene complex consists of 9 active first exons including *UGT1A10*, *1A9*, *1A7*, and *1A1* (in this order) and common exons 2 to 5. One of the 9 first exons can be used in conjunction with the common exons (Tukey and Strassburg, 2000). The UGT1A N-terminal domains (encoded by the first exons) determine substrate-binding specificity, and the C-terminal domain (encoded by exons 2 to 5) is important for binding to UDP-glucuronic acid. The 5′- or 3′-flanking region of each exon 1 is presumably involved in regulation of its expression. Substantial interindividual differences have been detected in mRNA and protein levels and enzymatic activity of the UGT1A isoforms (Fisher et al., 2000; Saito et al., 2007).

SN-38 glucuronidation is thought to be mediated mainly by UGT1A1, and its genetic polymorphisms affecting irinotecan pharmacokinetics and adverse reactions have been already identified. The TA-repeat polymorphism, −54_−39A(TA)$_6$TAA>A(TA)$_7$TAA (*UGT1A1*28* allele), is associated with lower promoter activity, resulting in reduced SN-38 glucuronidation (Beutler et al., 1998; Iyer et al., 1999). The single nucleotide polymorphism (SNP) 211G>A (Gly71Arg, **6* allele), found mainly in East Asians, causes reduced protein expression levels and SN-38 glucuronidation activity (Gagné et al., 2002; Jinno et al., 2003). Another SNP in the enhancer region of *UGT1A1*, −3279T>G (**60* allele), is also a causative factor for reduced expression (Sugatani et al., 2002). Allele frequencies have been reported for **28* (0.09–0.13), **6* (0.15–0.19), and **60* (0.26–0.32) in Japanese and Chinese populations and for **28* (0.30–0.39), **6* (−0), and **60* (0.44–0.55) in whites (Saito et al., 2007). In a previous study, in the Japanese population, we defined haplotype **28* as the haplotype harboring the **28* allele, haplotype **6* as that harboring the**6* allele, and haplotype **60* as that harboring the **60* allele (and without the **28* or **6* allele) (Sai et al., 2004; Saeki et al., 2006). Note that most of the **28* haplotypes concurrently harbored the **60* alleles, and that the **28* and **6* alleles were exclusively present on the different chromosomes (Sai et al., 2004; Saeki et al., 2006). We have also revealed that the haplotype **28*, **6*, or **60* was associated with reduced SN-38 glucuronide (SN-38G)/SN-38 area under concentration-time curve (AUC) ratios, an in vivo parameter for UGT1A activity (Minami et al., 2007).

In a recent study, an intronic SNP of *UGT1A9*, IVS1+399 (I399)C>T, has been shown to be associated with increased UGT1A9 protein levels and glucuronidation activities toward SN-38 and the UGT1A9 probe drug propofol (Girard et al., 2006). Elevation of

This study was supported in part by the program for the Promotion of Fundamental Studies in Health Sciences from the National Institute of Biomedical Innovation [Grant 05-25]; and a Health and Labour Sciences Research grant from the Ministry of Health, Labour and Welfare in Japan [Grant KHB1008].

Y.S. and K.S. contributed equally to this work.

Article, publication date, and citation information can be found at http://dmd.aspetjournals.org.

doi:10.1124/dmd.108.024208.

ABBREVIATIONS: SN-38, 7-ethyl-10-hydroxycamptothecin; UGT, UDP-glucuronosyltransferase; SNP, single nucleotide polymorphism; SN-38G, SN-38 glucuronide; AUC, area under concentration-time curve; I399, *UGT1A9* IVS1+399; LD, linkage disequilibrium.

SN-38 glucuronidation activity by this SNP is significant among subjects without *UGT1A1*28*. Sandanaraj et al. (2008) have also reported that I399C/C patients showed higher SN-38 AUC than C/T and T/T patients. With the same *UGT1A1* diplotypes, patients with I399T/T (and *UGT1A9* $-126\_-118T_{10}/T_{10}$) have shown higher SN-38G C_{max} than I399C/T (and T_9/T_{10}) patients. *UGT1A9*1b* (*UGT1A9* $-126\_-118T_9>T_{10}$) has been shown to have no affect on UGT1A9 expression levels (Girard et al., 2006; Ramírez et al., 2007; Sandanaraj et al., 2008). Thus, two groups did suggest that I399T allele was associated with higher glucuronidation activity. However, using human liver microsomes, Ramírez et al. (2007) showed that I399C>T had no significant effect on both UGT1A9 mRNA levels and glucuronidation activities for two UGT1A9 substrates. Therefore, the roles of I399C>T in UGT1A9 activities as well as SN-38 glucuronidation remain inconclusive.

In the present report, we reveal the linkage of I399C>T with *UGT1A1*, *UGT1A7*, and *UGT1A9* polymorphisms and analyze its association with the SN-38G/SN-38 AUC ratio and SN-38 AUC/dose (per dose) to clarify its role in SN-38 glucuronidation.

Materials and Methods

Patients. One hundred and seventy-seven patients (81 lung, 63 colon, 19 stomach, and 14 other cancer patients) administered irinotecan at the National Cancer Center were enrolled in this study as described previously (Minami et al., 2007). 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. Eligibility criteria, patient profiles, and irinotecan regimens are summarized in our previous report (Minami et al., 2007). In brief, patients consisted of 135 males and 42 females with a mean age of 60.5 (26–78 years old), and their performance status was 0 (84 patients), 1 (89 patients), or 2 (4 patients). Irinotecan administrations were conducted according to the standard protocols in Japan as follows: i.v. 90-min infusion at a dose of 100 mg/m^2 weekly or 150 mg/m^2 biweekly in irinotecan monotherapy; and 60 mg/m^2 weekly with cisplatin in most combination therapies.

Genotyping and Haplotype Analysis. Genomic DNA was extracted from whole blood of 177 irinotecan-administered patients (Saeki et al., 2006). *UGT1A9* IVS1+399C>T (rs2741049) was genotyped using the TaqMan SNP Genotyping Assay kit (C_9096281_10) according to the manufacturer's instructions (Applied Biosystems, Foster City, CA). The *UGT1A1*28* allele [$-54\_-39A(TA)_6TAA>A(TA)_7TAA$], *UGT1A1*6* allele [211G>A (Gly71Arg)], *UGT1A1*60* allele ($-3279T>G$), *UGT1A7*2* haplotype [387T>G, 391C>A and 392G>A (Asn129Lys and Arg131Lys)], *UGT1A7*3* haplotype [387T>G, 391C>A, 392G>A, and 622T>C (Asn129Lys, Arg131Lys, and Trp208Arg)], and *UGT1A9*1b* allele ($-126\_-118T_9>T_{10}$) were determined previously (Saeki et al., 2006). Hardy-Weinberg equilibrium analysis of I399C>T, linkage disequilibrium (LD) analysis of the *UGT1A9*, *UGT1A7*, and *UGT1A1* polymorphisms, and haplotype estimation with an expectation-maximization algorithm were performed using SNPAlyze version 7.0 software (Dynacom, Chiba, Japan).

Pharmacokinetics. Pharmacokinetic data for the 176 irinotecan-treated patients (data for one patient was unavailable) were described previously (Minami et al., 2007). In brief, heparinized blood was collected before irinotecan administration and at 0, 0.33, 1, 2, 4, 8, and 24 h after termination of the first infusion of irinotecan. SN-38 and SN-38G plasma concentrations were determined by high-performance liquid chromatography, and AUC was calculated using the trapezoidal method in WinNonlin version 4.01 (Pharsight, Mountain View, CA).

Statistical Analysis. Gene dose effects of I399C>T and *UGT1A1* haplotypes (**28*, **6*, *or *60*) were assessed by the Jonckheere-Terpstra test using StatExact version 6.0 (Cytel Inc., Cambridge, MA). Multiplicity adjustment was conducted with the false discovery rate. The significant difference was set at $p = 0.05$ (two-tailed).

Results

Linkages of *UGT1A9* IVS1+399 (I399)C>T with Other Polymorphisms. In our patients, I399C>T was detected at a 0.636 allele frequency, which is almost the same as those in the HapMap data (rs2741049) for Japanese (0.663) and Han Chinese (0.633) populations, but higher than those for Europeans (0.383) and Sub-Saharan Africans (Yoruba) (0.417). Genotype distribution for this SNP was in Hardy-Weinberg equilibrium ($p = 0.418$). LD analysis was performed between I399C>T and the previously determined genotypes, *UGT1A9*1b*, *UGT1A7*2* and **3*, and *UGT1A1*28*, **6*, and **60*, which were detected at >0.1 frequencies in Japanese populations (Saeki et al., 2006). When assessed by the |D'| value, I399C>T was in complete LD with *UGT1A7* 387T>G, 391C>A and 392G>A (*UGT1A7*2*, |D'| = 1.000); in strong LD with *UGT1A9* $-126\_-118T_9>T_{10}$ (*UGT1A9*1b*, 0.987), *UGT1A7* 622T>C (*UGT1A7*3*, 0.977), and *UGT1A1* 211G>A (*UGT1A1*6*, 0.864); and in moderate LD with *UGT1A1* $-3279T>G$ (*UGT1A1*60*, 0.554), but weakly associated with *UGT1A1* $-54\_-39A(TA)_6TAA>A(TA)_7TAA$ (*UGT1A1*28*, 0.252). In r^2 values, the I399C>T was in strong LD with *UGT1A7*2* ($r^2 = 0.976$) and *UGT1A9*1b* (0.916), in moderate LD with *UGT1A7*3* (0.478), but in weak LD with *UGT1A1*6* (0.261) and *UGT1A1*60* (0.208), and in little LD with *UGT1A1*28* (0.018).

Haplotype Analysis. Haplotype analysis was performed using the 9 polymorphisms including I399C>T. As shown in Fig. 1, 95% (123/129) of the I399C alleles were linked with the *UGT1A9* -126_-118T_9 alleles, and 100% (225/225) of the T alleles were linked with the T_{10} alleles (*UGT1A9*1b*). The I399C alleles were completely (129/129) linked with the *UGT1A7* 387G, 391A, and 392A alleles, and most T alleles (223/225) were linked with the 387T, 391C, and 392G alleles. The 40% (51/129) and 60% (78/129) of the I399C alleles were linked with *UGT1A7*2* and *UGT1A7*3* haplotypes, respectively. We also found that 98% (126/129) of the I399C alleles were linked with the *UGT1A1*6* (211G>A), **28* [$-54\_-39A(TA)_6TAA>A(TA)_7TAA$], or **60* ($-3279T>G$). According to the *UGT1A1* haplotype definition by Sai et al. (2004), 42% (54/129), 36% (46/129), 19% (25/129), and 1% (1/129) of the I399C alleles were linked with the *UGT1A1* haplotypes **6a* (harboring **6* allele), **60a* (harboring **60* allele), **28b* (harboring **60* and **28* alleles), and **28d* (harboring **28* allele), respectively. On the other hand, 85% (191/225) of the T alleles were linked with the *UGT1A1* wild-type haplotype **1*.

Association Analysis. The associations of I399C>T with irinotecan pharmacokinetic parameters were then analyzed using the estimated haplotypes. First, association with SN-38G/SN-38 AUC ratio, an in vivo parameter of UGT1A activity (Sai et al., 2004; Minami et al., 2007; Sandanaraj et al., 2008), was analyzed. *UGT1A7*2* had unchanged activity for SN-38 glucuronidation (Gagné et al., 2002), and neither *UGT1A9*1b* nor *UGT1A7*3* had significant effects on the SN-38G/SN-38 AUC ratio in our previous study (Minami et al., 2007). On the other hand, the *UGT1A1*6*, **28*, and **60* haplotypes were associated with the reduced SN-38G/SN-38 AUC ratios (Minami et al., 2007). Although effects of the haplotype **28* and **6* were more striking, haplotype *UGT1A1*60*, harboring only the **60* allele without the **28* allele, was weakly associated with the reduced ratio. To remove even this weak effect and clarify the real effect of I399C>T, *UGT1A1*60* was also considered as low-activity haplotype in this analysis. Namely, we analyzed the associations of I399C>T with the AUC ratio within the groups stratified by the *UGT1A1* haplotypes, *UGT1A1*28* (**28b* and **28d*), **6* (**6a*), and **60* (**60a*) (combined and shown as *UGT1A1*"+").

When stratified by the I399C>T genotype, a T allele-dependent

Gene		*UGT1A9*		*UGT1A7*[2]				*UGT1A1*[3]			Number	Frequency
Nucleotide change		-126_-118 T_9>T_{10}	IVS1+399 C>T	387 T>G	391 C>A	392 G>A	622 T>C	-3279 T>G	$(TA)_6$>$(TA)_7$	211 G>A		
Allele name		*1b		*2, *3	*2, *3	*2, *3	*3	*60, *28	*28	*6		
Haplotypes[1]	*1C-*3-*6a			■	■	■	■			■	47	0.133
	*1C-*2-*60a			■	■	■		■			44	0.124
	*1C-*3-*28b			■	■	■	■	■	■		21	0.059
	*1C-*2-*28b			■	■	■		■	■		4	0.011
	*1C-*3-*60a			■	■	■	■	■			2	0.006
	*1C-*3-*28d			■	■	■	■		■		1	0.003
	*1C-*2-*6a			■	■	■				■	1	0.003
	*1bC-*3-*6a	■		■	■	■	■			■	6	0.017
	*1C-*2-*1			■	■	■					2	0.006
	*1C-*3-*1			■	■	■	■				1	0.003
	*1bT-*1-*1	■	■								190	0.537
	*1bT-*3-*1	■	■	■	■	■	■				1	0.003
	*1bT-*1-*28b	■	■					■	■		22	0.062
	*1bT-*1-*60a	■	■					■			5	0.014
	*1bT-*1-*6a	■	■							■	5	0.014
	*1bT-*1-*28d	■	■						■		1	0.003
	*1bT-*2-*60a	■	■	■	■	■		■			1	0.003
Alelle frequency		0.653	0.636	0.370	0.370	0.370	0.223	0.280	0.138	0.167	354	1.000

FIG. 1. Haplotypes assigned by using common *UGT1A9*, *UGT1A7*, and *UGT1A1* polymorphisms. [1]Haplotypes were shown as *UGT1A9* haplotypes − *UGT1A7* haplotypes − *UGT1A1* haplotypes. Major allele, white blocks; minor allele, gray blocks. **1C*, T_9 and I399C; **1bC*, T_{10} and I399C; **1bT*, T_{10} and I399T in *UGT1A9*. [2]*UGT1A7*2* and *3 are the haplotypes harboring the three and four *UGT1A7* alleles, respectively. [3]*UGT1A1* $(TA)_6$>$(TA)_7$ indicates −54_−39A$(TA)_6$TAA>A$(TA)_7$TAA.

increase in the SN-38G/SN-38 AUC ratio was observed ($p < 0.0001$, Jonckheere-Terpstra test) (Fig. 2A). However, this trend was obviously dependent on biased distributions of *UGT1A1* haplotypes; e.g., 96% of the I399C/C patients were homozygotes for *UGT1A1*28*, *6, *or *60*; and "*UGT1A1*28*, *6, or *60*"-dependent reduction of SN-38G/SN-38 AUC ratio was found within the I399T/T genotypes ($p < 0.05$). As shown in Fig. 2B, *UGT1A1*28*, *6, or *60* (*UGT1A1*+)-dependent reduction in the SN-38G/SN-38 ratio was observed when patients were stratified by these three haplotypes. However, no significant effect of I399C>T was found within the stratified patients ($p > 0.05$ within the −/−, −/+, or +/+ patient group in Fig. 2B). As for SN-38 AUC/dose (SN-38 AUC values adjusted by the doses used), a similar *UGT1A1* haplotype dependence was observed. Although the I399T-dependent reduction of SN-38 AUC/dose was detected ($p < 0.0001$), biased distributions of the *UGT1A1*28*, *6, or *60* were again evident, and the *UGT1A1* + haplotypes-dependent increase was significant within the I399 C/T and T/T patients ($p < 0.01$ and $p < 0.05$, respectively) (Fig. 2C). Moreover, no significant effect of I399C>T on SN-38 AUC/dose was found when stratified by the *UGT1A1* haplotypes ($p > 0.05$ within the −/−, −/+, or +/+ patient group in Fig. 2D).

Discussion

In the present study, LD between I399C>T and *UGT1A1*, *UGT1A7*, or *UGT1A9* polymorphisms in Japanese populations was shown for the first time. Moreover, the apparent effect of I399C>T on SN-38 glucuronidation in Japanese cancer patients was suggested to result from its close association with *UGT1A1*28*, *6, or *60*.

As for the influence of I399C>T on UGT1A9 activity, conflicting results have been reported. Girard et al. (2006) have shown that I399C>T was associated with increased UGT1A9 protein levels and enzyme activity toward an UGT1A9 probe drug propofol using 48 human liver microsomes derived mainly from whites. In contrast, using human liver microsomes from 46 white subjects, Ramírez et al. (2007) have revealed that the I399C>T had no significant effects on UGT1A9 mRNA levels and in vitro glucuronidation activities toward the two UGT1A9 substrates, flavopiridol and mycophenolic acid. Furthermore, another report has demonstrated that I399C>T had no influence on the pharmacokinetic parameters (such as AUC and C_{max}) of mycophenolic acid in 80 Japanese renal transplant recipients (Inoue et al., 2007). Thus, these latter two studies did suggest that the I399C>T polymorphism has no effect on UGT1A9 enzymatic activity. Note that, at least for Japanese populations, no study has reported that I399C>T affects UGT1A9 activity.

As for the influence of I399C>T on SN-38 glucuronidation, a possible enhancing effect has been suggested. Girard et al. (2006) have shown an increasing effect of I399C>T on SN-38 glucuronidation, and that this SNP did not show any close linkages with the *UGT1A1*28* or *60 allele ($r^2 < 0.06$). In addition, Sandanaraj et al. (2008) have reported that in 45 Asians consisting of Chinese (80%), Malay (18%), and others (2%), I399C/C patients had higher SN-38 AUC than C/T and T/T patients. Again, this SNP was not in LD with the *UGT1A1*28*, *6, or *60 allele (r^2 were <0.09). Furthermore, association of I399T with increased SN-38G C_{max} has been observed even after stratified patients by *UGT1A1* genotypes, although the study sample size was small. These findings suggest that the I399T

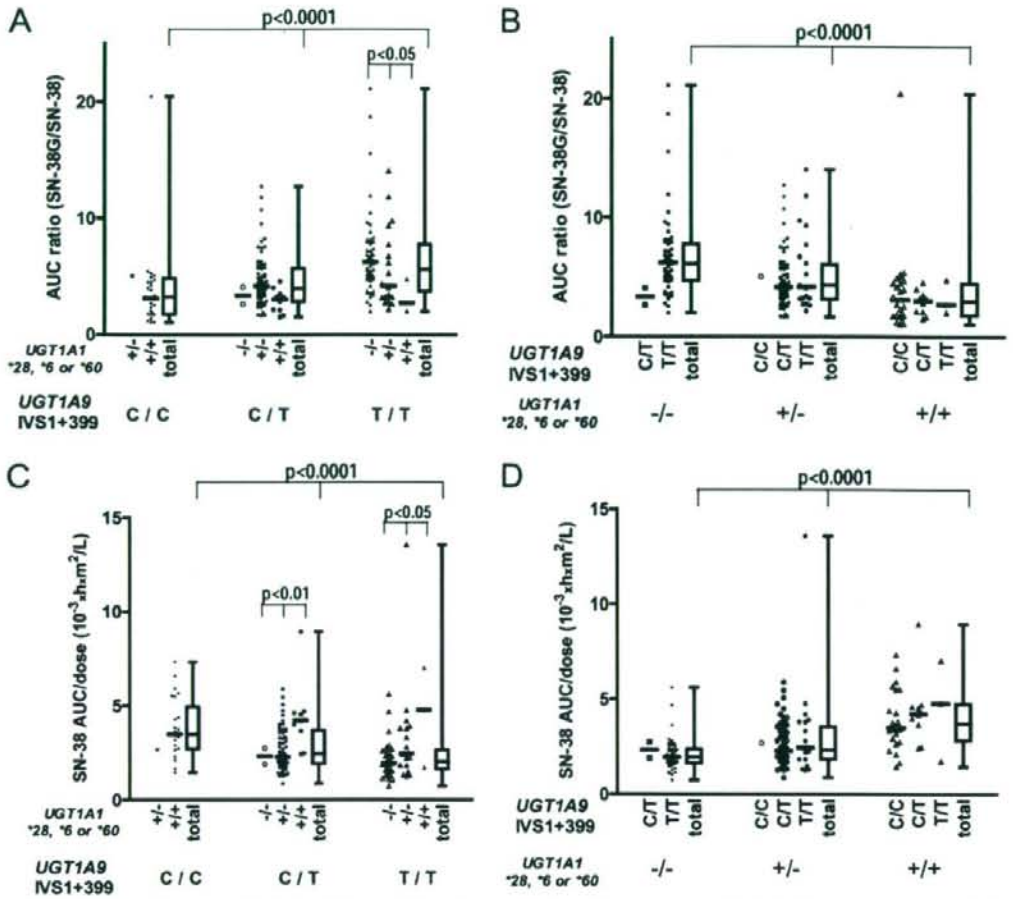

FIG. 2. Association analysis of *UGT1A9* IVS1+399 (I399)C>T with SN-38G/SN-38 AUC ratio (A and B) and SN-38 AUC/dose (C and D). A and C, I399 C/C, C/T, and T/T patients were further divided by the presence of *UGT1A1*28*, *6, or *60* haplotypes: −/−, no *UGT1A1*28*, *6, or *60*; −/+, heterozygotes for either *UGT1A1*28*, *6, or *60*; +/+, homozygotes or compound heterozygotes for either *UGT1A1*28*, *6, or *60*. B and D, *UGT1A1* −/−, −/+, and +/+ patients were further divided by I399 C/C, C/T, and T/T genotypes. Gene dose effects of I399C>T and the *UGT1A1* + haplotype were assessed by the Jonckheere-Terpstra test.

allele was associated with increased glucuronidation activity for SN-38 without linkages with the *UGT1A1* polymorphisms. Our data demonstrate that an increase in SN-38G/SN-38 AUC ratio (i.e., increased glucuronidation activity) was also found with I399C>T; however, after stratified patients by the *UGT1A1*6*, *28, or *60* haplotypes (haplotype+) showing reduced SN-38 glucuronidation activity (Sai et al., 2004; Minami et al., 2007), any significant effect of the I399C>T was no longer observed. Thus, no direct effect of I399C>T on SN-38 glucuronidation was shown in the current study in Japanese populations. The discrepancy between our study and others might be derived from ethnic and/or population differences in haplotype distribution. In fact, in our Japanese population, 98% of the I399C alleles were linked with either *UGT1A1 *6, *28, or *60*, whereas 85% of the T alleles were linked with *UGT1A1*1*. On the other hand, in Sandanaraj's report (in Chinese + Malay), 84% of the I399C alleles were linked with *UGT1A1 *6, *28, or *60*, whereas only 67% of the T alleles were linked with *UGT1A1*1* (Sandanaraj et al., 2008).

In irinotecan therapies, genetic polymorphisms leading to increases in SN-38 AUC, which closely correlates with increased risk of severe neutropenia (Minami et al., 2007), are clinically important. The current study also demonstrated no significant influence of I399C>T on SN-38 AUC/dose after stratified patients by *UGT1A1* haplotypes. Consistent with this finding, no influence of this SNP was observed on the incidence of grade 3 or 4 neutropenia after irinotecan therapy in our population (data not shown). Recently, genetic testing of *UGT1A1*6* and *28, which are related to severe neutropenia in Japanese populations, has been approved for clinical application in Japan. This study indicates that there is no clinical necessity for additional genotyping of I399C>T, at least in Japanese populations.

In conclusion of this study, the apparent influence of I399 (*UGT1A9* IVS1+399)C>T on SN-38 glucuronidation is attributable to its close association with *UGT1A1*6, *28, or *60* in the Japanese population. Furthermore, additional genotyping of I399C>T for personalized irinotecan therapy seems to be clinically irrelevant for Japanese populations.

Acknowledgments. We thank Chie Sudo for secretarial assistance.

Project Team for Pharmacogenetics (Y.S., K.Sa., K.M., N.K., J.S.), YOSHIRO SAITO
KIMIE SAI
Division of Functional Biochemistry and Genomics (Y.S., K.Sa., K.M., J.S.), KEIKO MAEKAWA
NAHOKO KANIWA
Division of Medicinal Safety Science (N.K.), KUNIAKI SHIRAO[1]
National Institute of Health Sciences, Tokyo, Japan; TETSUYA HAMAGUCHI
NOBORU YAMAMOTO
Gastrointestinal Oncology Division (K.Sh., T.H., Y.Y.), HIDEO KUNITOH
YUICHIRO OHE
Thoracic Oncology Division (N.Y., H.K., Y.O., T.T.), YASUHIDE YAMADA
TOMOHIDE TAMURA
National Cancer Center Hospital, Genetics Division (T.Y.), TERUHIKO YOSHIDA
HIRONOBU MINAMI[2]
National Cancer Center Research Institute, National Cancer Center, Tokyo, Japan; ATSUSHI OHTSU
YASUHIRO MATSUMURA
Division of Oncology/Hematology (H.M.), Division of Gastrointestinal Oncology/Digestive Endoscopy (A.O.), Investigative Treatment Division, Research Center for Innovative Oncology (Y.M.), NAGAHIRO SAIJO
Deputy Director (N.S.), National Cancer Center Hospital East, Kashiwa, Japan JUN-ICHI SAWADA

[1] Current affiliation: Department of Medical Oncology, Oita University Faculty of Medicine, Yufu, Japan.

[2] Current affiliation: Medical Oncology, Department of Medicine, Kobe University Hospital and Graduate School of Medicine, Kobe, Japan.

References

Beutler E, Gelbart T, and Demina A (1998) Racial variability in the UDP-glucuronosyltransferase 1 (UGT1A1) promoter: a balanced polymorphism for regulation of bilirubin metabolism? *Proc Natl Acad Sci U S A* **95**:8170–8174.

Fisher MB, Vandenbranden M, Findlay K, Burchell B, Thummel KE, Hall SD, and Wrighton SA (2000) Tissue distribution and interindividual variation in human UDP-glucuronosyltransferase activity: relationship between UGT1A1 promoter genotype and variability in a liver bank. *Pharmacogenetics* **10**:727–739.

Gagné JF, Montminy V, Belanger P, Journault K, Gaucher G, and Guillemette C (2002) Common human UGT1A polymorphisms and the altered metabolism of irinotecan active metabolite 7-ethyl-10-hydroxycamptothecin (SN-38). *Mol Pharmacol* **62**:608–617.

Girard H, Villeneuve L, Court MH, Fortier LC, Caron P, Hao Q, von Moltke LL, Greenblatt DJ, and Guillemette C (2006) The novel UGT1A9 intronic I399 polymorphism seems as a predictor of 7-ethyl-10-hydroxycamptothecin glucuronidation levels in the liver. *Drug Metab Dispos* **34**:1220–1228.

Inoue K, Miura M, Satoh S, Kagaya H, Saito M, Habuchi T, and Suzuki T (2007) Influence of UGT1A7 and UGT1A9 intronic I399 genetic polymorphisms on mycophenolic acid pharmacokinetics in Japanese renal transplant recipients. *Ther Drug Monit* **29**:299–304.

Iyer L, Hall D, Das S, Mortell MA, Ramírez J, Kim S, Di Rienzo A, and Ratain MJ (1999) Phenotype-genotype correlation of in vitro SN-38 (active metabolite of irinotecan) and bilirubin glucuronidation in human liver tissue with UGT1A1 promoter polymorphism. *Clin Pharmacol Ther* **65**:576–582.

Jinno H, Tanaka-Kagawa T, Hanioka N, Saeki M, Ishida S, Nishimura T, Ando M, Saito Y, Ozawa S, and Sawada J (2003) Glucuronidation of 7-ethyl-10-hydroxycamptothecin (SN-38), an active metabolite of irinotecan (CPT-11), by human UGT1A1 variants, G71R, P229Q, and Y486D. *Drug Metab Dispos* **31**:108–113.

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

Ramírez J, Liu W, Mirkov S, Desai AA, Chen P, Das S, Innocenti F, and Ratain MJ (2007) Lack of association between common polymorphisms in UGT1A9 and gene expression and activity. *Drug Metab Dispos* **35**:2149–2153.

Saeki M, Saito Y, Jinno H, Sai K, Ozawa S, Kurose K, Kaniwa N, Komamura K, Kotake T, Morishita H, et al. (2006) Haplotype structures of the UGT1A gene complex in a Japanese population. *Pharmacogenomics J* **6**:63–75.

Sai K, Saeki M, Saito Y, Ozawa S, Katori N, Jinno H, Hasegawa R, Kaniwa N, Sawada J, Komamura K, et al. (2004) UGT1A1 haplotypes associated with reduced glucuronidation and increased serum bilirubin in irinotecan-administered Japanese patients with cancer. *Clin Pharmacol Ther* **75**:501–515.

Saito Y, Maekawa K, Ozawa S, and Sawada J (2007) Genetic polymorphisms and haplotypes of major drug metabolizing enzymes in East Asians and their comparison with other ethnic populations. *Curr Pharmacogenomics* **5**:49–78.

Sandanaraj E, Jada SR, Shu X, Lim R, Lee SC, Zhou Q, Zhou S, Goh BC, and Chowbay B (2008) Influence of UGT1A9 intronic I399C>T polymorphism on SN-38 glucuronidation in Asian cancer patients. *Pharmacogenomics J* **8**:174–185.

Smith NF, Figg WD, and Sparreboom A (2006) Pharmacogenetics of irinotecan metabolism and transport: an update. *Toxicol In Vitro* **20**:163–175.

Sugatani J, Yamakawa K, Yoshinari K, Machida T, Takagi H, Mori M, Kakizaki S, Sueyoshi T, Negishi M, and Miwa M (2002) Identification of a defect in the UGT1A1 gene promoter and its association with hyperbilirubinemia. *Biochem Biophys Res Commun* **292**:492–497.

Tukey RH and Strassburg CP (2000) Human UDP-glucuronosyltransferases: metabolism, expression, and disease. *Annu Rev Pharmacol Toxicol* **40**:581–616.

Address correspondence to: Dr. Yoshiro Saito, Division of Functional Biochemistry and Genomics, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan. E-mail: yoshiro@nihs.go.jp

Efficacy and Safety of Erlotinib Monotherapy for Japanese Patients with Advanced Non-small Cell Lung Cancer

A Phase II Study

Kaoru Kubota, MD, PhD, Yutaka Nishiwaki, MD, Tomohide Tamura, MD, Kazuhiko Nakagawa, MD, Kaoru Matsui, MD, Koshiro Watanabe, MD, PhD, Toyoaki Hida, MD, Masaaki Kawahara, MD, Nobuyuki Katakami, MD, Koji Takeda, MD, Akira Yokoyama, MD, Kazumasa Noda, MD, Masahiro Fukuoka, MD, and Nagahiro Saijo, MD, PhD

Introduction: The aim of this study was to evaluate the efficacy and safety of Erlotinib in Japanese patients with previously treated non-small cell lung cancer (NSCLC). Available tumor biopsy samples were analyzed to examine relationships between biomarkers and clinical outcome.

Methods: This open-label phase II trial enrolled stage III/IV NSCLC patients who had progressive disease after at least one prior platinum-based chemotherapy regimen. Erlotinib was administered at a dose of 150 mg/d orally until disease progression or intolerable toxicity. Analysis of epidermal growth factor receptor gene mutations in exon 18–21 by direct sequencing was performed in tumor tissue specimens obtained at the first diagnosis.

Results: Sixty-two patients were enrolled and 60 patients were evaluable for efficacy. Objective response rate and disease control rate were 28.3% and 50.0%; median time to progression and overall survival were 77 days and 14.7 months, respectively. In logistic regression analysis, only smoking history was proved to be a statistically significant predictive factor for response (odds ratio: 0.06, $p < 0.001$). Only 7 patients had samples available for mutation analysis. Three patients who had deletion mutations on exon 19 (del E746-A750 or del S752-I759) exhibited objective response. Common toxicities were rash (98%), dry skin (81%), and diarrhea (74%). Discontinuation due to adverse events occurred in 11 patients (18%). Four patients (6%) experienced interstitial lung disease-like events, one of whom died.

Conclusion: Erlotinib is efficacious in Japanese patients with previously treated NSCLC. The toxicity profile was similar to that in Western patients, except for a somewhat higher incidence of skin disorders and interstitial lung disease. Further studies are needed to determine the relationship between epidermal growth factor receptor mutations and outcomes with Erlotinib in Japanese patients.

National Cancer Center Hospital East, Kashiwa, Chiba, Japan.

Disclosure: Kazuhiko Nakagawa had served as an adviser for pre-approval consulting of this drug. Masahiro Fukuoka was paid an honorarium as the chairman of the meeting and as medical advisor for clinical trial in relation to this drug. Nagahiro Saijo had received research grant in relation to this drug. The other authors declare no conflicts of interest.

Address for correspondence: Kaoru Kubota, MD, PhD, National Cancer Center Hospital East, 6-5-1 Kashiwanoha, Kashiwa, Chiba 277-8577, Japan. E-mail: kkubota@east.ncc.go.jp

ISSN: 1556-0864/08/0312-1439

Key Words: Non-small cell lung cancer, Erlotinib, Molecular target therapy, EGFR-TKIs, *EGFR* mutation.

(*J Thorac Oncol.* 2008;3: 1439–1445)

Lung cancer affects approximately 1.2 million people annually, and is the leading cause of cancer death in the world.[1] More than 80% of affected patients are diagnosed with non-small cell lung cancer (NSCLC). The standard first-line treatment for metastatic NSCLC is a combination of platinum chemotherapy with a third-generation agent such as docetaxel, paclitaxel, gemcitabine, vinorelbine, and irinotecan.[2,3] Although patients with stage II, IIIA, or IIIB NSCLC receive platinum-based chemotherapy as part of combined modality treatment with thoracic radiotherapy or surgery, many will be candidates for second or third-line chemotherapy. Docetaxel is the only cytotoxic agent with a proven survival advantage over supportive care in patients with disease progression after cisplatin-based chemotherapy for NSCLC.[4] The other agent for which a survival benefit has been demonstrated in this setting is erlotinib,[5] which was approved in Japan for the treatment of relapsed NSCLC in October 2007. Erlotinib is a selective, orally active epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI). In contrast to the experience with the cytotoxic chemotherapeutic agents, response to treatment with EGFR-TKIs has been reported to be influenced by gender, histological type, race or ethnic origin, and smoking status.[5–8]

Tumor molecular markers, including *EGFR* gene mutations and protein expression, have been widely studied in patients with NSCLC, and there is strong evidence that the presence of *EGFR* gene mutations is a predictor of tumor response and resistance.[9–12] However, few prospective studies have evaluated molecular markers as predictors of outcome, and their clinical usefulness is unproven.

This report presents the results of the first phase II study of erlotinib conducted in Japanese patients with NSCLC. The purpose was to evaluate the efficacy and safety of erlotinib in this population. Where available, tumor biopsy samples were analyzed for EFGR-related markers.

PATIENTS AND METHODS

This phase II, multicenter, open-label study recruited patients at 11 hospitals in Japan. The primary end point was the objective response rate (ORR) to erlotinib treatment (150 mg/d). Secondary endpoints were disease control rate (DCR), response duration, time to progression, overall survival (OS), quality of life (QoL), and safety. The protocol was approved by the ethics review boards of all participating institutions, and conducted in accordance with Japanese Good Clinical Practice guidelines.

Patient Selection

Patients with histologically or cytologically documented stage IIIB or IV NSCLC at study entry (not curable with surgery or radiotherapy) that was recurrent or refractory to treatment with one or more chemotherapy regimens (including at least one platinum-containing regimen), were enrolled into this study. Additional eligibility criteria included: the presence of measurable lesions by Response Evaluation Criteria in Solid Tumors (RECIST); age $\geq$20, $<$75 years; Eastern Cooperative Oncology Group performance status (ECOG PS) of 0–2, and adequate bone marrow, hepatic, and renal function, i.e., aspartate aminotransferase and alanine aminotransferase (ALT) levels $\leq$2.5 times the upper limit of normal and total bilirubin of $\leq$1.5 times the upper limit of normal. Patients with existing or previous interstitial lung disease (ILD) were excluded, although a history of radiation pneumonitis (limited to the field of radiation treatment) was permitted. Concomitant anticancer treatment and prophylactic medication for adverse events (AEs) were not permitted, nor was prior use of anti-EGFR or anti human epidermal growth factor receptor (HER2) agents (small molecules and monoclonal antibodies). Written informed consent was obtained from all patients.

Treatment Procedure

After completion of the baseline assessments (see below), all patients received erlotinib (150 mg orally) each morning, 1 hour before breakfast, until the occurrence of progressive disease (PD) or unacceptable toxicity (all AEs were graded using the National Cancer Institute Common Toxicity Criteria Version 2.0). In the event of treatment-related toxicity, 2 dose reductions of 50 mg were permitted per patient, and dosing could also be interrupted for up to 14 days. For grade 3 or intolerable grade 2 rash, treatment was withheld until the rash improved to grade 2 or less, when a lower dose of erlotinib was initiated. For grade 3 diarrhea, treatment was withheld until the diarrhea was grade 1 or less, when a lower dose was started. For ILD of any grade, or any grade 4 toxicity, treatment was immediately and permanently discontinued.

Evaluation of Efficacy

Objective tumor response was assessed in accordance with RECIST.[13] Tumor assessments were performed at baseline, then every 4 weeks until week 16, and then every 8 weeks thereafter. Confirmation of complete or partial responses (PR) was required, by means of a second assessment conducted 28 days or more after the initial assessment. Stable disease (SD) was defined as disease control (absence of progression) maintained for at least 6 weeks. An independent response evaluation committee consisting of 2 oncologists and a radiologist reviewed images of patients with complete response, PR, and SD. Individual survival times were determined from the survival status of each patient during the study period and at the post study follow-up survey conducted in June-July 2005 and May-July 2006. OS was defined as the time from first administrated to death.

Quality of Life Evaluation

The Functional Assessment of Cancer Therapy-Lung (FACT-L) questionnaire (Version 4-A)[14] was used to assess QoL. The full FACT-L questionnaire was administered at baseline and then every 28 days. In addition, the Lung Cancer Subscale (LCS), an independently validated component of FACT-L, was administered weekly during the treatment period. Best responses on the LCS were analyzed for all patients with a baseline LCS score of 24 or less (out of a possible 28 points) and symptomatic improvement was defined as an increase from the baseline score of 2 or more points, sustained for at least 4 weeks.

Evaluation of Safety

Baseline assessment included a full patient history, physical examination, standard laboratory tests, electrocardiography, chest radiography, pregnancy test, and ophthalmologic tests (vision test and slit-lamp examination). Every week until week 8 and every 2 weeks thereafter, vital signs and ECOG PS were monitored and blood samples were taken for hematology and blood chemistry tests. A radiograph examination to assess pulmonary toxicity was conducted weekly until week 4 and every 2 weeks thereafter. Ophthalmologic examinations were repeated at week 8 and at the end of the study. Observation and evaluation of AEs was conducted as appropriate throughout the study period. All AEs were graded using National Cancer Institute Common Toxicity Criteria Version 2.0. For all ILD-like events, the data safety monitoring board (which consisted of oncologists and pneumonologists) reviewed the clinical data and images; the images were also examined by a review committee of radiologists with expertise in drug-induced pulmonary disorders.

Biomarker Analysis

EGFR mutations and EGFR and HER2 protein expression were assessed in patients with suitable tumor tissue specimens at first diagnosis or surgery; these assessments were done only with separate written consent. Tumor samples were obtained from each center as formalin-fixed and paraffin-embedded blocks, or as thinly sliced tissue sections mounted on glass microscope slides. For the mutation analysis, the tissue was microdissected by Targos Molecular Pathology (Kassel, Germany) and direct sequencing was conducted at the Roche Centre of Medical Genomics (Basel, Switzerland), using a nested polymerase chain reaction of exon 18–21. EGFR protein expression was analyzed by Lab Corp (Mechelen, Belgium). EGFR expression analysis was conducted by immunohistochemistry using Dako EGFR PharmDx™ kits (Dako, Carpinteria, CA). A positive test was

defined as membranous staining in ≥10% of the tumor cells. HER2 protein expression was measured using HercepTest™ (Dako, Carpinteria, CA), and a score of 1+ or above (possible scores were: 0, 1+, 2+, 3+) was regarded as positive.

Statistical Analysis

Given an expected ORR of 20%, a Fisher's exact test was performed (one-sided $\alpha = 2.5\%$). Based on 50 patients, the power to test the null hypothesis (ORR = 5%) was 89.66%. The target sample size of 60 patients was chosen on the expectation that a proportion of patients would prove to be ineligible for the study. The main analysis of efficacy was conducted on the full analysis set (FAS), which was produced by omitting ineligible patients. The 95% confidence interval (CI) for ORR, DCR, and symptom improvement rate was calculated by the Clopper-Pearson method. The time-to-event variables were estimated by the Kaplan-Meier method. Logistic regression and Cox proportional hazards regression analysis was conducted on best response and survival time, respectively. In both cases, univariate and multivariate analyses were used to evaluate the effects of 11 factors relating to patient and disease characteristics, and previous treatment.

RESULTS

Patient Characteristics

A total of 62 patients were enrolled between December 2003 and January 2005. All were evaluable for safety and 60 were evaluable for efficacy (FAS). Two patients did not have a measurable lesion according to RECIST. The baseline characteristics of the patients, including their treatment history, are shown in Table 1. The median age was 60.5 years (range: 28–74 years), and 71% of patients were male. Fifty-seven patients (92%) had adenocarcinoma, and 20 (32%) were never-smokers. Twenty-seven patients (44%) had received only one previous chemotherapy regimen.

Efficacy

Tumor response rates in the FAS (as assessed by extrainstitutional review) are shown in Table 2. Seventeen patients were assessed as having a PR and 13 as having SD. The ORR was 28.3% (95% CI: 17.5–41.4%) and the DCR was 50% (95% CI: 36.8–63.2%). In three patients, objective response could not be adequately confirmed, because each discontinued treatment early in the study due to AEs. The median duration of response was 278 days (95% CI: 203–422 days), and time to progression was 77 days (95% CI: 55–166 days). OS was determined based on information collected until the follow-up survey conducted in May–July 2006. The median survival time was 14.72 months (95% CI: 11.07–20.57 months; 19 censored cases) and the 1-year survival rate was 56.5% (95% CI: 43.9–69.1%) (Figure 1). The median OS of patients with PD was 9.95 months. The symptom improvement rate measured using the LCS was 42.1% (24/57; 95% CI: 29.1–55.9%).

The overall response rate was higher in women (58.8%; 10/17) than in men (16.3%; 7/43, χ^2 test: $p = 0.0029$), and in never-smokers (63.2%; 12/19) than in current or former smokers (12.2%; 5/41, $p = 0.0002$). There was no statistically significant difference between the response rate in patients with adenocarcinoma (28.6%; 16/56) and nonadenocarcinoma histology (25.0%; 1/4, $p = 1.0000$). The response

TABLE 1. Summary of Baseline Patient Characteristics and Demographics

Patient and Disease characteristics	No. of Patients ($n = 62$)	%
Age (yr)		
Median	60.5	
Range	28–74	
Sex		
Female	18	29
Male	44	71
Performance status		
0	20	32
1	41	66
2	1	2
Histology		
Adenocarcinoma	57	92
Squamous cell	4	6
Unclassified	1	2
Stage		
IIIB	8	13
IV	54	87
Smoking history		
Never smoked	20	32
Current- or former smoker	42	68
Time since initial diagnosis (d)		
Median	304.0	
Range	2–2353	
Prior chemotherapy regimens		
1	27	44
2	23	37
≥3	12	19
Prior taxanes		
No	10	16
Yes	52	84
Time since last regimen (d)		
Median	80.0	
Range	29–528	

TABLE 2. Response Assessment

Parameter	n	(%)
Partial response	17	28.3
Stable disease	13	21.7
Progressive disease	27	45.0
Not assessable	3	5.0
Response rate (%) (95% CI)	28.3 (17.5–41.4)	
Disease control rate (%) (95% CI)	50.0 (36.8–63.2)	
Duration of response (median: days)[a] (95% CI)	278 (203.0–422.0)	
Time to progression (median: days)[a] (95% CI)	77 (55–166)	

[a] Kaplan–Meier method.
CI, confidence intervals.

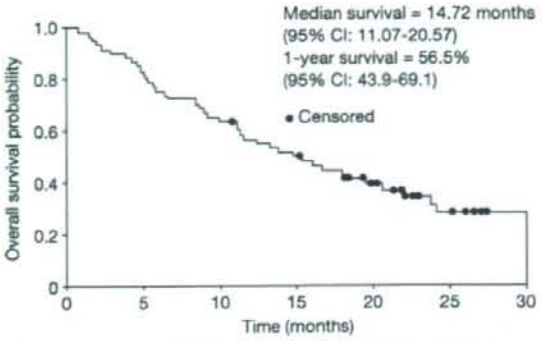

FIGURE 1. Kaplan-Meier plot showing overall survival.

rate was not affected by the number of previous chemotherapy regimens, however, being 27% for patients with one previous regimen (7/26) and 29% for those with 2 or more regimens (10/34). No statistically significant differences were found between other patient subgroups. In a multivariate logistic regression analysis, only smoking history was found to be a statistically significant predictor of response. A multivariate Cox regression analysis showed that both smoking history and ECOG PS were significant predictors for OS (Table 3).

Safety

All 62 patients who received erlotinib were assessed for safety. Treatment-related AEs were observed in all patients, and there were 24 serious AEs in 18 patients (29%). AEs led to discontinuation of erlotinib in 11 patients (18%), including 3 due to ILD-like events, 2 due to ALT elevation, and one each due to rash, paronychia, punctate keratitis, dyspnea/hypoxia, pneumonia and fever/inflammatory neck swelling, and to dose interruptions in 30 patients (48.4%). While the main reasons for the dose interruptions were rash (n = 15; 24.2%) and diarrhea (n = 4; 6.5%), only one patient with rash

TABLE 3. Logistic and Cox Regression Analysis

	Odds Ratio[b]	(95% CI)	p
Logistic regression analysis of response			
Univariate analysis			
Sex (female vs male)	0.14	0.04–0.48	0.002
Age (<65 vs ≥65)	1.26	0.38–4.13	0.704
Histology (non-AD vs AD)	1.20	0.12–12.41	0.878
Smoking history (never vs current or former)	0.08	0.02–0.30	<0.001
Performance status (0 vs ≥1)	0.62	0.19–1.98	0.420
Prior regimens (1 vs ≥2)	1.13	0.36–3.53	0.832
Stage (IIIB vs IV)	0.99	0.17–5.65	0.988
KL-6 (baseline) (<median [496.5 U/ml[a]] vs ≥median)	1.64	0.53–5.12	0.392
Best response to previous chemotherapy (non-PR vs PR)	0.90	0.24–3.33	0.869
Prior taxanes (no vs yes)	0.43	0.10–1.84	0.253
Time since initial diagnosis (≤12 mo vs >12 mo)	1.02	0.31–3.30	0.976
Multivariate analysis			
Smoking history (never vs current or former)	0.06	0.02–0.28	<0.001
Time since initial diagnosis (<12 mo vs ≥12 mo)	2.22	0.49–10.20	0.304
Cox regression analysis of survival			
Univariate analysis			
Sex (female vs male)	1.76	0.85–3.61	0.126
Age (<65 vs ≥65)	0.86	0.44–1.71	0.675
Histology (non-AD vs AD)	0.55	0.19–1.55	0.255
Smoking history (never vs current or former)	1.90	0.93–3.90	0.079
Performance status (0 vs ≥1)	2.31	1.12–4.73	0.023
Prior regimens (1 vs ≥2)	0.93	0.50–1.75	0.833
Stage (IIIB vs IV)	1.38	0.49–3.89	0.542
KL-6 (baseline) (<median [496.5 U/ml[a]] vs ≥median)	1.64	0.87–3.06	0.125
Best response to previous chemotherapy (non-PR vs PR)	0.66	0.31–1.44	0.300
Prior taxanes (no vs yes)	2.09	0.74–5.90	0.163
Time since initial diagnosis (≤12 mo vs >12 mo)	0.76	0.40–1.47	0.418
Multivariate analysis			
Smoking history (never vs current or former)	2.20	1.06–4.56	0.035
Performance status (0 vs ≥1)	2.59	1.25–5.37	0.011

[a] Or 629 ng/ml.
[b] Left site of 'vs' indicates reference group.
PR, partial response; AD, adenocarcinoma; CI, confidence interval.

TABLE 4. Major Treatment-Related Adverse Events and Interstitial Lung Disease-Like Events

Event[a]	*n*	%	NCI-CTC Grade (*n*)			
			1	2	3	>4
Rash	61	98.4	18	41	2	0
Dry skin	50	80.6	44	6	—	—
Diarrhea	46	74.2	33	10	3	0
Pruritus	45	72.6	38	7	0	—
Stomatitis	24	38.7	19	4	1	0
Fatigue	21	33.9	15	6	0	0
Anorexia	19	30.6	11	6	2	0
Paronychia	18	29.0	12	5	1	0
C-reactive protein increased	15	24.2	8	7	0	0
Alanine aminotransferase increased	15	24.2	11	2	2	0
Total bilirubin increased	15	24.2	8	7	0	0
Weight loss	13	21.0	13	0	0	—
ILD-like events	4	6.5	1	0	2	1[b]

Case	Sex	Age	Smoking History	Brinkman Index	Performance Status	Histology	Onset (day)	Outcome	Relation to Erlotinib[c]
1	Male	75	Former	640	1	Adenocarcinoma	52	Recovery	Probable
2	Male	67	Never	—	1	Adenocarcinoma	103	Death (145)	Possible
3	Female	39	Never	—	0	Adenocarcinoma	85	Recovery	Probable
4	Male	69	Former	1000	1	Adenocarcinoma	13	Recovery	Unlikely

[a] Categorized by MedDra Ver.7.1 (except for event).
[b] Grade 5.
[c] Judged by ILD review committee.
NCI-CTC, National Cancer Institute Common Toxicity Criteria; ILD, interstitial lung disease.

had to discontinue treatment, and no patients had to discontinue because of diarrhea or any other digestive toxicity. Fourteen patients (23%) had dose reductions due to AEs, mostly due to rash ($n = 9$; 15%). Treatment-related AEs with an incidence of 20% or more are shown in Table 4; the main events were rash (98%), dry skin (81%), and diarrhea (74%). Elevated laboratory test values related to liver function were found in some patients (total bilirubin: 24%, ALT: 24%), and grade 3 ALT elevation led to treatment discontinuation in 2 patients. Four patients had ILD-like events, including worsening of radiation pneumonitis in one patient, and one died (Table 4). All four (three men; one woman) had an ECOG PS of 0–1 and 2 were former smokers. The patient who died was a 67-year-old man with adenocarcinoma and no history of smoking who discontinued treatment on day 84 due to PD. He developed interstitial pneumonia on day 103 and received 3 days of palliative thoracic irradiation from day 99, after completing the study (3 Gy × 3 days). A computed tomography scan showed characteristic features of ILD (cryptogenic organizing pneumonia-like pattern), and the ILD review committee decided that use of erlotinib could not be excluded as the cause. For the patient with worsening of radiation pneumonitis (case 4), the committee concluded that there was a possible influence of previous radiation therapy, and that this could be seen in the computed tomography scan on day 1. There was, therefore, little reason to suspect that the use of erlotinib had been the cause. Rather, it appeared that the radiation pneumonitis had worsened according to the normal course of illness.

Biomarker Analysis

Tissue samples for measurement of *EGFR* mutations were available for 16 of the 60 patients evaluated for efficacy. For 7 patients, all base sequences were successfully identified in the 4 segments of exons 18–21. All seven (three men, four women) had adenocarcinoma; three were never-smokers, three former smokers and one a current smoker. Three had PR, two SD and two PD. Five of the seven patients had *EGFR* gene mutations and, in all, seven different mutations were detected. The 3 patients with PR all had deletion mutations in exon 19 (del E746-A750 or del S752-I759). One of the 2 patients with PD had no mutations and the other had 2 substitution mutations: L858R in exon 21 and the resistance mutation T790M in exon 20 (Table 5).

Paraffin-embedded tissue samples for immunohistochemistry were available from 12 patients, among whom, 11 had successful determinations of immunohistochemical staining (including 3 patients with PR). Six of the 11 were found to be EGFR-positive and 4 were HER2-positive. However, there were no notable relationships between the EGFR and HER2 expression status and either tumor response or patient characteristics such as sex, histological type or smoking history (data not shown).

DISCUSSION

The present study was conducted on the basis of results from a phase I study of erlotinib in Japanese patients with solid tumors,[15] which showed erlotinib to be well tolerated at

TABLE 5. *EGFR* Mutation Analysis

Response	TTP (d)	Survival (d)	Sex	Histology	Smoking history	Mutation status	Exon	Type of Mutation
PR	222	546	Female	Adenocarcinoma	Never	+	19	del E746–A750
PR	230	811+	Male	Adenocarcinoma	Current	+	19	del S752 –I759 and T751N
PR	278+	911	Female	Adenocarcinoma	Never	+	19	V786M, del E746–A750
SD	224	649+	Male	Adenocarcinoma	Former	+	21	del V834-
SD	77	737	Female	Adenocarcinoma	Former	−	—	—
PD	60	604+	Female	Adenocarcinoma	Never	+	20, 21	L858R, T790M
PD	19	347	Male	Adenocarcinoma	Former	−	—	—

TTP, time to progression; PR, partial response; SD, stable disease; PD, progressive disease.

a dose of 150 mg/d, as well as a phase II study of erlotinib in NSCLC conducted in the United States.[16] In this study, erlotinib achieved an ORR of 28.3%, which was higher than expected, and a DCR of 50%. The response rate was higher than that determined in the above-mentioned phase II study[16] and in keeping with the rate seen in the Japanese subgroup in the phase II study of gefitinib (IDEAL1; 27.5%).[6] Assessment of QoL using the LCS demonstrated a clinically meaningful rate of symptom improvement of 42.1%.

The characteristics of the patients in this study were generally similar to those of NSCLC patients as a whole, in terms of their demographics and disease and treatment history, with the exception of a particularly high proportion of patients with adenocarcinoma (92%). The possibility of enrollment bias on the basis of histological type cannot be ruled out, in part because enrollment coincided with the emergence of reports that the efficacy of EGFR-TKI therapy was greater in patients with adenocarcinoma.[17] However, we also observed one PR and two SDs among three patients with squamous cell carcinoma (FAS population), and our results do not rule out the efficacy of erlotinib in any patient subtype. A multivariate logistic regression analysis showed that smoking status was significantly associated with tumor response, in agreement with previous studies of predictive factors for response to EGFR-TKIs.[5,18,19]

The median survival time with erlotinib was an encouraging 14.7 months. One of the reasons for this long survival may be the high proportion of never-smokers and patients with adenocarcinoma compared with those of other studies, particularly the multinational phase III erlotinib study (BR.21).[5] On the other hand, the presence of *EGFR* gene mutations is currently regarded as an important determinant of treatment response to EGFR-TKIs[20,21] and may be the most important factor in relation to the favorable results seen in the present study. However, it is important to recognize that the potential prognostic effect of mutation status cannot be excluded. The sample size of this and previous trials limits the interpretation of this effect, which will be adequately assessed only by means of appropriately powered trials specifically designed to examine these factors.

Assessment of the presence or absence of *EGFR* gene mutation was possible in only seven patients in the present study. Despite this, the results were consistent with the results of some previous studies. All three of the patients who had a PR (including a male current smoker) had an in-frame deletion in exon 19, which is considered to be the most frequent mutation site in the EGFR-TK domain.[22] One of the 2 patients with PD had a point substitution mutation (L858R) in exon 21, the second most frequent mutation site,[22] and a point mutation (T790M) in exon 20, which is suggested to be involved in tolerance to EGFR-TKI.[12,23,24] It would be valuable to conduct further prospective randomized studies on the association between these markers and survival during treatment with erlotinib in Japanese patients.

Rash and diarrhea were the main AEs reported by patients on erlotinib treatment, as reported in previous studies.[5,15,16] Rash was observed in almost all patients, and was the main reason for treatment interruptions or dose reductions. Although the protocol allowed treatment to be interrupted for grade 3 rash (or intolerable grade 2 rash), grade 3 rash only occurred in 2 patients, leading to discontinuation of treatment in one. Most cases of rash responded to symptomatic treatment and either interruption or dose reduction of erlotinib. Despite suggestions in some reports that the presence of erlotinib-related rash is associated with treatment efficacy and can be used to predict response,[25] no supportive evidence was found in the present study.

The incidence of ILD, which is the most clinically problematic AE associated with erlotinib, tended to be higher than that reported in other clinical studies of erlotinib.[5,26] This is in keeping with this class of agent, and is not unexpected in the Japanese population.

We would recommend that careful screening of patients for ILD risk factors, particularly signs of interstitial pneumonia and pulmonary fibrosis, is done before erlotinib therapy is initiated. Individuals with any previous history of ILD were excluded from this study.

In conclusion, erlotinib (150 mg/d) was shown to have promising antitumor efficacy in Japanese patients with previously treated NSCLC, leading to clinically meaningful improvements in symptoms and an encouraging median survival time. Despite, as expected, a high rate of rash and diarrhea, erlotinib was well tolerated at a dose of 150 mg/d by the majority of patients.

REFERENCES

1. Parkin MD. Global cancer statistics in the year 2000. *Lancet Oncol* 2001;2:533–543.
2. Schiller JH, Harrington D, Belani CP, et al. Comparison of four chemotherapy regimens for advanced non-small cell lung cancer. *N Engl J Med* 2002;346:92–98.

3. Ohe Y, Ohashi Y, Kubota K, et al. Randomized phase III study of cisplatin plus irinotecan versus carboplatin plus paclitaxel, cisplatin plus gemcitabine, and cisplatin plus vinorelbine for advanced non-small-cell lung cancer: four-arm cooperative study in Japan. *Ann Oncol* 2007;18: 317–323.
4. Shepherd FA, Dancey J, Ramlau R, et al. Prospective randomized trial of docetaxel versus best supportive care in patients with non-small cell lung cancer previously treated with platinum-based chemotherapy. *J Clin Oncol* 2000;18:2095–2103.
5. Shepherd F, Rodrigues J, Ciuleanu T, et al. Erlotinib in previously treated non-small cell lung cancer. *N Engl J Med* 2005;353:123–132.
6. Fukuoka M, Yano S, Giaccone G, et al. Multi-institutional randomized phase II trial of gefitinib for previously treated patients with advanced non-small-cell lung cancer. *J Clin Oncol* 2003;21:2237–2246.
7. Chang A, Parikh P, Thongprasert S, et al. Gefitinib (IRESSA) in patients of Asian origin with refractory advanced non-small cell lung cancer: subset analysis from the ISEL study. *J Thorac Oncol* 2006;1:847–855.
8. Miller VA, Kris MG, Shah N, et al. Bronchioloalveolar pathologic subtype and smoking history predict sensitivity to gefitinib in advanced non-small-cell lung cancer. *J Clin Oncol* 2004;22:1103–1109.
9. Paez JG, Jänne PA, Lee JC, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science* 2004;304: 1497–1500.
10. Lynch TJ, Bell DW, Sordella R, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N Engl J Med* 2004;350:2129–2139.
11. Kobayashi S, Boggon TJ, Dayaram T, et al. EGFR mutation and resistance of non-small-cell lung cancer to gefitinib. *N Engl J Med* 2005;352:786–792.
12. Pao W, Miller V, Zakovski M, et al. EGF receptor gene mutations are common in lung cancers from "never smokers" and are associated with sensitivity of tumors to gefitinib and erlotinib. *Proc Natl Acad Sci USA* 2004;101:13306–13311.
13. Therasse P, Arbuck SG, Eisenhauer EA, et al. New guidelines to evaluate the response to treatment in solid tumors. *J Natl Cancer Inst* 2000;92:205–216.
14. Cella DF, Bonomi AE, Lloyd SR, et al. Reliability and validity of the functional assessment of cancer therapy-lung (FACT-L) quality of life instrument. *Lung Cancer* 1995;12:199–220.
15. Yamamoto N, Horiike A, Fujisaka Y, et al. Phase I dose-finding and pharmacokinetic study of the oral epidermal growth factor receptor tyrosine kinase inhibitor Ro50–8231 (erlotinib) in Japanese patients with solid tumors. *Cancer Chemother Pharmacol* 2008;61:489–496.
16. Pérez-Soler RS, Chachoua A, Hammond LA, et al. Determinants of tumor response and survival with erlotinib in patients with non-small-cell lung cancer. *J Clin Oncol* 2004;22:3238–3247.
17. Kaneda H, Tamura K, Kurata T, et al. Retrospective analysis of the predictive factors associated with the response and survival benefit of gefitinib in patients with advanced non-small-cell lung cancer. *Lung Cancer* 2004;46:247–254.
18. Thatcher N, Chang A, Parikh P, et al. Gefitinib plus best supportive care in previously treated patients with refractory advanced non-small cell lung cancer: results from a randomised, placebo-controlled, multicentre study (Iressa Survival Evaluation in Lung Cancer). *Lancet* 2005;366: 1527–1537.
19. Clark GM, Zborowski DM, Santabárbara P, et al. Smoking history and epidermal growth factor receptor expression as predictors of survival benefit from erlotinib for patients with non-small-cell lung cancer in the National Cancer Institute of Canada Clinical Trials Group study BR. 21. *Clin Lung Cancer* 2006;7:389–394.
20. Toyooka S, Matsuo K, Shigematsu H, et al. The impact of sex and smoking status on the mutational spectrum of epidermal growth factor receptor gene in non small cell lung cancer. *Clin Cancer Res* 2007;13: 5763–5768.
21. Mitsudomi T, Kosaka T, Endoh H, et al. Mutations of the epidermal growth factor receptor gene predict prolonged survival after gefitinib treatment in patients with non-small cell lung cancer with postoperative recurrence. *J Clin Oncol* 2005;23:2513–2520.
22. Sequist LV, Bell DW, Lynch TJ, et al. Molecular predictors of response to epidermal growth factor receptor antagonists in non-small-cell lung cancer. *J Clin Oncol* 2007;25:587–595.
23. Pao W, Miller VA, Politi KA, et al. Acquired resistance of lung adenocarcinomas to gefitinib or erlotinib is associated with a second mutation in the EGFR kinase domain. *PLoS Med* 2005;2:e73.
24. Tokumo M, Toyooka S, Ichihara S, et al. Double mutation and gene copy number of EGFR in gefitinib refractory non-small cell lung cancer. *Lung Cancer* 2006;53:117–121.
25. Wacker B, Nagrani T, Weinberg J, et al. Correlation between development of rash and efficacy in patients treated with the epidermal growth factor receptor tyrosine kinase inhibitor erlotinib in two large phase III studies. *Clin Cancer Res* 2007;13:3913–3921.
26. Tsuboi M, Le Chevalier T. Interstitial lung disease in patients with non-small cell lung cancer treated with epidermal growth factor receptor inhibitors. *Med Oncol* 2006;23:161–170.

Lung Cancer (2008) 62, 316–320

available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/lungcan

Mutational status of *EGFR* and *KIT* in thymoma and thymic carcinoma

Kiyotaka Yoh[a,*], Yutaka Nishiwaki[a], Genichiro Ishii[b], Koichi Goto[a], Kaoru Kubota[a], Hironobu Ohmatsu[a], Seiji Niho[a], Kanji Nagai[a], Nagahiro Saijo[a]

[a] *Division of Thoracic Oncology, National Cancer Center Hospital East, 6-5-1 Kashiwanoha, Kashiwa, Chiba 277-8577, Japan*
[b] *Pathology Division, Research Center for Innovative Oncology, National Cancer Center Hospital East, 6-5-1 Kashiwanoha, Kashiwa, Chiba 277-8577, Japan*

Received 13 February 2008; received in revised form 16 March 2008; accepted 19 March 2008

KEYWORDS
Thymoma;
Thymic carcinoma;
EGFR;
KIT;
Mutation;
Treatment

Summary This study was conducted to evaluate the prevalence of *EGFR* and *KIT* mutations in thymomas and thymic carcinomas as a means of exploring the potential for molecularly targeted therapy with tyrosine kinase inhibitors. Genomic DNA was isolated from 41 paraffin-embedded tumor samples obtained from 24 thymomas and 17 thymic carcinomas. *EGFR* exons 18, 19, and 21, and *KIT* exons 9, 11, 13, and 17, were analyzed for mutations by PCR and direct sequencing. Protein expression of EGFR and KIT was evaluated immunohistochemically. *EGFR* mutations were detected in 2 of 20 thymomas, but not in any of the thymic carcinomas. All of the *EGFR* mutations detected were missense mutations (L858R and G863D) in exon 21. EGFR protein was expressed in 71% of the thymomas and 53% of the thymic carcinomas. The mutational analysis of *KIT* revealed only a missense mutation (L576P) in exon 11 of one thymic carcinoma. KIT protein was expressed in 88% of the thymic carcinomas and 0% of the thymomas. The results of this study indicate that *EGFR* and *KIT* mutations in thymomas and thymic carcinomas are rare, but that many of the tumors express EGFR or KIT protein.

1. Introduction

Thymic epithelial tumors are uncommon neoplasms and there are two major histological types: thymoma and thymic carcinoma [1]. Surgical resection is the preferred treatment option for all subtypes of thymoma and thymic carcinoma. However, thymic carcinomas and some thymomas tend to behave in a malignant manner clinically, and in many cases dissemination or distant metastasis has already occurred at presentation. Patients with metastatic or unresectable tumors are candidates for systemic chemotherapy, but no standard chemotherapy has been established because of the rarity of both tumors [2–5], and alternative therapeutic molecular targets are needed.

* Corresponding author. Tel.: +81 4 7133 1111; fax: +81 4 7131 4724.
E-mail address: kyoh@east.ncc.go.jp (K. Yoh).

0169-5002/$ – see front matter © 2008 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.lungcan.2008.03.013

Receptor tyrosine kinases such as epidermal growth factor receptor (EGFR) and KIT, contribute to a number of processes related to the survival and growth activity of many solid tumors, making them promising targets for cancer therapy [6–8]. Recent studies have shown that the presence of kinase domain mutations in the EGFR gene in non-small cell lung cancer (NSCLC) tissue predicts a significant clinical response to small-molecule tyrosine kinase inhibitors (TKIs) of EGFR, such as gefitinib and erlotinib [9], and it is widely known that there is an association between exon 11 mutations of the KIT gene in gastrointestinal stromal tumors (GISTs) and greater responsiveness to imatinib as a small-molecule TKI of KIT [10].

Several immunohistochemical studies have shown overexpression of EGFR protein in both thymoma and thymic carcinoma [11,12], and in thymic carcinoma, immunohistochemical studies have shown a high frequency of KIT overexpression but that thymomas express hardly any KIT [13,14]. Two interesting cases have recently been reported. One was a case of thymic carcinoma with an activating *KIT* mutation that responded to imatinib, reported by Strobel et al. [15], and the other was a case of thymic carcinoma with *EGFR* mutations that was responsive to gefitinib, reported by Yamaguchi et al. [16]. However, because of the rarity of these tumors, information on the mutational status of *EGFR* and *KIT* in thymomas and thymic carcinomas has been limited to only a few reports, and the prevalence of *EGFR* and *KIT* mutations remains unknown.

In this study, we investigated the status of *EGFR* and *KIT* mutations in thymoma and thymic carcinoma patients to explore the potential for molecularly targeted therapy with TKIs. We also investigated the relation between protein expression assessed by immunohistochemistry and the mutational status of *EGFR* and *KIT*.

2. Patients and methods

2.1. Patients

The tumor samples used in this study were obtained from paraffin-embedded surgical specimens from 41 cases of thymoma or thymic carcinoma treated surgically at the National Cancer Center Hospital East between 1993 and 2005. All samples were reviewed to confirm the diagnosis of thymoma or thymic carcinoma. The clinical data of all patients was collected from their medical records. This study was approved by the Institutional Review Board of our institution.

The characteristics of all of the patients are listed in Table 1. Patient age ranged from 21 to 77 years, and their median age was 61 years. The specimens used were from 24 thymomas and 17 thymic carcinomas. According to the World Health Organization (WHO) classification of thymic epithelial tumors, the histological subtype of the thymomas was type A in 7 cases, type AB in 7 cases, type B1 in 6 cases, and type B2 in 4 cases. The histological subtype of the thymic carcinomas was squamous cell carcinoma in 14 cases, and adenocarcinoma, adenosquamous carcinoma, and nonspecified in 1 case each. According to the system described by Masaoka et al. [17], the clinical stage was stage I in 15 patients, stage II in 8 patients, stage III in 9 patients, stage IVa in 1 patient, and stage IVb in 8 patients. All patients had undergone total resection ($n = 36$) or partial resection ($n = 5$) after obtaining their informed consent in accordance with institutional guidelines.

Table 1 Patient characteristics

	Patients ($n = 41$)
Age, years	
Median	61
Range	21–77
Gender	
Female	20
Male	21
Histology	
Thymoma	24
Thymic carcinoma	17
Stage	
I	15
II	8
III	9
IVa	1
IVb	8
Surgical procedure	
Total resection	36
Partial resection	5
Smoking history	
Never	19
Former	11
Current	11

2.2. Mutational analysis of *EGFR* and *KIT*

Tumor genomic DNA was isolated from paraffin-embedded samples of a total of 41 tumors, 24 thymomas and 17 thymic carcinomas. To ensure that tumor-cell-rich areas of tissues were isolated, hematoxylin and eosin stained slides were prepared from each selected paraffin-embedded block. Polymerase chain reaction (PCR) was performed to amplify exons 18, 19, and 21 of *EGFR* and exons 9, 11, 13, and 17 of *KIT* by using previously described primers [9,18], and the PCR products were directly sequenced with an ABI 3100 DNA Sequencer (Applied Biosystems, Foster City, CA, USA). All sequencing reactions were performed in both forward and reverse directions. A series of mutational analyses was performed at Mitsubishi Chemical Safety Institute Ltd.

2.3. Immunohistochemistry

Protein expression of EGFR and KIT was evaluated immunohistochemically in representative paraffin-embedded sections. EGFR staining was performed by using the DAKO (Carpinteria, CA, USA) pharmDX kit for EGFR according to the manufacturer's instructions, and immunostaining for KIT was performed by using a polyclonal rabbit antibody (A 4502; Dako, Glostrup, Denmark) according to the manufacturer's instructions. Staining of both markers was considered posi-

tive if more than 50% of the tumor cells stained. All slides were examined and scored independently by two observers (G.I. and K.Y.).

2.4. Statistical analysis

The variables measured in the study were tested for associations by Fisher's exact test. P values <0.05 were considered statistically significant.

3. Results

3.1. EGFR analysis of thymomas and thymic carcinomas

Sequencing of the *EGFR* tyrosine kinase domain encoded by exons 18, 19, and 21 was successful in 29 of the 41 tumors (Table 2). *EGFR* mutations were detected in 2 of the 20 thymomas, but direct sequencing showed no evidence of mutations in any of the 9 thymic carcinomas. All of the *EGFR* mutations detected were missense mutations in exon 21 (L858R or G863D), and no mutations were detected in exons 18 and 19. Examination of 21 thymomas and 17 thymic carcinomas for EGFR protein expression by immunohistochemistry revealed EGFR expression in 15 (71%) of the 21 thymomas and 9 (53%) of the 17 thymic carcinomas. The difference in EGFR expression between the thymomas and thymic carcinomas was not significant ($P = 0.31$).

3.2. KIT analysis of thymomas and thymic carcinomas

It was possible to analyze the *KIT* mutation status of 22 thymomas and 11 thymic carcinomas by direct sequencing (Table 3). A missense mutation in exon 11 (L576P) was found in only one thymic carcinoma, and direct sequencing of *KIT* exons 9, 13, and 17 revealed no mutations in any of the tumors analyzed. Immunohistochemistry showed KIT protein expression in 15 (88%) of the 17 thymic carcinomas, but no KIT expression in any of the 24 thymomas ($P < 0.0001$).

Table 4 summarizes the data of all patients whose tumors were positive for *EGFR* or *KIT* mutations. Exon 21 mutations in the *EGFR* gene were found in two thymomas (Fig. 1A and B), and an exon 11 mutation was identified in the *KIT* gene of 1 thymic carcinoma (Fig. 1C). Because these mutations were not detected in the normal lung tissues from the same patients, they were considered to be somatic mutations. Both patients whose tumors were positive for *EGFR* mutation were never smokers. All three patients had undergone surgical resection, and they are currently alive and relapse-free.

Table 2 EGFR status of thymomas and thymic carcinomas

EGFR mutation	Thymoma ($n = 20$)	Thymic carcinoma ($n = 9$)	
Exon 18	0	0	
Exon 19	0	0	
Exon 21	2	0	
No mutation	18	9	
EGFR expression	Thymoma ($n = 21$)	Thymic carcinoma ($n = 17$)	P
Positive	15 (71%)	9 (53%)	0.31

Table 3 KIT status of thymomas and thymic carcinomas

KIT mutation	Thymoma ($n = 22$)	Thymic carcinoma ($n = 11$)	
Exon 9	0	0	
Exon 11	0	1	
Exon 13	0	0	
Exon 17	0	0	
No mutation	22	10	
KIT expression	Thymoma ($n = 24$)	Thymic carcinoma ($n = 17$)	P
Positive	0 (0%)	15 (88%)	< 0.0001

4. Discussion

In this study, *EGFR* mutations were observed in the DNA sequences of 2 thymomas of 29 tumors analyzed, and analysis of the *KIT* mutation status of 22 thymomas and 11 thymic carcinomas by direct sequencing revealed a missense mutation in exon 11 in only 1 thymic carcinoma. By contrast, 71% of the thymomas and 53% of the thymic carcinomas expressed EGFR protein, and overexpression of KIT was observed in 88% of the thymic carcinomas and 0% of the thymomas. The results show that the EGFR and KIT protein expression in the thymomas and thymic carcinomas was not associated with *EGFR* or *KIT* mutations.

A review of the medical literature retrieved reports of two studies that investigated *EGFR* mutations in thymomas or thymic carcinomas [19,20] and of one study that tested thymic carcinomas for *KIT* mutations [13]. Suzuki et al. reported that direct sequencing did not reveal any *EGFR* missense mutations in a total of 38 thymoma samples obtained from Japanese patients [19]. Meister et al. reported detecting no mutations in the tyrosine kinase domain of *EGFR* in 20 DNA samples from 17 thymomas and 3 thymic carcinomas analyzed by direct sequencing [20]. Pan et al. performed a mutation analysis of *KIT* by direct DNA sequencing in 21 thymic carcinomas, but found none [13]. To date, *EGFR* mutations (double missense mutations: G719A in exon 18 and L858R in exon 21) have been reported in one case of thymic carcinoma [16], and a *KIT* mutation (V560del in exon 11) in one case of thymic carcinoma [15]. The results of our study and review of the literature suggest that *EGFR* or *KIT* mutations are rare in thymomas and thymic carcinomas but that expression of EGFR and KIT is frequently present. Mutations that activate receptor tyrosine kinases contribute to the development of human carcinomas, and the activation of a mutation in the *KIT* gene is thought to be the most important factor in the pathogenesis of GISTs [7,8]. However, we speculate that *EGFR* or *KIT* mutations may not be implicated in the carcinogenesis of thymomas and thymic

Table 4 Summary of thymoma and thymic carcinoma patients with *EGFR* or *KIT* mutations in their tumors

Clinical characteristics						Mutation				IHC
No.	Age/sex	Smoking status	Masaoka stage	Histology	Clinical outcome	Gene	Exon	Nucleotide change	Amino acid change	
1	65/F	Never	II	Thymoma (type A)	3 years of disease-free survival after complete resection	*EGFR*	21	2573T > G	L858R	EGFR (+)
2	69/F	Never	III	Thymoma (type B1)	5 years of disease-free survival after complete resection	*EGFR*	21	2588G > A	G863D	EGFR (−)
3	59/M	Former (20 pack-years)	I	Thymic carcinoma (Sq)	6 years of disease-free survival after complete resection	*KIT*	11	1748T > C	L576P	KIT (+)

Abbreviations: Sq, squamous cell carcinoma; IHC, immunohistochemistry.

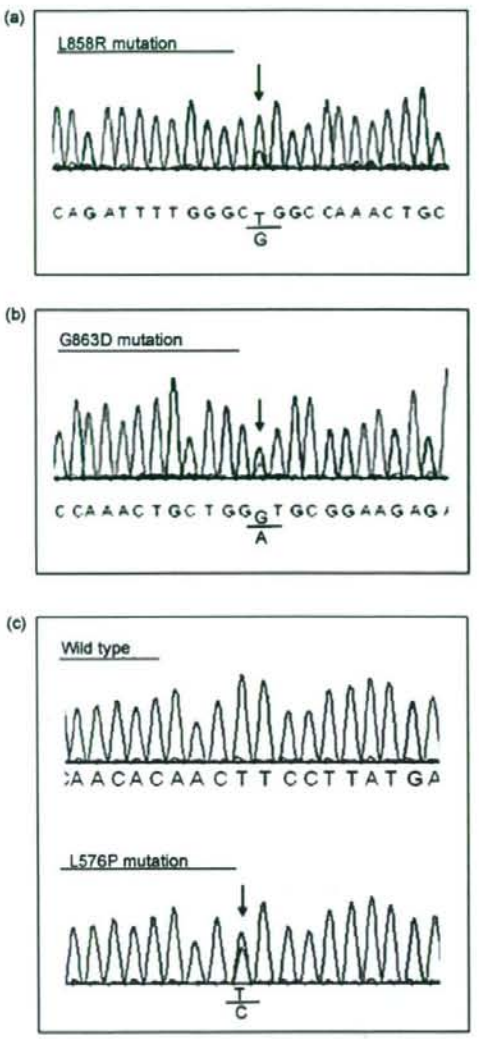

Fig. 1 Electropherograms of the products of direct sequencing of *EGFR* and *KIT*. (a and b) Two thymomas contained a single missense point mutation in exon 21 of *EGFR*. (c) One thymic carcinoma contained a single missense point mutation in exon 11 of *KIT*.

carcinomas because of the low frequency of *EGFR* or *KIT* mutations in these tumors.

Remarkably, the *EGFR* mutations (L858R and G863D, respectively, in exon 21) observed in the 2 thymomas in our study were similar to the active mutations in NSCLC that have been reported to be predictors of a therapeutic response to EGFR-TKI by NSCLCs [9,21]. Moreover, the *KIT* mutation (L576P in exon 11) identified in the 1 thymic carcinoma in our study had previously been described as one of the mutations that predicts a clinical response of GISTs to