Available online at www.sciencedirect.com
ScienceDirect
Cancer Letters 261 (2008) 165–171
CANCER Letters
www.elsevier.com/locate/canlet

Importance of *UDP-glucuronosyltransferase 1A1*6* for irinotecan toxicities in Japanese cancer patients

Kimie Sai [a,*], Yoshiro Saito [b], Hiromi Sakamoto [c], Kuniaki Shirao [d], Koichi Kurose [e], Mayumi Saeki [f], Shogo Ozawa [g], Nahoko Kaniwa [e], Setsuo Hirohashi [h], Nagahiro Saijo [i], Jun-ichi Sawada [b], Teruhiko Yoshida [c]

[a] *Division of Biosignaling, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan*
[b] *Division of Biochemistry and Immunochemistry, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan*
[c] *Genetics Division, National Cancer Center Research Institute, 5-1-5 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan*
[d] *Division of Internal Medicine, National Cancer Center Hospital, 5-1-5 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan*
[e] *Division of Medical Safety Science, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan*
[f] *Project Team for Pharmacogenetics, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan*
[g] *Division of Pharmacology, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan*
[h] *Pathology Division, National Cancer Center Research Institute, 5-1-5 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan*
[i] *Deputy Director, National Cancer Center Hospital East, 6-5-1 Kashiwanoha, Kashiwa, Chiba 277-8577, Japan*

Received 31 July 2007; received in revised form 31 October 2007; accepted 9 November 2007

Abstract

Recent pharmacogenetic studies on irinotecan have revealed the impact of *UDP glucuronosyltransferase (UGT) 1A1*28* on severe irinotecan toxicities. Although the clinical role of *UGT1A1*6*, which is specifically detected in East Asian patients, in irinotecan toxicities is suggested, clear evidence remains limited. To examine the impact of *6, the association of *UGT1A1* genotypes with severe irinotecan toxicities was retrospectively investigated in Japanese cancer patients. A significant *6-dependent increase in the incidence of grade 3 or 4 neutropenia was observed in 49 patients on irinotecan monotherapy ($p = 0.012$). This study further clarifies the clinical importance of *6 in irinotecan therapy in East Asians.
© 2007 Elsevier Ireland Ltd. All rights reserved.

Keywords: UGT1A1; Pharmacogenetics; Irinotecan; SN-38

1. Introduction

Irinotecan, an anticancer prodrug, is widely applied for a broad range of carcinomas, including colorectal and lung cancers. The active metabolite, SN-38 (7-ethyl-10-hydroxycamptothecin), a topoisomerase I inhibitor, is generated by hydrolysis of the parent compound by carboxylesterases [1]. SN-38 is subsequently glucuronidated by uridine diphosphate glucuronosyltransferase 1As (UGT1As) such as 1A1, 1A7, 1A9 and 1A10, to form the inactive metabolite, SN-38 glucuronide (SN-38G) [2–5]. Among the UGT

* Corresponding author. Tel.: +81 3 3700 9478; fax: +81 3 3707 6950.
E-mail address: sai@nihs.go.jp (K. Sai).

0304-3835/$ - see front matter © 2007 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.canlet.2007.11.009

isoforms, UGT1A1 is thought to be a predominant contributor to SN-38G formation [2,6]. The dose-limiting toxicities in irinotecan therapy are severe diarrhea and leucopenia [7], and lowered UGT activity is well correlated with severe irinotecan toxicities [8]. Since Ando et al. first reported the significant relevance of *UGT1A1*28* – a repeat polymorphism in the TATA box (−40_−39insTA) – to severe neutropenia/diarrhea [9], a number of clinical studies, primarily conducted in Caucasian patients, have shown associations between *UGT1A1*28* and lowered SN-38G formation or severe neutropenia/diarrhea [10–13]. Based on these findings, the Food and Drug Administration (FDA) of the United States approved a revision of the label for Camptosar (irinotecan HCl) (NDA 20-571/S-024/S-027/S-028), recommending "a reduction in the starting dose by at least one level of irinotecan for the *UGT1A1*28* homozygous patients". Subsequently, the clinical application of *UGT1A1*28* testing was put into practice for irinotecan therapy in the United States.

To implement personalized irinotecan therapy in Asian countries, the racial differences in *UGT1A1* polymorphisms among Caucasians, African-Americans, and Asians must be taken into consideration [14]. For East Asians, the frequency of *28 is one third of that of Caucasians or African-Americans, and another low-activity allele *6 [211G>A(G71R)], which is not detected in Caucasians or African-Americans, shows the same frequency as the *28 allele. Clinical studies in Japanese cancer patients have demonstrated that significantly low area under concentration-time curve (AUC) ratios of SN-38G to SN-38 are observed in patients having *6 and/or *28 [15–17], suggesting the necessity of typing *6 in addition to *28. A recent report on Korean lung cancer patients who received a combination therapy of irinotecan and cisplatin, showed a significant association of *6 homozygotes with severe neutropenia [18]. However, data on the role of *6 in irinotecan toxicities is still limited in terms of the various irinotecan-containing regimens. In the first study by Ando et al. on Japanese cancer patients, the association of *6 with irinotecan toxicities was not evident, but a possible enhancement of *28-related toxicities by *6 was suggested [9]. Other studies in Japanese patients showed an additive effect of *6 on the lowered UGT activity by *28 [15–17]. A significant association of the genetic marker "*6 or *28" with severe neutropenia was also shown in our previous study, but due to a lack of *6 homozygotes in our patient population, the effect of *6 alone was not confirmed [17].

In this study, to further demonstrate the clinical importance of *6 alone, *UGT1A1* genotypes were determined using DNA extracted from paraffin-embedded specimens (non-cancerous tissues) from 75 Japanese cancer patients by the pyrosequencing method [19,20], and the associations between *UGT1A1* genotype and severe irinotecan toxicities and serum total bilirubin levels were retrospectively analyzed.

2. Materials and methods

2.1. Patients and irinotecan treatment

In a post-marketing surveillance study conducted by Daiichi Pharmaceutical Co., Ltd. (currently Daiichi Sankyo Co., Ltd., Tokyo, Japan), irinotecan was prescribed to 297 patients with various types of cancers from 1995 to 2000 at the National Cancer Center Hospital. The patients were selected through standard clinical practice according to the drug label for indications and contraindications. Methanol-fixed, paraffin-embedded archival tissue specimens, which were necessary for high-quality extraction of DNA greater than 2 kb in size [21], were available for 75 of the 297 patients and were analyzed in this study. Irinotecan was administered by intravenous 30-min infusion as a single agent or in combination chemotherapy at a dose of 60 mg/m^2 (weekly or biweekly), 100 mg/m^2 (biweekly), or 150 mg/m^2 (biweekly). Profiles of the patients in this study, including cancer type, treatment history, and regimens, are summarized in Table 1. The pre-treatment levels of serum total bilirubin were determined by a kit (VL T-BIL, Azwell Inc., Osaka, Japan) according to an enzymatic method using bilirubin oxidase [22]. Toxicities were monitored during irinotecan therapy and graded according to the Common Toxicity Criteria version 2 of the National Cancer Institute.

Because the samples in this study were residual specimens remaining after histopathological diagnosis in the hospital and not collected specifically for research purposes, the samples and their clinical information were anonymized in an unlinkable fashion according to the Ethics Guidelines for Human Genome/Gene Analysis Research by the Ministry of Education, Culture, Sports, Science and Technology, Ministry of Health, Labour and Welfare, and Ministry of Economy, Trade and Industry of Japan. This study was approved by the ethics committees of the National Cancer Center and the National Institute of Health Sciences.

2.2. DNA extraction from paraffin-embedded tissue sections and genotyping of UGT1A1 polymorphisms

Three sections (20 μm of pathologically normal tissues around tumors) were deparaffinized twice by treat-

Table 1
Profiles of cancer patients in this study

		No. of patients
Patients genotyped		75
(Male/female)		(51/24)
Age		
Mean/range (y)	50.7/34–75	
Performance Status[a]		
	0/1/2	18/48/8
Previous treatment		
Surgery[a]	+/–	71/3
Chemotherapy[b]	+/–	63/10
Radiotherapy[b]	+/–	9/64
Combination therapy and tumor type [dose of irinotecan (mg/m^2)/(w or 2w)[c]]		
Irinotecan monotherapy	Lung (60/w or 100/2w)	4
	Stomach (100/2w or 150/2w)	5
	Colon (100/2w or 150/2w)	40
With cisplatin	Lung (60/w or 100/2w)	4
	Stomach (60/2w)	11
With mitomycin C (MMC)	Stomach (150/2w)	8
	Breast (120/2w)	1
With 5-fluorouracil (5-FU)	Colon (150/2w)	2
Available data on serum bilirubin levels		37

[a] Data from one patient is lacking.
[b] Data from two patients are lacking.
[c] Weekly or biweekly.

ment with 1.5 ml of xylene at room temperature. After centrifugations, the residual pellet was then washed twice with 1.5 ml of ethanol. Finally, the pellet was dried at 37 °C for 15 min. DNA extraction was performed using a QIAamp tissue kit (QIAGEN K.K., Tokyo, Japan) according to the manufacturer's instructions with some modifications. Briefly, 540 μl of ATL lysis buffer and 60 μl of proteinase K (Qiagen) were added to each pellet, mixed thoroughly, and incubated at 56 °C for 3 h with a rotator. Any remaining tissue debris was removed by centrifugation, and the resulting supernatant was used for the extraction. Twelve microliters of RNase A (100 mg/ml) was added to the supernatant and incubated for 2 min at room temperature. Next, 600 μl of buffer AL was added and mixed thoroughly, and the mixture was incubated at 70 °C for 10 min. Six-hundred microliters of ethanol was added to the solution and mixed well, followed by extraction of DNA using a Qiagen DNA extraction column. The DNA was eluted in a final elution volume of 150 μl. The yield was determined using a NanoDrop spectrophotometer (NanoDrop Technology, Inc, Rockland, DE, USA) and the size of the extracted DNA was checked by agarose gel electrophoresis.

Genotyping of *UGT1A1**6* (211G>A, G71R), **28* (–364C>T, which is perfectly linked with –40_–39insTA in Japanese), and **60* (–3279T>G) were performed by pyrosequencing as described previously [19,20].

2.3. Association analysis and statistics

For association analysis, we focused on incidences of severe diarrhea and neutropenia (grade 3 or greater) observed during irinotecan-therapy. The incidence of severe diarrhea was very low, and the incidence of neutropenia was higher in combination therapy. Therefore, the association of neutropenia with *UGT1A1* genotypes was primarily evaluated in 49 patients with irinotecan monotherapy. As a parameter for in vivo UGT1A1 activity, serum total bilirubin levels taken at baseline from 37 patients were also used.

Statistical analysis for evaluation of the relationship between *UGT1A1* genotypes and severe neutropenia was performed using the chi-square test for trend using Prism version 4.0 (GraphPad Prism Software Inc., San Diego, CA). The gene-dose effect of the genetic marker "**6 or *28*" on serum total bilirubin levels was analyzed using the Jonckheere–Terpstra (JT) test in the SAS system (version 5.0, SAS Institute, Inc., Cary, NC). The *P*-value of 0.05 (two-tailed) was set as a significant level. Multivariate logistic regression analysis on neutropenia (grade 3 or greater) was performed using JMP software (version 6.0.0, SAS Institute, Inc., Cary, NC), including variables for age, sex, body surface area, performance status, concomitant disease, history of adverse reaction, irinotecan dosage, dosing interval, and *UGT1A1* genotypes. The variables in the final model for neutropenia were chosen using the forward and backward stepwise procedure at the significance level of 0.1.

3. Results

3.1. UGT1A1 diplotypes/haplotypes

The diplotypes and haplotypes (**1, *60, *6* and **28*) of *UGT1A1* exon 1 were analyzed in 75 Japanese cancer patients (Table 1) and their frequencies were summarized (Table 2). The haplotypes were assigned according to our previous definition [15]. It should be noted that the **60* haplotype does not harbor the **28* allele (–40_–39insTA), but most of the **28* haplotype does harbor the **60* allele (–3279T>G). In this study, the **28* homozygote was not present, and the frequency of haplotype **28* (0.113) was slightly lower than that found in our previous study (0.138) [17]. In contrast, the frequency of haplotype **6* (0.213) was higher than that found in the previous study (0.167) [17].

Table 2
Frequencies of *UGT1A1* diplotypes (A) and haplotypes (B) for cancer patients in this study

		Frequency
(A) Diplotype	No. of patients (N = 75)	
*1/*1	21	0.280
*1/*60	9	0.120
*60/*60	2	0.027
*6/*1	14	0.187
*6/*60	8	0.107
*6/*6	4	0.053
*28/*1	12	0.160
*28/*60	3	0.040
*28/*6	2	0.027
*28/*28	0	0.000
(B) Haplotype[a]	No. of chromosomes (N = 150)	
*1	77	0.513
*60	24	0.160
*6	32	0.213
*28	17	0.113

[a] Haplotype definition follows the previous report [15]; *60, −3279T>G without −40_−39insTA; *6, 211G>A(G71R); *28, −40_−39insTA.

3.2. Association of UGT1A1 genotypes with serum total bilirubin levels

Serum total bilirubin levels at baseline, a parameter of in vivo UGT1A1 activity, were available from 37 patients (treated by various regimens), and we analyzed their association with *UGT1A1* genotypes (Fig. 1). The median values of total bilirubin in *60/*1, *28/*1 and *6/*1 heterozygotes were not significantly different from that of the wild type (*1/*1). Higher median values were observed for the *6 homozygotes (*6/*6) and the double heterozygotes of *6 and *28 (*6/*28) than that of the wild type (*1/*1), with increases of 1.9-fold and 2.2-fold, respectively. Since *6 and *28 are mutually independent and their reducing effects on UGT activity are equivalent [15,17], diplotypes were classified by the presence of "*6 or *28" (indicated by "+" in Fig. 1). As shown in Fig. 1, a significant "*6 or *28"-dependent increase in total bilirubin levels was observed ($p = 0.0088$, Jonckheere–Terpstra test).

3.3. Severe toxicities observed in this study

Incidences of severe diarrhea and neutropenia (grade 3 or greater) are shown in Table 3 for each irinotecan-containing regimen. Grade 3 diarrhea was observed in only 4 of the 75 subjects, and since the incidence of diarrhea was low (5.3%), an association analysis on diarrhea was not conducted. Regarding neutropenia, 26 patients experienced grade 3 or 4 neutropenia. Of these 26 patients, 90% experienced neutropenia within 2 months after starting irinotecan-therapy, and 70% within 2 weeks. Significant differences in neutropenia incidences were observed among the regimens used, and considerably high incidences were observed in the combination therapies. Accordingly, association of the *UGT1A1* genotypes with severe neutropenia was analyzed primarily in the patients who received irinotecan-monotherapy.

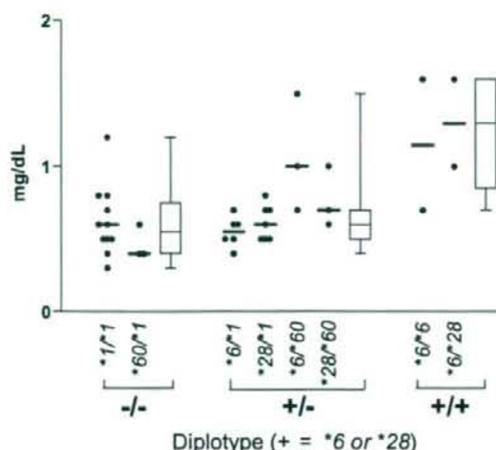

Fig. 1. Effects of *UGT1A1* genotypes on serum total bilirubin levels at baseline in Japanese cancer patients ($N = 37$). Each point represents a patient, and the median value of each diplotype is shown with a bar. All diplotypes are classified into −/−, +/−, and +/+ by the genetic marker, "*UGT1A1**6 or *28", indicated by "+", and their distributions are shown by a box representing the 25–75 percentiles with a bar at the median and lines representing the highest and lowest values. A significant "*6 or *28"-dependent increase in total bilirubin levels was observed ($p = 0.0088$, Jonckheere–Terpstra test).

Table 3
Severe toxicities observed in Japanese cancer patients

Treatment	Diarrhea[a]/total (%)	Neutropenia[b]/total (%)
Total patients	4/75 (5.3)	26/75 (34.7)
Irinotecan alone	1/49 (2.0)	6/49 (12.2)
With CDDP	2/15 (13.3)	11/15 (73.3)
With MMC	1/9 (11.1)	8/9 (88.9)
With 5-FU	0/2 (0.0)	1/2 (50.0)
P-value[c]	NS	<0.0001

[a] Grade 3.
[b] Grade 3 or 4.
[c] Chi-squre test.

3.4. Association of UGT1A1 genotypes with neutropenia

Since significant associations of *UGT1A1**6 and *28 with increased total bilirubin levels (decreased UGT-activity) were once again confirmed in this study, we assessed the clinical relevance of these haplotypes, focusing on the effect of *6 on severe neutropenia. In the 49

patients who received irinotecan monotherapy, the incidence of grade 3 or 4 neutropenia was *6-dependently increased ($p = 0.012$ in the chi-square test for trend). Namely, incidences of severe neutropenia in the *6 heterozygotes (*6/*1,*6/*60, and *6/*28) and homozygotes (*6/*6) were 2.3-fold and 15-fold higher, respectively, than that seen in the non-*6 bearing patients (*1/*1, *60/*1, *28/*1, and *28/*60) (Table 4). In this study, no *28 heterozygotes (*28/*1 and *28/*60) experienced any severe neutropenia, and there were no *28 homozygotes enrolled. Therefore, the effect of *28 could not be determined. For the *60-bearing patients without *6 or *28 (only heterozygote, *60/*1), one patient among six experienced severe neutropenia, and no significant *60-dependent increase was observed (data not shown). Although no statistically significant association of the *28 heterozygotes with severe neutropenia was confirmed in this study, the incidence of discontinuation of irinotecan monotherapy was higher in the *28-bearing patients (91%, $N = 11$) than that in the non-*28 subjects (79%, N = 38), while *60- or *6-dependent increased discontinuation rates were not found (data not shown). For the patients with cisplatin-combination therapy, a higher incidence of severe neutropenia was observed in the *6-bearing patients (*6/*1, *6/*60, and *6/*6) (100%, $N = 3$) than that in the non-*6 bearing subjects (*1/*1, *60/*1, *60/*60, and *28/*1) (66.7%, $N = 12$).

3.5. Multivariate analysis of neutropenia

In order to further clarify the clinical impact of *6 on irinotecan toxicities, multivariate logistic regression analysis on grade 3 or 4 neutropenia was conducted using variables, including *UGT1A1* genotypes and patient background factors, described in Section 2. The final model revealed a significant association of *6 with the incidence of grade 3 or 4 neutropenia at an odds ratio of 5.87 (Table 5).

4. Discussion

The clinical application of the genetic test for *UGT1A1**28 prior to irinotecan therapy has been in practice in the United States since 2005, which was based on cumulative evidence supporting the significant association of *28 with severe irinotecan toxicity [9–13]. Most of the evidence was obtained in Caucasian patients, where *28 is relatively frequent (30–40%) [14]. Although additive effects of another low activity allele, *6, which is specific for East Asians, has been also suggested [9,15–17], direct evidence in Japanese patients has remained limited. In this study, we clearly showed the significant correlation of *6 to grade 3 or 4 neutropenia in Japanese cancer patients who received irinotecan monotherapy. An increased incidence of severe neutropenia was also observed in the *6-bearing patients using cisplatin combination therapy. This finding is in accordance with a report on Korean lung cancer patients who received a combination therapy of irinotecan and cisplatin, which showed a significant association of *6 homozygotes with grade 4 neutropenia [18]. Since combination therapies using irinotecan may cause higher incidences of severe toxicities, the *UGT1A1* polymorphisms should be carefully considered in regimens that include irinotecan.

Since the alleles *6 and *28 are mutually independent [15] and their effects on the UGT activities were shown to be equivalent, the usefulness of the genetic marker "*6 or *28" for personalized irinotecan therapies has been suggested [17]. This was also supported in the current study, which showed a "*6 or *28"-dependent increase in serum total bilirubin levels (Fig. 1). Because of the low frequency of *28 without homozygotes among our subjects, the influence of *28 on toxicities was not clearly demonstrated, as in the case of the Korean patients where the allele frequency of *1A1**6 (23.5%) was much higher than that of *1A1**28 (7.3%) [18]. However, in the current study, the double heterozygotes of *6 and *28 (*6/*28) showed increases in serum total bilirubin levels (Fig. 1). Moreover, a higher incidence of severe neutropenia in the *6/*28 patients was observed, although the patient number was small ($N = 2$) (Table 4). This finding also indi-

Table 4
Association of *UGT1A1* genotypes with severe neutropenia (grade 3 or 4) in irinotecan monotherapy

Diplotype[b]	Neutropenia[a]/total (%)	Effect of *6 (%)	
–/–	1/20 (5.0)	non-*6/non-*6	(3.4)
*28/–	0/9 (0.0)		
*6/–	3/16 (18.8)	*6/non-*6	(22.2)
*6/*28	1/2 (50.0)		
*6/*6	1/2 (50.0)	*6/*6	(50.0)
P-value[c]		0.012	

[a] Grade 3 or 4.
[b] "–" represents "*1 or *60".
[c] Chi-square test for trend.

Table 5
Multivariate logistic regression analyasis of severe neutropenia (grade 3 or 4) in irinotecan monotherapy

Variable	Coefficient	SE	*P*-value	Odds ratio	(95% Confidence limit)
*UGT1A1**6	1.77	0.809	0.0289	5.87	(1.37–39.6)

$R^2 = 0.157$, Intercept = 3.15, $N = 49$.

cates the importance of "*6 or *28" in severe neutropenia, and in fact, a gene-dose effect of "*6 or *28" ($p = 0.04$ in the chi-square test for trend) and its significant contribution in multivariate analysis ($p = 0.0326$) were also confirmed (data not shown).

For the *60 haplotype (-3279T>G without -40_-39insTA), no association of *60 with severe neutropenia was observed in this study, which coincides with reports of other studies on Japanese cancer patients [17,23]. As for the *27 allele [686C>A(P229Q)], it was linked with the *28 allele and the haplotype was defined as the *28 subtype, *28c [15]. One *28c-heterozygous patient with irinotecan monotherapy showed no severe neutropenia, suggesting a small contribution of the *27 allele (data not shown).

In this study, the association between *UGT1A1* genotypes and antitumor activity was difficult to evaluate because of the small number of subjects stratified into each tumor type. Further clinical studies are needed to establish methods for selection of the appropriate regimen or dosage based on the *UGT1A1* genotypes, where a balance between toxicity and antitumor effect should be considered.

In conclusion, this study demonstrated the significant association of *UGT1A1*6* with severe irinotecan-mediated neutropenia. The current data also supported the usefulness of the genetic marker "*6 or *28" for personalized irinotecan therapy in Japanese, and likely East Asian, patients.

Acknowledgements

This study was supported in part by the Program for the Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation, and by the Program for the Promotion of Studies in Health Sciences of the Ministry of Health, Labor and Welfare of Japan. We thank Daiichi Pharmaceutical Co., Ltd. (currently Daiichi Sankyo Co., Ltd.) and Yakult Honsha Co., Ltd. for providing useful information and technical advice on the analysis of the adverse event data of this study. We also thank Ms. Chie Sudo for her administrative assistance.

References

[1] J.G. Slatter, P. Su, J.P. Sams, L.J. Schaaf, L.C. Wienkers, Bioactivation of the anticancer agent CPT-11 to SN-38 by human hepatic microsomal carboxylesterases and the in vitro assessment of potential drug interactions, Drug Metab. Dispos. 25 (1997) 1157–1164.

[2] L. Iyer, C.D. King, P.F. Whitington, M.D. Green, S.K. Roy, T.R. Tephly, et al., Genetic predisposition to the metabolism of irinotecan (CPT-11). Role of uridine diphosphate glucuronosyltransferase isoform 1A1 in the glucuronidation of its active metabolite (SN-38) in human liver microsomes, J. Clin. Invest. 15 (1998) 847–854.

[3] M. Ciotti, N. Basu, M. Brangi, I.S. Owens, Glucuronidation of 7-ethyl-10-hydroxycamptothecin (SN-38) by the human UDP-glucuronosyltransferases encoded at the UGT1 locus, Biochem. Biophys. Res. Commun. 260 (1999) 199–202.

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

[5] T. Oguri, T. Takahashi, M. Miyazaki, T. Isobe, N. Kohno, P.I. Mackenzie, et al., UGT1A10 is responsible for SN-38 glucuronidation and its expression in human lung cancers, Anticancer Res. 24 (2004) 2893–2896.

[6] N. Hanioka, S. Ozawa, H. Jinno, M. Ando, Y. Saito, J. Sawada, Human liver UDP-glucuronosyltransferase isoforms involved in the glucuronidation of 7-ethyl-10-hydroxycamptothecin, Xenobiotica 31 (2001) 687–699.

[7] M. de Forni, R. Bugat, G.G. Chabot, S. Culine, J.M. Extra, A. Gouyette, et al., Phase I and pharmacokinetic study of the camptothecin derivative irinotecan, administered on a weekly schedule in cancer patients, Cancer Res. 54 (1994) 4347–4354.

[8] E. Gupta, T.M. Lestingi, R. Mick, J. Ramirez, E.E. Vokes, M.J. Ratain, Metabolic fate of irinotecan in humans: correlation of glucuronidation with diarrhea, Cancer Res. 54 (1994) 3723–3725.

[9] Y. Ando, H. Saka, M. Ando, T. Sawa, K. Muro, H. Ueoka, et al., Polymorphisms of UDP-glucuronosyltransferase gene and irinotecan toxicity: a pharmacogenetic analysis, Cancer Res. 60 (2000) 6921–6926.

[10] L. Iyer, S. Das, L. Janisch, M. Wen, J. Ramirez, T. Karrison, et al., UGT1A1*28 polymorphism as a determinant of irinotecan disposition and toxicity, Pharmacogenomics J. 2 (2002) 43–47.

[11] F. Innocenti, S.D. Undevia, L. Iyer, P.X. Chen, S. Das, M. Kocherginsky, et al., Genetic variants in the UDP-glucuronosyltransferase 1A1 gene predict the risk of severe neutropenia of irinotecan, J. Clin. Oncol. 22 (2004) 1382–1388.

[12] E. Marcuello, A. Altes, A. Menoyo, E. Del Rio, M. Gomez-Pardo, M. Baiget, UGT1A1 gene variations and irinotecan treatment in patients with metastatic colorectal cancer, Br. J. Cancer 91 (2004) 678–682.

[13] E. Rouits, M. Boisdron-Celle, A. Dumont, O. Guerin, A. Morel, E. Gamelin, Relevance of different UGT1A1 polymorphisms in irinotecan-induced toxicity: a molecular and clinical study of 75 patients, Clin. Cancer Res. 10 (2004) 5151–5159.

[14] N. Kaniwa, K. Kurose, H. Jinno, T. Tanaka-Kagawa, Y. Saito, M. Saeki, et al., Racial variability in haplotype frequencies of UGT1A1 and glucuronidation activity of a novel single nucleotide polymorphism 686C> T (P229L) found in an African-American, Drug Metab. Dispos. 33 (2005) 458–465.

[15] K. Sai, M. Saeki, Y. Saito, S. Ozawa, N. Katori, H. Jinno, et al., UGT1A1 haplotypes associated with reduced glucuronidation and increased serum bilirubin in irinotecan-administered Japanese patients with cancer, Clin. Pharmacol. Ther. 75 (2004) 501–515.

[16] K. Araki, K. Fujita, Y. Ando, F. Nagashima, W. Yamamoto, H. Endo, et al., Pharmacogenetic impact of polymorphisms in the coding region of the UGT1A1 gene on SN-38 glucuronidation in Japanese patients with cancer, Cancer Sci. 97 (2006) 1255–1259.

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

[18] J.Y. Han, H.S. Lim, E.S. Shin, Y.K. Yoo, Y.H. Park, J.E. Lee, et al., Comprehensive analysis of UGT1A polymorphisms predictive for pharmacokinetics and treatment outcome in patients with non-small-cell lung cancer treated with irinotecan and cisplatin, J. Clin. Oncol. 24 (2006) 2237–2244.

[19] M. Saeki, Y. Saito, H. Jinno, M. Tohkin, K. Kurose, N. Kaniwa, et al., Comprehensive UGT1A1 genotyping in a Japanese population by pyrosequencing, Clin. Chem. 49 (2003) 1182–1185.

[20] M. Saeki, Y. Saito, K. Sai, K. Maekawa, N. Kaniwa, J. Sawada, et al., A combinatorial haplotype of the UDP-glucuronosyltransferase 1A1 gene (#60-#IB) increases total bilirubin concentrations in Japanese volunteers, Clin. Chem. 53 (2007) 356–358.

[21] M. Noguchi, S. Furuya, T. Takeuchi, S. Hirohashi, Modified formalin and methanol fixation methods for molecular biological and morphological analyses, Pathol. Int. 47 (1997) 685–691.

[22] S. Otsuji, K. Mizuno, S. Ito, S. Kawahara, M. Kai, A new enzymatic approach for estimating total and direct bilirubin, Clin. Biochem. 21 (1988) 33–38.

[23] C. Kitagawa, M. Ando, Y. Ando, Y. Sekido, K. Wakai, K. Imaizumi, et al., Genetic polymorphism in the phenobarbital-responsive enhancer module of the UDP-glucuronosyltransferase 1A1 gene and irinotecan toxicity, Pharmacogenet. Genomics 15 (2005) 35–41.

VOLUME 26 · NUMBER 4 · FEBRUARY 1 2008

JOURNAL OF CLINICAL ONCOLOGY

ORIGINAL REPORT

Phase II Trial of Preoperative Chemoradiotherapy Followed by Surgical Resection in Patients With Superior Sulcus Non–Small-Cell Lung Cancers: Report of Japan Clinical Oncology Group Trial 9806

Hideo Kunitoh, Harubumi Kato, Masahiro Tsuboi, Taro Shibata, Hisao Asamura, Yukito Ichonose, Nobuyuki Katakami, Kanji Nagai, Tetsuya Mitsudomi, Akihide Matsumura, Ken Nakagawa, Hirohito Tada, and Nagahiro Saijo

From the Department of Medical Oncology and Division of Thoracic Surgery, National Cancer Center Hospital; Department of Thoracic Surgery, Tokyo Medical University; Japan Clinical Oncology Group Data Center, Center for Cancer Control and Information Services, National Cancer Center; Department of Thoracic Surgery, Cancer Institute Hospital, Tokyo; Department of Chest Surgery, National Kyushu Cancer Center, Fukuoka; Pulmonary Unit, Kobe City Medical Center General Hospital, Kobe; Department of Thoracic Surgery, National Cancer Center Hospital East, Kashiwa; Department of Thoracic Surgery, Aichi Cancer Center Hospital, Nagoya; Department of Surgery, National Hospital Organization Kinki-Chuo Chest Medical Center, Sakai; and Department of Thoracic Surgery, Osaka City General Hospital, Osaka, Japan.

Submitted September 1, 2007; accepted October 25, 2007.

Supported by the Grant-in-Aid for Cancer Research from the Ministry of Health, Labour and Welfare of Japan (Grants No. 11S-2, 11S-4, 14S-2, 14S-4, 17S-2, and 17S-5).

Presented in part at the 39th Annual Meeting of the American Society of Clinical Oncology, May 31-June 3, 2003, Chicago, IL, and at the 11th World Conference on Lung Cancer, July 3-6, 2005, Barcelona, Spain.

Authors' disclosures of potential conflicts of interest and author contributions are found at the end of this article.

Corresponding author: Hideo Kunitoh, MD, Department of Medical Oncology, National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan; e-mail: hkkunito@ncc.go.jp.

© 2008 by American Society of Clinical Oncology

0732-183X/08/2604-644/$20.00

DOI: 10.1200/JCO.2007.14.1911

ABSTRACT

Purpose
To evaluate the safety and efficacy of preoperative chemoradiotherapy followed by surgical resection for superior sulcus tumors (SSTs).

Patients and Methods
Patients with pathologically documented non–small-cell lung cancer with invasion of the first rib or more superior chest wall were enrolled as eligible; those with distant metastasis, pleural dissemination, and/or mediastinal node involvement were excluded. Patients received two cycles of chemotherapy every 4 weeks as follows; mitomycin 8 mg/m^2 on day 1, vindesine 3 mg/m^2 on days 1 and 8, and cisplatin 80 mg/m^2 on day 1. Radiotherapy directed at the tumor and the ipsilateral supraclavicular nodes was started on day 2 of each course, at the total dose of 45 Gy in 25 fractions, with a 1-week split. Thoracotomy was undertaken 2 to 4 weeks after completion of the chemoradiotherapy. Those with unresectable disease received boost radiotherapy.

Results
From May 1999 to November 2002, 76 patients were enrolled, of whom 20 had T4 disease; 75 patients were fully assessable. Chemoradiotherapy was generally well tolerated. Fifty-seven patients (76%) underwent surgical resection, and pathologic complete resection was achieved in 51 patients (68%). There were 12 patients with pathologic complete response. Major postoperative morbidity, including chylothorax, empyema, pneumonitis, adult respiratory distress syndrome, and bleeding, was observed in eight patients. There were three treatment-related deaths, including two deaths owing to postsurgical complications and one death owing to sepsis during chemoradiotherapy. The disease-free and overall survival rates at 3 years were 49% and 61%, respectively; at 5 years, they were 45% and 56%, respectively.

Conclusion
This trimodality approach is safe and effective for the treatment of patients with SSTs.

J Clin Oncol 26:644-649. © 2008 by American Society of Clinical Oncology

INTRODUCTION

Superior sulcus tumors (SSTs), involving structures at the thoracic inlet, represent a small subtype of non–small-cell lung carcinoma (NSCLC). These SSTs, first described by Henry Pancoast[1,2] and thus also called Pancoast tumors, have posed a challenging problem for surgeons, radiation oncologists, and medical oncologists alike, ever since they were first described.[3]

Preoperative radiotherapy has long been the community standard in the management of SSTs.[4-17] However, both the complete resection rate (approximately 50%) and long-term survival rate (approximately 30%) have remained poor and unchanged over the last 40 years, since the first treatment strategy was reported in the 1960s. Local control has remained the main problem,[15,17,18] adversely affecting quality of life as well as survival of patients. Presence of mediastinal lymph node metastasis (N2 status) has been reported to be associated with a particularly poor prognosis.[9,18]

However, a series of clinical trials over the last two decades have shown concurrent chemoradiotherapy to be beneficial in the treatment of unresectable stage III NSCLC.[19-21] The addition of chemotherapy to thoracic radiotherapy seems to suppress distant micrometastases,[22,23] and giving

Information downloaded from jco.ascopubs.org and provided by KOKURITSU GAN CENTER on March 9, 2009 from 160.190.160.15.
Copyright © 2008 by the American Society of Clinical Oncology. All rights reserved.

concurrent chemotherapy with radiotherapy has been shown to yield improved local control[19,24] with survival benefit.

Encouraged by the promising data of concurrent chemoradiotherapy for N2 NSCLC, the Southwest Oncology Group (SWOG) applied this modality as preoperative therapy for patients with SSTs (SWOG 9416, Intergroup Trial 0160), and reported favorable results.[25]

The Japan Clinical Oncology Group (JCOG) launched another trial of this preoperative concurrent chemoradiotherapy, or the trimodality approach, for the treatment of SSTs in 1999, before the first report of SWOG 9416 was published. Our study was initiated to evaluate the safety and efficacy of this treatment strategy in this rare subset of patients with NSCLC. As the induction treatment, we used mitomycin, vindesine, and cisplatin (MVP) combination chemotherapy, which has been demonstrated to be safe and effective for concurrent chemotherapy with thoracic radiotherapy in Japanese trials.[19]

PATIENTS AND METHODS

Eligibility Criteria

Patients with untreated histologically or cytologically documented NSCLC involving the superior sulcus with clinical stage T3 or T4 disease were eligible for entry onto this study. T4 diseases included tumor invasion to the spine (including to a transverse process of vertebra), aorta, or superior vena cava; invasion to the chest wall or subclavian vessels was included in T3 disease. Involvement of the superior sulcus was confirmed by computed tomographic (CT) or magnetic resonance imaging (MRI) evidence of tumor invasion of the first rib or more superior chest wall. Patients with pleural or pericardial dissemination, malignant effusion, and/or distant metastasis (M1) were excluded. Those with clinical N2 disease (mediastinal node involvement) were also excluded; all mediastinal nodes measuring ≥ 1.0 cm in size on CT images were required to be biopsied and documented to be negative for metastasis before patient enrollment. However, those with ipsilateral supraclavicular node involvement (N3) were eligible, unless it was accompanied by mediastinal node metastasis. Each patient was required to fulfill the following criteria: 15 to 74 years of age, Eastern Cooperative Oncology Group performance status of 0 to 1; adequate organ function (ie, leukocyte count ≥ 4,000/μL, platelet count ≥ 10^5/μL, hemoglobin ≥ 11.0 g/dL, serum creatinine less than 1.5 mg/dL, creatinine clearance ≥ 60 mL/min, serum bilirubin less than 1.5 mg/dL, serum ALT and AST less than double the upper limit of the institutional normal range, arterial partial pressure of oxygen ≥ 70 mmHg, and predicted postoperative forced expiratory volume in 1 second ≥ 0.8 L. From July 2001, when the protocol was revised after the death of a patient from septic shock during chemoradiotherapy, those patients with systemic use of corticosteroids were excluded.

Patient eligibility was confirmed by the JCOG Data Center before patient registration. This study was approved by the institutional review boards at each participating center, and written informed consent was obtained from all patients.

Treatment Plan

Induction chemotherapy. Patients received two courses of MVP combination chemotherapy with a 4-week interval in between. Mitomycin was administered at 8 mg/m^2 on chemotherapy day 1, and vindesine was administered at 3 mg/m^2 on days 1 and 8; both were administered as bolus injections. Cisplatin was administered at 80 mg/m^2 as a 2-hour infusion on day 1, with ample hydration and antiemetic administration.

The second cycle of chemotherapy was postponed until all the severe toxicities recovered to grade 1 or 0. If the second cycle could not be started within 2 weeks of the due date, it was canceled, and the patient received only preoperative radiotherapy, if possible.

Induction radiotherapy. Thoracic radiotherapy was started with a linear accelerator (≥ 4 MeV) on chemotherapy day 2. The first session was scheduled to be given with the first chemotherapy cycle at 27 Gy in 15 fractions over 3 weeks. Then the second session was started after a week's interval until day 2 of the second course of chemotherapy. The second session, given with the second cycle of MVP, was administered at 18 Gy in 10 fractions over 2 weeks. The total radiation dose was thus 45 Gy in 25 fractions administered over 6 weeks, including the 1-week split, or interval, between the two sessions; this schedule, including the split, basically followed that of the original method reported by Furuse et al.[19] The radiation field included the primary tumor and the ipsilateral supraclavicular nodes. The mediastinal and hilar nodes were not irradiated, even in cases with hilar node involvement (clinical N1 cases).

Surgery. After the induction chemoradiotherapy, each case was reevaluated to determine the clinical response and resectability. The resectability of the tumor was determined by the multimodality team of each institution, irrespective of the clinical response (tumor shrinkage). Surgical resection of the tumor was performed 2 to 4 weeks after the completion of the induction therapy. The surgical procedures undertaken included lobectomy or pneumonectomy, with systematic node dissection. Standard systematic node dissection, ND2, includes complete removal of the hilar and mediastinal nodes. Less complete dissection includes ND0 (ie, no systematic dissection with or without lymph node sampling) or ND1 (ie, hilar node dissection with or without mediastinal lymph node sampling).

Boost therapy. For unresected or incompletely resected cases, boost radiotherapy of 21.6 Gy in 12 fractions was given. Those who were judged to have undergone complete resection were followed up without additional therapy until clinical evidence of recurrence.

Patient Evaluation and Follow-Up

Before enrollment onto the study, each patient underwent complete medical history taking and physical examination, blood cell count determinations, serum biochemistry testing, arterial blood gas analysis, chest x-ray, ECG, CT scan of the chest, bronchoscopy, CT scan or ultrasound of the upper abdomen, whole-brain CT or MRI, and an isotope bone scan. Chest MRI was recommended for evaluation of the local tumor status but was not mandatory. Blood cell counts, serum biochemistry testing, and chest x-ray were performed weekly during each course of chemotherapy. Chest CT was performed every 3 to 4 weeks during the induction therapy.

Chemotherapy toxicity was evaluated according to the JCOG Toxicity Criteria,[26] modified from the National Cancer Institute Common Toxicity Criteria version 1. Tumor responses were assessed radiographically according to the standard, two-dimensional WHO criteria[27] and were classified into complete response (CR), partial response, no change, progressive disease (PD), and not assessable. Response confirmation at 4 weeks or longer intervals was not necessitated. After curative resection and/or definitive boost radiotherapy, the patients were followed up with periodic re-evaluation, including with chest CT, as well as a systemic survey every 6 months for the first 3 years.

Central Review

Radiographic reviews for eligibility of the enrolled patients and the clinical responses were performed at the time of the JCOG Lung Cancer Surgical Study Group meeting, held every 3 to 4 months. The study coordinator (H.K., a medical oncologist), the group coordinator (M.T., a surgical oncologist), and a few selected investigators of the group reviewed the radiographic films. The clinical response data presented below were all confirmed by this central review.

Statistical Considerations

The primary end point of the study was the survival rate at 3 years. The sample size calculation was performed, as described in Appendix 1 (online only).

Secondary end points included the objective tumor response to chemotherapy, complete resection rate, and postsurgical morbidity/mortality. Both overall survival (OS) and progression-free survival (PFS) were calculated from the date of enrollment by the Kaplan-Meier method. For exploratory analysis to identify prognostic factors, the OS or PFS of subgroups was compared by two-sided log-rank tests. All analyses were performed with the SAS software version 8.2 (SAS Institute, Cary, NC).

Information downloaded from jco.ascopubs.org and provided by KOKURITSU GAN CENTER on March 9, 2009 from 160.190.160.15.
Copyright © 2008 by the American Society of Clinical Oncology. All rights reserved.

RESULTS

Patient Characteristics

From May 1999 to November 2002, 76 patients from 19 institutions were enrolled onto the study. Three patients were ineligible. One patient was found to have concomitant anemia and did not receive the protocol treatment. Two others were found ineligible by the central review, after completion of the protocol therapy; the tumor was judged not to involve the first rib in one case, and in the other, a mediastinal node was judged to be enlarged on chest CT, without confirmation by mediastinoscopy. These two cases were included in the analysis. Therefore, 75 patients were analyzed to determine the toxicities, response rates, surgical and pathologic results, PFS, and OS. All 76 patients were included in the analysis of the patient characteristics, as shown in Table 1. In each of the T4 cases, the tumor was judged to have involved the spine. Nodal status was clinically determined and was pathologically confirmed in only a few cases.

Induction Therapy Delivery and Toxicity

The study schema with the actual numbers of patients receiving the protocol therapy is shown in Appendix Figure A1 (online only).

Table 1. Patient Characteristics (n = 76)

Characteristic	No. of Patients	%
Sex		
Male	67	88
Female	9	12
Age, years		
Median	57.5	
Range	34-74	
ECOG performance status		
0	30	39
1	46	61
Clinical T stage		
T3	56	74
T4	20	26
Clinical N stage		
N0	59	78
N1	9	12
N2*	1	1
N3	7	9
Smoking history		
No	4	5
Yes	72	95
Median smoking history	1.5 packs for 37 years	
Body weight loss within 6 months		
≤ 5%	61	80
5-10%	7	9
> 10%	5	7
Missing	3	4
Histology		
Adenocarcinoma	34	45
Squamous cell carcinoma	27	36
Others/unclassified	15	20
Primary site		
Right	39	51
Left	37	49

Abbreviation: ECOG, Eastern Cooperative Oncology Group.
*Found ineligible by central review but included in the subsequent analyses.

The induction therapy could be completed in 71 (95%) of the 75 patients. The treatment was terminated in the remaining four patients after only one course of chemotherapy (owing to the development of adverse events in two cases, patient refusal in one case, and early toxicity-related death in one case).

Table 2 lists the major toxicities of the protocol therapy. They were mainly hematologic, and although more than 80% of the patients experienced neutropenia/leukopenia, they were generally transient and not complicated by infection/fever. Overall, toxicities were well tolerated. There was one toxic death on chemoradiotherapy day 6 as a result of severe myelosuppression and subsequent development of septic shock.

Clinical Response to the Induction Therapy

The clinical responses of the 75 eligible patients to induction therapy were judged radiologically and confirmed by the central review. The responses were as follows: CR, 0 patients; partial response, 46 patients; no change, 22 patients; PD, five patients; not assessable, two patients. The overall response rate was 61% (95% CI, 49% to 72%).

Surgical and Pathologic Results

Thoracotomy was performed in 57 (76%) of the 75 patients who received the induction therapy. The surgical procedures undertaken

Table 2. Major Toxicities of Induction Therapy (N = 75) and Postsurgical Complications

Toxicity or Complication	No. of Patients			% Grade 3/4
	Grade 1/2	Grade 3	Grade 4	
Acute toxicity*				
Leukopenia	1/11	37	26†	84
Neutropenia	3/9	26	36†	83
Anemia	19/47	5	0	7
Thrombocytopenia	14/12	9	2†	15
ALT	27/5	2	0	3
Creatinine	18/2	0	0	0
PaO_2	37/6	0	0	0
Emesis	32/25	2	— (not defined)	3
Diarrhea	7/5	1	0	1
Constipation	22/3	1	0	1
Esophagitis	22/9	0	0	0
Infection	10/9	6	1†	9
Neuropathy	8/0	0	— (not defined)	0
Skin toxicity	16/2	1	0	1
Fever	25/19	1	1	3
Postsurgical complications‡				
ARDS	0	1	1 (grade 5)	
Empyema	0	2	0	
Cylothorax	1	1	0	
Pneumonitis	0	1	0	
Late complications‡				
Pneumonitis	0	1	0	
Bleeding	0	0	1 (grade 5)	

Abbreviations: PaO_2, alveolar-arterial difference in partial pressure of oxygen; ARDS, adult respiratory distress syndrome.
*During induction therapy.
†Includes one patient with toxic death owing to septic shock.
‡Report of each complication was evaluated by National Cancer Institute Common Toxicity Criteria version 3.0.

Information downloaded from jco.ascopubs.org and provided by KOKURITSU GAN CENTER on March 9, 2009 from 160.190.160.15.
Copyright © 2008 by the American Society of Clinical Oncology. All rights reserved.

were as follows: lobectomy, 53 patients; partial resection, three patients; exploratory thoracotomy, one patient; none of the cases required pneumonectomy. Combined resection of the chest wall was undertaken in 51 of the 57 patients. Complete mediastinal lymph node dissection (ND2) was performed in 42 patients, and the remaining 15 patients underwent less extensive dissection or sampling (ND0 or ND1).

The results of thoracotomy were as follows: gross residual tumor (R2 resection, including one with probe thoracotomy), three patients; microscopically residual tumor on pathologic review (R1 resection), three patients; complete surgical and pathologic resection (R0 resection), 51 patients. Pathologic downstaging of the tumor as compared with the clinical stage before induction therapy was achieved in 23 patients (40% of the patients who underwent surgery); this is an inherently inaccurate figure and should be interpreted as such, owing to the lack of pathologic confirmation of the c stage at presentation. Pathologic CR, with no residual viable tumor cells in the resected specimens, was achieved in 12 patients (16% of the 75 treated patients). Table 3 lists the surgical and pathologic results according to the initial clinical T factor.

The major postoperative morbidities included adult respiratory distress syndrome (ARDS) in two patients, empyema in two patients, chylothorax in two patients, and pneumonitis in two patients. One patient died of sudden major bleeding on postoperative day 24. The bleeding was identified at autopsy as being from an intercostal artery. Another patient died of ARDS after off-protocol pneumonectomy. The patient had been judged to have PD in response to the induction therapy as a result of emergence of intrapulmonary metastases. The attending surgeon and the patient agreed to salvage surgery, and the patient developed postoperative ARDS.

Thus the total number of toxic deaths was three, including one caused by septic shock during the induction, one by delayed postoperative bleeding, and one by the development of ARDS after off-protocol, salvage surgery.

Table 3. Surgical and Pathologic Results According to Initial Clinical T Stage

Characteristic	c-T3	c-T4
No. of patients	55	20
No surgery performed		
No.	7	11
%	13	55
Reason for no surgery		
Protocol violation	0	1
Toxic death	0	1
Adverse event	0	1
Progressive disease	2	2
Judged unresectable	0	3
Patient refusal	5	3
Surgical procedures		
Thoracotomy		
No.	48	9
%	87	45
Pneumonectomy	0	0
Lobectomy	45	8
Probe thoracotomy	1	0
Other	2	1
With combined resection	44	7
Rib	38	6
Parietal pleura	4	1
Vertebra	3	3
Major vessel	3	0
Clavicle	1	0
Completeness of resection		
R2 operation	2	1
R1 operation	3	0
R0 operation		
No.	43	8
%	78	40
Pathologic results		
Downstaging	18	5
Pathologic complete response	9	3

Boost Therapy

Boost radiotherapy was given to 15 patients, including 12 of the 15 patients in whom thoracotomy was not performed after the completion of induction chemoradiotherapy. One patient received boost radiotherapy after grossly incomplete resection, and another received boost radiotherapy after gross complete resection with microscopically residual disease. In 12 of the 15 patients, boost radiotherapy was completed with a total dose of 66.6 Gy.

PFS and OS

Figures 1 and 2 show the PFS and OS curves, updated in November 2006. Forty-one patients were alive, with a median follow-up period of 68 months. The median PFS time was 28 months. The PFS rates at 3 and 5 years were 49% and 45%, respectively. The median OS has not yet been reached. The OS at 3 and 5 years were 61% and 56%, respectively. Subset analysis (Appendix Figs A2 through A5, online only) revealed that clinical T stage was a prognostic factor (Appendix Fig A2). Patients with clinical T3 disease had better outcome than those with clinical T4 disease (the survival rates at 3 and 5 years were 69% and 61%, respectively, versus 40% and 40%, respectively; log-rank $P = .031$). The clinical N stage and histologic type of the tumor did not significantly affect the OS (Appendix Figs A3 and A4) or PFS. As expected, the survival rate was good in patients in whom complete resection could be achieved, with a projected 5-year OS of 70% as compared with 24% in whom complete resection could not be

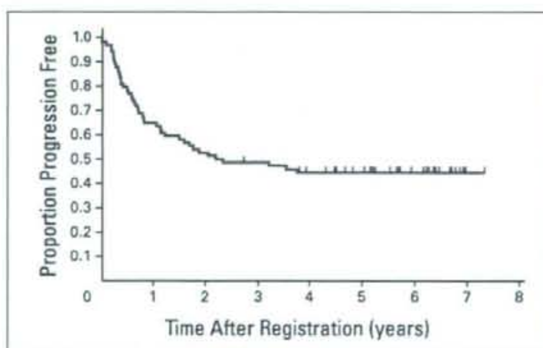

Fig 1. Progression-free survival (PFS) of the 75 eligible patients. PFS at 3 years and 5 years was 49% (95% CI, 38% to 60%) and 45% (95% CI, 34% to 56%), respectively, with a median PFS of 27.7 months.

Information downloaded from jco.ascopubs.org and provided by KOKURITSU GAN CENTER on March 9, 2009 from 160.190.160.15.
Copyright © 2008 by the American Society of Clinical Oncology. All rights reserved.

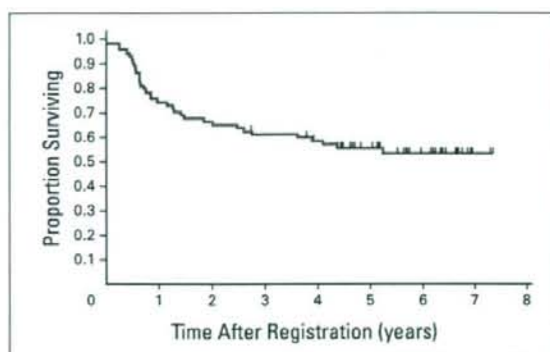

Fig 2. Overall survival (OS) of the 75 eligible patients. OS at 3 years and 5 years was 61% (95% CI, 49% to 71%) and 56% (95% CI, 44% to 66%), respectively. The median OS has not been reached.

achieved (Appendix Fig A5). The survival of the 12 patients with pathologic CR was especially favorable (Appendix Fig A6, online only).

Pattern of Relapse

So far, 39 patients have experienced tumor relapse. Table 4 lists the initial relapse sites, according to the curative extent of the surgical resection. For unresected or incompletely resected cases, locoregional relapse was predominant. To the contrary, for completely resected cases, relapse at distant sites was the most frequent relapse pattern, with some brain-only relapse patients.

DISCUSSION

We conducted a multi-institutional phase II trial of a trimodality approach, namely, preoperative chemoradiotherapy followed by surgical resection, in patients with SSTs. Because of the rarity of this subtype of NSCLC, no randomized trial has been conducted previously.[28] Our report is the second of a large-scale, prospective trial after SWOG 9416/INT 0160 and reproduced its favorable outcomes.[25]

The long-term results of the SWOG 9416/INT 0160 trial were recently published.[29] Although the chemotherapy regimens used were different, a standard classic platinum-based combination was used in both. The preoperative radiotherapy doses were also identical (45 Gy), although a 1-week split (interval between two sessions) was included in our protocol (but not in the SWOG trial). Boost chemotherapy was planned after curative resection in the SWOG trial, but the compliance rate was poor,[25] as in other perioperative therapy reports; we had anticipated that the majority of the patients would not be fit enough for additional toxic therapy after a major thoracic surgery and did not include it in our protocol.

Table 4. Initial Relapse Sites

Relapse Site	Patients With Complete Resection (n = 51)	Patients Without Complete Resection (n = 24)	Total (N = 75)
Locoregional* only	2	8	10
Distant only	14	6	20
Brain only	4	1	5
Both	4	5	9
Total	20	19	39

*Locoregional = surgical margin, within radiation field, hilar lymph nodes, mediastinal lymph nodes, supraclavicular lymph nodes.

Despite these minor differences, the results of the two trials were strikingly similar (Table A1, online only). The radiologic response rate was higher, whereas the pathologic CR rate was lower in our trial, but the differences were probably not clinically relevant, considering interobserver differences in the response evaluation and the well-known discrepancy between clinical versus pathologic effects. The intensive trimodality approach was found to be feasible in both reports, with a reasonably low toxic death rate of 4%. The resection rate, which had remained unchanged at approximately 50% for almost 40 years with conventional preoperative radiotherapy, was approximately 70% in both studies. Particularly noteworthy was the reproducibility of the favorable survival data, with a 5-year OS rate of 44% in the United States trial and 56% in our trial, which were clearly superior to the historical value of 30%.[3,25]

A shift in the trend of clinical problems also became clear.[25,28,29] The relapse patterns changed from predominantly locoregional[17,18] to mainly distant recurrences in cases with complete resection,[25,28,29] and a significant number of such patients suffered from metastasis in the brain as the initial site of relapse.[29] To the contrary, complete resection could be achieved in less than half of the patients with c-T4 disease, and neither local control nor long-term survival was satisfactory in those in whom it could not be achieved. It seems that there might be room for improvement in radiotherapy.

Several questions remain unresolved. One is that of management of patients with mediastinal node involvement. These clinical N2 cases have been known to have the poorest prognosis[9,18] and were excluded from both the SWOG and JCOG trials. Although trimodality approaches have been reported in cases with clinical N2 stage NSCLC,[30,31] inclusion of the hilar and mediastinal nodes in the irradiation field increased the toxicity risk to an unacceptable level in our prior phase II trial (JCOG 9805).[32]

In addition to the unresolved questions above, our study also had a critical limitation. Although this was a prospective, large-scale, and multi-institutional trial, no definite conclusions could be obtained from the single-arm phase II study. As repeatedly pointed out, however, a phase III trial would be unrealistic due to the rarity of SSTs. Possibly, clinical questions common with other patient subsets could be tested in a phase III trial targeting a broader patient population; for example, patients with SSTs and other stage III NSCLC could be enrolled onto a phase III trial of prophylactic cranial irradiation after definitive induction therapy.[33]

In conclusion, we report a favorable outcome of preoperative chemoradiotherapy in patients with SSTs, confirming the results of the previous SWOG/Intergroup trial. We believe that this strategy may be acceptable as standard for the treatment of this disease and also serves as a reference for future trials.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest.

Information downloaded from jco.ascopubs.org and provided by KOKURITSU GAN CENTER on March 9, 2009 from 160.190.160.15.
Copyright © 2008 by the American Society of Clinical Oncology. All rights reserved.

AUTHOR CONTRIBUTIONS

Conception and design: Hideo Kunitoh, Harubumi Kato, Nagahiro Saijo
Financial support: Nagahiro Saijo
Administrative support: Nagahiro Saijo
Provision of study materials or patients: Hideo Kunitoh, Harubumi Kato, Masahiro Tsuboi, Hisao Asamura, Yukito Ichonose, Nobuyuki Katakami, Kanji Nagai, Tetsuya Mitsudomi, Akihide Matsumura, Ken Nakagawa, Hirohito Tada
Collection and assembly of data: Masahiro Tsuboi, Taro Shibata
Data analysis and interpretation: Taro Shibata
Manuscript writing: Hideo Kunitoh, Taro Shibata
Final approval of manuscript: Hideo Kunitoh, Harubumi Kato, Masahiro Tsuboi, Taro Shibata, Hisao Asamura, Yukito Ichonose, Nobuyuki Katakami, Kanji Nagai, Tetsuya Mitsudomi, Akihide Matsumura, Ken Nakagawa, Hirohito Tada, Nagahiro Saijo

REFERENCES

1. Pancoast HK: Importance of careful roentgenray investigation of apical chest tumors. JAMA 83:1407-1411, 1924
2. Pancoast H: Superior pulmonary sulcus tumor: Tumor characterized by pain, Horner's syndrome, destruction of bone and atrophy of hand muscles. JAMA 99:1391-1396, 1932
3. Arcasoy SM, Jett JR: Superior pulmonary sulcus tumors and Pancoast's syndrome. N Engl J Med 337:1370-1376, 1997
4. Shaw RR, Paulson DL, Kee JL Jr: Treatment of superior sulcus tumor by irradiation followed by resection. Ann Surg 154:29-40, 1961
5. Paulson DL: The survival rate in superior sulcus tumors treated by presurgical irradiation. JAMA 196:342, 1966
6. Hilaris BS, Luomanen RK, Beattie EJ Jr: Integrated irradiation and surgery in the treatment of apical lung cancer. Cancer 27:1369-1373, 1971
7. Paulson DL: Carcinomas in the superior pulmonary sulcus. J Thorac Cardiovasc Surg 70:1095-1104, 1975
8. Devine JW, Mendenhall WM, Million RR, et al: Carcinoma of the superior pulmonary sulcus treated with surgery and/or radiation therapy. Cancer 57:941-943, 1986
9. Anderson TM, Moy PM, Holmes EC: Factors affecting survival in superior sulcus tumors. J Clin Oncol 4:1598-1603, 1986
10. Hilaris BS, Martini N, Wong GY, et al: Treatment of superior sulcus tumor (Pancoast tumor). Surg Clin North Am 67:965-977, 1987
11. Shahian DM, Neptune WB, Ellis FH Jr: Pancoast tumors: Improved survival with preoperative and postoperative radiotherapy. Ann Thorac Surg 43:32-38, 1987
12. Neal CR, Amdur RJ, Mendenhall WM, et al: Pancoast tumor: Radiation therapy alone versus preoperative radiation therapy and surgery. Int J Radiat Oncol Biol Phys 21:651-660, 1991
13. Maggi G, Casadio C, Pischedda F, et al: Combined radiosurgical treatment of Pancoast tumor. Ann Thorac Surg 57:198-202, 1994
14. Komaki R, Roth JA, Walsh GL, et al: Outcome predictors for 143 patients with superior sulcus tumors treated by multidisciplinary approach at the University of Texas M. D. Anderson Cancer Center. Int J Radiat Oncol Biol Phys 48:347-354, 2000
15. Martinod E, D'Audiffret A, Thomas P, et al: Management of superior sulcus tumors: Experience with 139 cases treated by surgical resection. Ann Thorac Surg 73:1534-1539, 2002
16. Muscolino G, Valente M, Andreani S: Pancoast tumours: Clinical assessment and long-term results of combined radiosurgical treatment. Thorax 52:284-286, 1997
17. Ginsberg RJ, Martini N, Zaman M, et al: Influence of surgical resection and brachytherapy in the management of superior sulcus tumor. Ann Thorac Surg 57:1440-1445, 1994
18. Rusch VW, Parekh KR, Leon L, et al: Factors determining outcome after surgical resection of T3 and T4 lung cancers of the superior sulcus. J Thorac Cardiovasc Surg 119:1147-1153, 2000
19. Furuse K, Fukuoka M, Kawahara M, et al: Phase III study of concurrent versus sequential thoracic radiotherapy in combination with mitomycin, vindesine, and cisplatin in unresectable stage III non-small-cell lung cancer. J Clin Oncol 17:2692-2699, 1999
20. Curran WJ Jr: Treatment of locally advanced non-small cell lung cancer: What we have and have not learned over the past decade. Semin Oncol 32:S2-S5, 2005
21. Zatloukal P, Petruzelka L, Zemanova M, et al: Concurrent versus sequential chemoradiotherapy with cisplatin and vinorelbine in locally advanced non-small cell lung cancer: A randomized study. Lung Cancer 46:87-98, 2004
22. Sause W, Kolesar P, Taylor SI, et al: Final results of phase III trial in regionally advanced unresectable non-small cell lung cancer: Radiation Therapy Oncology Group, Eastern Cooperative Oncology Group, and Southwest Oncology Group. Chest 117:358-364, 2000
23. Dillman RO, Seagren SL, Propert KJ, et al: A randomized trial of induction chemotherapy plus high-dose radiation versus radiation alone in stage III non-small-cell lung cancer. N Engl J Med 323:940-945, 1990
24. Schaake-Koning C, van den Bogaert W, Dalesio O, et al: Effects of concomitant cisplatin and radiotherapy on inoperable non-small-cell lung cancer. N Engl J Med 326:524-530, 1992
25. Rusch VW, Giroux DJ, Kraut MJ, et al: Induction chemoradiation and surgical resection for non-small cell lung carcinomas of the superior sulcus: Initial results of Southwest Oncology Group Trial 9416 (Intergroup Trial 0160). J Thorac Cardiovasc Surg 121:472-483, 2001
26. Tobinai K, Kohno A, Shimada Y, et al: Toxicity grading criteria of the Japan Clinical Oncology Group. Jpn J Clin Oncol 23:250-257, 1993
27. Miller AB, Hoogstraten B, Staquet M, et al: Reporting results of cancer treatment. Cancer 47:207-214, 1981
28. Wright CD, Menard MT, Wain JC, et al: Induction chemoradiation compared with induction radiation for lung cancer involving the superior sulcus. Ann Thorac Surg 73:1541-1544, 2002
29. Rusch VW, Giroux D, Kraut MJ, et al: Induction chemoradiation and surgical resection for superior sulcus non-small-cell lung carcinomas: Long-term results of Southwest Oncology Group trial 9416 (Intergroup trial 0160). J Clin Oncol 25:313-318, 2007
30. Albain KS, Rusch VW, Crowley JJ, et al: Concurrent cisplatin/etoposide plus chest radiotherapy followed by surgery for stages IIIA (N2) and IIIB non-small-cell lung cancer: Mature results of Southwest Oncology Group phase II study 8805. J Clin Oncol 13:1880-1892, 1995
31. Albain KS, Swann RS, Rusch VR, et al: Phase III study of concurrent chemotherapy and radiotherapy (CT/RT) vs CT/RT followed by surgical resection for stage IIIA(pN2) non-small cell lung cancer (NSCLC): Outcomes update of North American Intergroup 0139 (RTOG 9309). Proc Am Soc Clin Oncol 23:624S, 2005 (abstr 7014)
32. Kunitoh H, Saijo N, Tsuboi M, et al: A pilot trial of preoperative MVP-combined chemoradiotherapy: Mitomycin C (MMC), vindesine (VDS), and cisplatin (CDDP), concurrently given with thoracic radiotherapy (TRT) in N2 non-small cell lung cancer (NSCLC). Proc Am Soc Clin Oncol 19:530a, 2000 (abstr 2085)
33. Stuschke M, Eberhardt W, Pottgen C, et al: Prophylactic cranial irradiation in locally advanced non-small-cell lung cancer after multimodality treatment: Long-term follow-up and investigations of late neuropsychologic effects. J Clin Oncol 17:2700-2709, 1999

■ ■ ■

Acknowledgment

We thank Mieko Imai for data management and Takashi Asakawa and Naoki Ishizuka, PhD, for statistical analyses.

Appendix

The Appendix is included in the full-text version of this article, available online at www.jco.org. It is not included in the PDF version (via Adobe® Reader®).

Information downloaded from jco.ascopubs.org and provided by KOKURITSU GAN CENTER on March 9, 2009 from 160.190.160.15.
Copyright © 2008 by the American Society of Clinical Oncology. All rights reserved.

British Journal of Cancer (2008) 98, 693–696
© 2008 Cancer Research UK All rights reserved 0007–0920/08 $30.00
www.bjcancer.com

Short Communication

Randomised phase II trial of irinotecan plus cisplatin *vs* irinotecan, cisplatin plus etoposide repeated every 3 weeks in patients with extensive-disease small-cell lung cancer

I Sekine*[,1], H Nokihara[1], K Takeda[2], Y Nishiwaki[3], K Nakagawa[4], H Isobe[5], K Mori[6], K Matsui[7], N Saijo[3] and T Tamura[1]

[1]*Division of Internal Medicine and Thoracic Oncology, National Cancer Center Hospital, Tokyo, Japan;* [2]*Department of Clinical Oncology, Osaka City General Hospital, Osaka, Japan;* [3]*Division of Thoracic Oncology, National Cancer Center Hospital East, Kashiwa, Japan;* [4]*Department of Medical Oncology, Kinki University School of Medicine, Sayama, Japan;* [5]*Department of Pulmonary Disease, National Hospital Organization Hokkaido Cancer Center, Sapporo, Japan;* [6]*Department of Thoracic Diseases, Tochigi Prefectural Cancer Center, Utsunomiya, Japan;* [7]*Department of Internal Medicine, Osaka Prefectural Medical Center for Respiratory and Allergic Diseases, Habikino, Japan*

Patients with previously untreated extensive-disease small-cell lung cancer were treated with irinotecan 60 mg m^{-2} on days 1 and 8 and cisplatin 60 mg m^{-2} on day 1 with ($n = 55$) or without ($n = 54$) etoposide 50 mg m^{-2} on days 1–3 with granulocyte colony-stimulating factor support repeated every 3 weeks for four cycles. The triplet regimen was too toxic to be considered for further studies.
British Journal of Cancer (2008) **98,** 693–696. doi:10.1038/sj.bjc.6604233 www.bjcancer.com
Published online 5 February 2008
© 2008 Cancer Research UK

Keywords: small-cell lung cancer; chemotherapy; irinotecan; etoposide; three drug combination

Small-cell lung cancer (SCLC), which accounts for approximately 14% of all malignant pulmonary tumours, is an aggressive malignancy with a propensity for rapid growth and early widespread metastases (Jackman and Johnson, 2005). A combination of cisplatin and etoposide (PE) has been the standard treatment, with response rates ranging from 60 to 90% and median survival times (MSTs) from 8 to 11 months in patients with extensive disease (ED)-SCLC (Fukuoka *et al*, 1991; Roth *et al*, 1992). A combination of irinotecan and cisplatin (IP) showed a significant survival benefit over the PE regimen (MST: 12.8 *vs* 9.4 months, $P = 0.002$) in a Japanese phase III trial for ED-SCLC (Noda *et al*, 2002), although another phase III trial comparing these regimens failed to show such a benefit (Hanna *et al*, 2006). Thus, irinotecan, cisplatin and etoposide are the current key agents in the treatment of SCLC. A phase II trial of the three agents, IPE combination, in patients with ED-SCLC showed a promising antitumour activity with a response rate of 77%, complete response (CR) rate of 17% and MST of 12.9 months (Sekine *et al*, 2003).

We have developed these IP and IPE regimens in a 4-week schedule where irinotecan was given on days 1, 8 and 15. The dose of irinotecan on day 15, however, was frequently omitted because of toxicity in both regimens (Noda *et al*, 2002; Sekine *et al*, 2003). The objectives of this study were to evaluate the toxicities and antitumour effects of IP and IPE regimens in the 3-week schedule in patients with ED-SCLC and to select the right arm for subsequent phase III trials.

PATIENTS AND METHODS

Patient selection

Patients were enrolled in this study if they met the following criteria: (1) a histological or cytological diagnosis of SCLC; (2) no prior treatment; (3) measurable disease; (4) ED, defined as having distant metastasis or contralateral hilar lymph node metastasis; (5) performance status of 0–2 on the Eastern Cooperative Oncology Group (ECOG) scale; (6) predicted life expectancy of 3 months or longer; (7) age between 20 and 70 years; (8) adequate organ function as documented by a white blood cell (WBC) count $\geqslant 4.0 \times 10^3\,\mu l^{-1}$, neutrophil count $\geqslant 2.0 \times 10^3\,\mu l^{-1}$, haemoglobin $\geqslant 9.5\,g\,dl^{-1}$, platelet count $\geqslant 100 \times 10^3\,\mu l^{-1}$, total serum bilirubin $\leqslant 1.5\,mg\,dl^{-1}$, hepatic transaminases $\leqslant 100\,IU\,l^{-1}$, serum creatinine $\leqslant 1.2\,mg\,dl^{-1}$, creatinine clearance $\geqslant 60\,ml\,min^{-1}$, and $PaO_2 \geqslant 60$ torr; and (9) providing written informed consent.

Patients were not eligible for the study if they had any of the following: (1) uncontrollable pleural, pericardial effusion or ascites; (2) symptomatic brain metastasis; (3) active infection; (4) contraindications for the use of irinotecan, including diarrhoea, ileus, interstitial pneumonitis and lung fibrosis; (5) synchronous active malignancies; (6) serious concomitant medical

*Correspondence: Dr I Sekine, Division of Internal Medicine and Thoracic Oncology, National Cancer Center Hospital, Tsukiji 5-1-1, Chuo-ku, Tokyo 104-0045, Japan; E-mail: isekine@ncc.go.jp
Received 15 October 2007; revised 2 January 2008; accepted 9 January 2008; published online 5 February 2008

illness, including severe heart disease, uncontrollable diabetes mellitus or hypertension; or (7) pregnancy or breast feeding.

Treatment schedule

In the IP arm, cisplatin, 60 mg m^{-2}, was administered intravenously over 60 min on day 1 and irinotecan, 60 mg m^{-2}, was administered intravenously over 90 min on days 1 and 8. Prophylactic granulocyte colony-stimulating factor (G-CSF) was not administered in this arm. In the IPE arm, cisplatin and irinotecan were administered at the same dose and schedule as the IP arm. In addition, etoposide, 50 mg m^{-2}, was administered intravenously over 60 min on days 1–3. Filgrastim 50 μg m^{-2} or lenograstim 2 μg kg^{-1} was subcutaneously injected prophylactically from day 5 to the day when the WBC count exceeded $10.0 \times 10^3 \mu l^{-1}$. Hydration (2500 ml) and a 5HT$_3$ antagonist were given on day 1, followed by an additional infusion if indicated in both arms. These treatments were repeated every 3 weeks for a total of four cycles.

Toxicity assessment, treatment modification and response evaluation

Toxicity was graded according to the NCI Common Toxicity Criteria version 2.0.

Doses of anticancer agents in the following cycles were modified according to toxicity in the same manner in both arms. Objective tumour response was evaluated according to the Response Evaluation Criteria in Solid Tumors (RECIST) (Therasse *et al*, 2000).

Study design, data management and statistical considerations

This study was designed as a multi-institutional, prospective randomised phase II trial. This study was registered on 6 September 2005 in the University hospital Medical Information Network (UMIN) Clinical Trials Registry in Japan (http://www.umin.ac.jp/ctr/index.htm), which is acceptable to the International Committee of Medical Journal Editors (ICMJE) (http://www.icmje.org/faq.pdf). The protocol and consent form were approved by the Institutional Review Board of each institution. Patient registration and randomisation were conducted at the Registration Center. No stratification for randomisation was performed in this study. The sample size was calculated according to the selection design for pilot studies based on survival (Liu *et al*, 1993). Assuming that (1) the survival curve was exponential for survivals; (2) the MST of the worse arm was 12 months and that of the better arm was 12 months × 1.4; (3) the correct selection probability was 90%; and (4) additional follow-up in years after the end of accrual was 1 year, the estimated required number of patients was 51 for each arm. Accordingly, 55 patients for each arm and their accrual period of 24 months were planned for this study.

The dose intensity of each drug was calculated for each patient using the following formula as previously described:

$$\text{The dose intensity } (\text{mg m}^{-2}\,\text{week}^{-1}) = \frac{\text{Total milligrams of a drug in all cycles per body surface area}}{\text{Total days of therapy}/7}$$

where total days of therapy is the number of days from day 1 of cycle 1 to day 1 of the last cycle plus 21 days for both arms (Hryniuk and Goodyear, 1990).

Differences in the reason for termination of the treatment and the frequencies of grade 3–4 toxicities were assessed by χ tests. Survival was measured as the date of randomisation to the date of death from any cause or the date of the most recent follow-up for overall survival and to the date of disease progression or the date of death for progression-free survival (PFS). The survival of the arms was estimated by the Kaplan–Meier method and compared in an exploratory manner with log-rank tests (Armitage *et al*, 2002).

RESULTS

Patient characteristics

From March 2003 to May 2005, 55 patients were randomised to IP and 55 patients to IPE. One patient in the IP arm was excluded because the patient was ineligible and did not receive the study treatment. The remaining 109 patients were included in the analyses of toxicity, tumour response and patient survival. There were no differences between the two arms in any demographic characteristics listed (Table 1).

Treatment delivery

Treatment was well tolerated with respect to the number of cycles delivered in both arms (Table 2). Among reasons for termination of the treatment, disease progression was noted in nine (17%)

Table 1 Patient characteristics

	IP (n = 54)	IPE (n = 55)
Sex		
Female	11	8
Male	43	47
Age (years)		
Median (range)	63 (42–70)	62 (48–70)
PS		
0	11	12
1	42	41
2	1	2
Weight loss		
0–4%	38	43
5–9%	10	10
⩾10%	6	2

Table 2 Treatment delivery

	IP (n = 54) No. (%)	IPE (n = 55) No. (%)
Number of cycles delivered		
6[a]	—	1 (2)
4	41 (76)	36 (65)
3	6 (11)	6 (11)
2	3 (6)	6 (11)
1	4 (7)	6 (11)
Reasons for termination of the treatment[†]		
Completion	40 (74)	35 (64)
Disease progression	9 (17)	2 (4)
Toxicity	3 (6)	13 (24)
Patient refusal	2 (4)	4 (7)
Others	0 (0)	1 (2)
Total number of cycles delivered	192 (100)	186 (100)
Total number of omission on day 8	35 (18)	37 (17)
Total number of cycles with dose reduction	28 (15)	31 (17)

[†]P = 0.013 by χ^2 test. [a]Protocol violation.

© 2008 Cancer Research UK

patients in the IP arm and in two (4%) patients in the IPE arm, whereas toxicity was noted in three (6%) patients in the IP arm and 13 (24%) patients in the IPE arm ($P = 0.013$) (Table 2). The dose of irinotecan on day 8 was omitted in 35 (18%) cycles in the IP arm and 37 (17%) cycles in the IPE arm (Table 2). The total dose and dose intensity of cisplatin and etoposide were similar between the IP and IPE arms in the present study (Table 3).

Toxicity

The myelotoxicity was more severe in the IPE arm (Table 4). Grade 3 febrile neutropaenia was noted in 5 (9%) patients in the IP arm and 17 (31%) patients in the IPE arm ($P = 0.005$). Packed red blood cells were transfused in 4 (7%) patients in the IP regimen and 14 (26%) patients in the IPE regimen ($P = 0.011$). Platelet concentrates were needed in none in the IP regimen and 2 (4%) patients in the IPE regimen ($P = 0.16$). Grade 3–4 diarrhoea was observed in 8 (15%) patients in the IP arm and 13 (24%) patients in the IPE arm ($P = 0.262$). Grade 3–4 fatigue was more common in the IPE arm with marginal significance (2 *vs* 11%, $P = 0.054$). The severity of other non-haematological toxicities did not differ significantly between the arms. No treatment-related death was observed in this study.

Table 3 Total dose and dose intensity

	3-week regimens in this study		4-week regimen[a]
	IP ($n = 54$) Median (range)	IPE ($n = 55$) Median (range)	IPE ($n = 30$) Median (range)
Total dose ($mg\,m^{-2}$)			
Cisplatin	240 (60–240)	240 (60–360)	240 (60–240)
Irinotecan	420 (60–480)	390 (60–720)	563 (60–720)
Etoposide	0	600 (150–900)	600 (150–600)
Dose intensity ($mg\,m^{-2}\,week^{-1}$)			
Cisplatin	19 (14–25)	20 (16–34)	15 (12–15)
Irinotecan	33 (14–40)	35 (15–55)	35 (19–45)
Etoposide	0	48 (34–68)	37 (28–38)

[a]From our previous study (Sekine *et al*, 2003).

Table 4 Grade 3–4 toxicities

	IP ($n = 54$)			IPE ($n = 55$)		
	Grade 3	4	3+4 (%)	Grade 3	4	3+4 (%)
Leukocytopaenia	9	1	10 (19)	18	11	29 (53)*
Neutropaenia	17	11	28 (52)	24	28	52 (95)*
Anaemia	18	0	18 (25)	16	9	25 (45)
Thrombocytopaenia	2	0	2 (4)	13	0	13 (13)†
Febrile neutropaenia	5	0	5 (9)	17	0	7 (13)
Diarrhoea	8	0	8 (15)	11	2	13 (24)
Vomiting	4	0	4 (7)	3	0	3 (5)
Fatigue	1	0	1 (2)	5	1	6 (11)‡
Hyponatraemia	9	3	12 (22)	11	2	13 (24)
AST elevation	0	0	0 (0)	3	0	3 (5)
CRN elevation	1	0	1 (2)	0	0	0 (0)

*$P < 0.001$; †$P < 0.01$; and ‡$P = 0.054$ by χ^2 test.

Response, treatment after recurrence and survival

Four CRs and 37 partial responses (PRs) were obtained in the IP arm, resulting in the overall response rate of 76 with 95% confidence interval (CI) of 65–87%, whereas six CRs and 42 PRs were obtained in the IPE arm, and the overall response rate was 87% with a 95% CI of 79–96% ($P = 0.126$). Median PFS was 4.8 months (95% CI, 4.0–5.6) in the IP and 5.4 months (95% CI, 4.8–6.0) in the IPE arm ($P = 0.049$) (Figure 1A). After recurrence, 22 (44%) patients in the IP arm and 8 (16%) patients in the IPE arm received etoposide-containing chemotherapy. The MST and 1-year survival rate were 12.4 months (95% CI, 9.7–15.1) and 54.8% (95% CI, 41.4–68.2%) in the IP and 13.7 months (95% CI, 11.9–15.5) and 61.5% (95% CI, 48.6–74.4%) in the IPE arm ($P = 0.52$), respectively (Figure 1B).

DISCUSSION

This study showed that the IPE regimen in a 3-week schedule with CSF support produced a promising response rate, PFS and overall survival. Haematological toxicity in the IPE arm, however, was very severe in spite of the G-CSF support with the grade 3 febrile neutropaenia noted in 31% of patients.

In comparison between the 3-week IPE regimen in this study and the 4-week IPE regimen in the previous study, the delivery of cisplatin and etoposide was improved in the 3-week IPE regimen when compared with the 4-week IPE regimen at the cost of the irinotecan total dose. The response rate and MST were 87% and 13.7 months, respectively, in the 3-week IPE regimen and 77% and 12.9 months in the previous 4-week schedule, and toxicity profiles were comparable to each other (Sekine *et al*, 2003).

The MST of 12.4 months in the IP arm in this study was comparable to that of the previous phase III study, with an MST of 12.8 months (Noda *et al*, 2002). Thus, this study showed the reproducible excellent survival outcome of patients with ED-SCLC who were treated with the IP combination. In contrast, a recent American phase III study of the PE regimen *vs* IP regimen failed to show the superiority of the IP regimen to the PE regimen; the MST

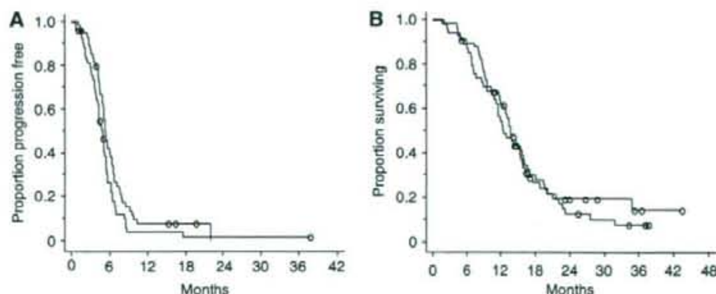

Figure 1 Progression-free survival (**A**) and overall survival (**B**). Thick line indicates the IPE regimen and thin line indicates the IP regimen.

© 2008 Cancer Research UK

for the PE regimen was 10.2 months and that for the IP regimen was 9.3 months (Hanna *et al*, 2006). The discrepancy between the Japanese and American trials may be explained by the different cisplatin dose schedules; cisplatin was delivered at a dose of $60\,mg\,m^{-2}$ on day 1 every 3 or 4 weeks in the Japanese trials, whereas cisplatin was delivered at a dose of $30\,mg\,m^{-2}$ on days 1 and 8 every 3 weeks in the American one. A platinum agent administered at divided doses was associated with poor survival in patients with ED-SCLC in our previous randomised phase II study (Sekine *et al*, 2003).

The issue of adding further agents to the standard doublet regimen has been investigated in patients with ED-SCLC. The addition of ifosfamide or cyclophosphamide and epirubicin to the cisplatin and etoposide combination produced a slight survival benefit, but at the expense of greater toxicity (Loehrer *et al*, 1995; Pujol *et al*, 2001). Phase III trials of cisplatin and etoposide with or without paclitaxel showed unacceptable toxicity with 6–13% toxic deaths in the paclitaxel-containing arm (Mavroudis *et al*, 2001; Niell *et al*, 2005). The results in these studies and the current study are consistent in the increased toxicity despite the G-CSF support and no definite survival benefit in the three or four drug combinations over the standard doublet in patients with ED-SCLC.

In conclusion, the IPE regimen was marginally more effective than the IP regimen, but was too toxic despite the administration of prophylactic G-CSF.

ACKNOWLEDGEMENTS

This study was supported, in part, by Grants-in-Aid for Cancer Research from the Ministry of Health, Labour and Welfare of Japan. We thank the following doctors for their care for patients and valuable suggestion and comments on this study: Takahiko Sugiura, Aichi Cancer Center; Yoshinobu Ohsaki, Asahikawa Medical College; Shinzo Kudoh, Osaka City University Medical School; Makoto Nishio, Cancer Institute Hospital; Hiroshi Chiba, Kumamoto Community Medical Center; Koichi Minato, Gunma Prefectural Cancer Center; Naoyuki Nogami, Shikoku Cancer Center; Hiroshi Ariyoshi, Aichi Cancer Center Aichi Hospital; Takamune Sugiura, Rinku General Medical Center; Akira Yokoyama, Niigata Cancer Center Hospital; and Koshiro Watanabe, Yokohama Municipal Citizen's Hospital. We also thank Fumiko Koh, Yuko Yabe and Mika Nagai for preparation of the paper.

REFERENCES

Armitage P, Berry G, Matthews J (2002) Survival analysis. In *Statistical Methods in Medical Research*, Armitage P, Berry G, Matthews J (eds), pp 568–590. Oxford: Blackwell Science Ltd

Fukuoka M, Furuse K, Saijo N, Nishiwaki Y, Ikegami H, Tamura T, Shimoyama M, Suemasu K (1991) Randomized trial of cyclophosphamide, doxorubicin, and vincristine *vs* cisplatin and etoposide *vs* alternation of these regimens in small-cell lung cancer. *J Natl Cancer Inst* **83:** 855–861

Hanna N, Bunn Jr PA, Langer C, Einhorn L, Guthrie Jr T, Beck T, Ansari R, Ellis P, Byrne M, Morrison M, Hariharan S, Wang B, Sandler A (2006) Randomized phase III trial comparing irinotecan/cisplatin with etoposide/cisplatin in patients with previously untreated extensive-stage disease small-cell lung cancer. *J Clin Oncol* **24:** 2038–2043

Hryniuk WM, Goodyear M (1990) The calculation of received dose intensity. *J Clin Oncol* **8:** 1935–1937

Jackman DM, Johnson BE (2005) Small-cell lung cancer. *Lancet* **366:** 1385–1396

Liu PY, Dahlberg S, Crowley J (1993) Selection designs for pilot studies based on survival. *Biometrics* **49:** 391–398

Loehrer Sr PJ, Ansari R, Gonin R, Monaco F, Fisher W, Sandler A, Einhorn LH (1995) Cisplatin plus etoposide with and without ifosfamide in extensive small-cell lung cancer: a Hoosier Oncology Group study. *J Clin Oncol* **13:** 2594–2599

Mavroudis D, Papadakis E, Veslemes M, Tsiafaki X, Stavrakakis J, Kouroussis C, Kakolyris S, Bania E, Jordanoglou J, Agelidou M, Vlachonicolis J, Georgoulias V (2001) A multicenter randomized clinical trial comparing paclitaxel–cisplatin–etoposide *vs* cisplatin–etoposide as first-line treatment in patients with small-cell lung cancer. *Ann Oncol* **12:** 463–470

Niell HB, Herndon II JE, Miller AA, Watson DM, Sandler AB, Kelly K, Marks RS, Perry MC, Ansari RH, Otterson G, Ellerton J, Vokes EE, Green MR (2005) Randomized phase III intergroup trial of etoposide and cisplatin with or without paclitaxel and granulocyte colony-stimulating factor in patients with extensive-stage small-cell lung cancer: Cancer and Leukemia Group B Trial 9732. *J Clin Oncol* **23:** 3752–3759

Noda K, Nishiwaki Y, Kawahara M, Negoro S, Sugiura T, Yokoyama A, Fukuoka M, Mori K, Watanabe K, Tamura T, Yamamoto S, Saijo N (2002) Irinotecan plus cisplatin compared with etoposide plus cisplatin for extensive small-cell lung cancer. *N Engl J Med* **346:** 85–91

Pujol JL, Daures JP, Riviere A, Quoix E, Westeel V, Quantin X, Breton JL, Lemarie E, Poudenx M, Milleron B, Moro D, Debieuvre D, Le Chevalier T (2001) Etoposide plus cisplatin with or without the combination of 4'-epidoxorubicin plus cyclophosphamide in treatment of extensive small-cell lung cancer: a French Federation of Cancer Institutes multicenter phase III randomized study. *J Natl Cancer Inst* **93:** 300–308

Roth BJ, Johnson DH, Einhorn LH, Schacter LP, Cherng NC, Cohen HJ, Crawford J, Randolph JA, Goodlow JL, Broun GO, Omura GA, Greco FA (1992) Randomized study of cyclophosphamide, doxorubicin, and vincristine *vs* etoposide and cisplatin *vs* alternation of these two regimens in extensive small-cell lung cancer: a phase III trial of the Southeastern Cancer Study Group. *J Clin Oncol* **10:** 282–291

Sekine I, Nishiwaki Y, Noda K, Kudoh S, Fukuoka M, Mori K, Negoro S, Yokoyama A, Matsui K, Ohsaki Y, Nakano T, Saijo N (2003) Randomized phase II study of cisplatin, irinotecan and etoposide combinations administered weekly or every 4 weeks for extensive small-cell lung cancer (JCOG9902-DI). *Ann Oncol* **14:** 709–714

Therasse P, Arbuck SG, Eisenhauer EA, Wanders J, Kaplan RS, Rubinstein L, Verweij J, Van Glabbeke M, van Oosterom AT, Christian MC, Gwyther SG (2000) New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. *J Natl Cancer Inst* **92:** 205–216

© 2008 Cancer Research UK

Identification of the H2-K^d-restricted cytotoxic T lymphocyte epitopes of a tumor-associated antigen, SPARC, which can stimulate antitumor immunity without causing autoimmune disease in mice

Yoshiaki Ikuta,[1,2,7] Yuki Hayashida,[1,3,7] Shinya Hirata,[1] Atsushi Irie,[1] Satoru Senju,[1] Tatsuko Kubo,[4] Tetsuya Nakatsura,[1,5] Mikio Monji,[1] Yutaka Sasaki,[3] Hideo Baba[2] and Yasuharu Nishimura[1,6]

Departments of [1]Immunogenetics, [2]Gastroenterological Surgery, [3]Gastroenterology and Hepatology and [4]Molecular Pathology, Graduate School of Medical Sciences, Kumamoto University, 1-1-1 Honjo, Kumamoto, Kumamoto 860-8556; [5]The Immunotherapy Section, Investigative Treatment Division, Center for Innovative Medicine, National Cancer Center East, 6-5-1 Kashiwanoha, Kashiwa, Chiba 277-8577, Japan

(Received August 12, 2008/Revised September 17, 2008/Accepted September 22, 2008/Online publication December 5, 2008)

We previously reported that the secreted protein acidic and rich in cystein (SPARC) was overexpressed in melanoma in humans, and the serum SPARC level was useful as a novel tumor marker for melanoma. SPARC was also reported to be overexpressed in various human cancers. In this study, we asked whether SPARC-specific cytotoxic T lymphocytes (CTL) could induce antitumor immunity to SPARC-expressing tumor in mice or not as a preclinical study of SPARC-directed anticancer immunotherapy. Because of similarities in the structural motifs of major histocompatibility complex-binding peptides between H2-K^d and HLA-A24 (*A*2402*), the most common human leukocyte antigen class I allele in the Japanese population, we attempted to identify the H2-K^d-restricted SPARC epitope for CTL in BALB/c mice and we found that the mouse SPARC$_{143-151}$ (DYIGPCKYI) and SPARC$_{225-234}$ (MYIFPVHWQF) peptides could induce peptide-reactive CTL in BALB/c mice without causing autoimmune diseases. The immunization of mice with SPARC$_{225-234}$ peptide-pulsed bone marrow-derived dendritic cells (BMDC) inhibited the growth of s.c. inoculated mouse mammary cancer cell line, N2C, expressing SPARC and these mice lived longer than the mice immunized with peptide-unpulsed BMDC. In conclusion, our study indicated that SPARC peptide-based cancer immunotherapy was effective and safe at least in a mouse tumor prevention model. (*Cancer Sci* 2009; 100: 132–137)

Secreted protein acidic and rich in cystein (SPARC), also called osteonectin or BM-40, was identified in 1981 as a major non-collagenous constituent of bovine bone.[1] SPARC is a matricellular glycoprotein secreted by many cells types,[2] that modulates cellular interaction with extracellular matrix during tissue remodeling.[3] SPARC plays an important role in wound repair, cell proliferation, cell migration, morphogenesis, cellular differentiation and angiogenesis.[2–5] Targeted disruption of the *SPARC* gene in mice results in early cataractogenesis,[6,7] osteopenia[8] and curly tails.[2]

SPARC was reported to be overexpressed in various human cancers,[9–12] including primary and metastatic melanomas. The overexpression of SPARC by melanoma cells was associated with an invasive phenotype *in vivo*.[13,14] We previously reported that serum SPARC levels observed in melanoma patients were higher than those observed in healthy donors.[15] Increased level of serum SPARC was observed in 33% of all melanoma patients, irrespective of the clinical stages and even in the sera of patients with stage 0 *in situ* melanoma. Moreover, the combined use of SPARC and glypican-3, which was reported by us as a novel tumor marker for melanoma,[16] enabled a 66.2% detection rate of melanoma patients at an early stage (0–II). Thus, SPARC is considered to be a useful tumor marker for melanoma. However, the usefulness of SPARC as a target for cancer immunotherapy has not been previously investigated.

One of the actual methods of the immunotherapy for cancer was vaccination of epitope peptides derived from tumor-associated antigen. Recently, several investigators have reported the effect of peptide vaccination on cancer.[17,18] However, the effect was partial, and more useful antigens were required. We previously identified several tumor-associated antigens, including glypican-3, heart shock protein 105, proliferation potential-related protein, KM-HN-1, cell division cycle associated 1 and cadherin-3/P-cadherin.[16,19–27] In addition, we identified several HLA-A2- or HLA-A24-restricted cytotoxic T lymphocytes (CTL) epitopes derived from these antigens. The immunization with these epitopes was effective in a mouse tumor model and some of these were applied to phase I clinical trials of cancer immunotherapy.

In this study, we identified the H2-K^d-restricted and SPARC-derived CTL epitopes useful for SPARC-directed immunotherapy, and the vaccination with these peptides elicited effective antitumor immunity with no evidence of autoimmune diseases in mice.

Materials and Methods

Cell lines. Mouse cancer cell lines, B16, B16F1, B16F10, EL4, MCA, NIH3T3, 3LL, BALB/3T3, Colon26, A20, RL male1 and MethA were provided by the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer Tohoku University (Sendai, Japan). A mouse mammary cancer cell line, N2C, was provided by Dr Sangaletti Sabina of the National Institute of Tumors (Milan, Italy). T2K^d, a TAP-deficient T2 cell transfected with K^d-gene expression vector, was provided by Dr Paul M. Allen of Washington University School of Medicine (St Louis, MO, USA). These cells were maintained *in vitro* in RPMI-1640 or Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (FCS). Expression of H2-K^d was examined with flow cytometry analysis by using a fluorescein isothiocyanate (FITC)-conjugated antimouse H2-K^d-specific antibody (clone SF1-1.1, mIgG2ak; BD Biosciences Pharmingen, San Diego, CA, USA).

Mice. Seven-week-old female BALB/c mice (H-2^d), purchased from Charles River Laboratories Japan (Yokohama, Japan),

[6]To whom correspondence should be addressed.
E-mail: mxnishim@gpo.kumamoto-u.ac.jp
[7]These two authors contributed equally.

doi: 10.1111/j.1349-7006.2008.01016.x
© 2008 Japanese Cancer Association

were kept in the Center for Animal Resources and Development of Kumamoto University and handled in accordance with the animal care policy of Kumamoto University.

Identification of the CTL epitopes of SPARC in BALB/c mice. Mouse SPARC-derived peptides (purity, >90%), sharing the amino acid sequences with human SPARC and carrying binding motifs for both H2-K^d and HLA-A24 (*A*2402*), were searched for using BIMAS software (BioInformatics and Molecular Analysis Section, Center for Information Technology, NIH, Bethesda, MD, USA), and we purchased four kinds of peptides (Table 1) from AnyGen (Gwangju, Korea). Identification of the CTL epitopes of SPARC was done using BALB/c mice as described.[19] In brief, the BALB/c mice were immunized i.p. with bone marrow-derived dendritic cells (BMDC) pulsed with the mixture of SPARC candidate peptides once a week for 2 weeks. Seven days after the last immunization, CD4$^-$ spleen cells collected from immunized BALB/c were stimulated with syngeneic BMDC pulsed with each peptide *in vitro*. Then, 6 days later, CD4$^-$ T cells were collected from the culture and the CTL-producing γ-interferon (IFN-γ) was detected by an enzyme-linked immunospot (ELISPOT) assay. Moreover, after 5 days culture *in vitro* under the same conditions, cytotoxic activities of these cells directed against target cells were tested by standard 6-h ^{51}Cr release assays as described previously.[19]

Table 1. SPARC-derived peptides conserved between human and mouse SPARC and predicted to bind to H2-K^d and HLA-A24

Designation	Position	Subsequence residue listing	Binding score	
			H2-K^d	HLA-A24
SPARC-1	143–151	DYIGPCKYI	4000	75
SPARC-2	123–131	HFFATKCTL	1382	20
SPARC-3	161–170	EFPLRMRDWL	960	30
SPARC-4	225–234	MYIFPVHWQF	120	210

These peptides were searched for using BIMAS (Bioinformatics and Molecular Sections, Center for Information Technology, NIH, Bethesda, MD, USA) software (http://www-bimas.cit.nih.gov/molbio/hla_bind/).

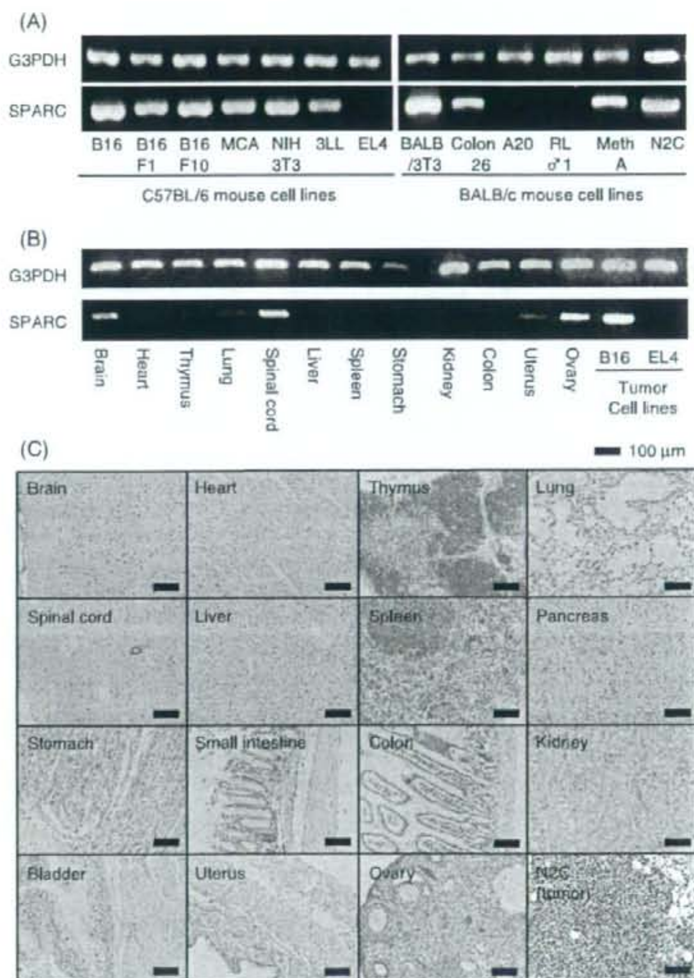

Fig. 1. The reverse transcription polymerase chain reaction (RT-PCR) analysis of *SPARC* mRNA expression in mouse cancer cell lines and normal tissues. (A) Various C57BL/6 and BALB/c mouse-derived cancer cell lines showed strong expression of *SPARC* except EL4, A20 and RL male 1. (B) Mouse *SPARC* gene was expressed in the normal tissues including ovary, spinal cord, brain and uterus by the RT-PCR analysis. (C) SPARC was not detected in the normal tissues by immunohistochemical staining.

© 2008 Japanese Cancer Association

Histological and immunohistochemical analysis. Immunohistochemical detections of SPARC was done as described previously.[21] We purchased Human, Normal Organs, and Cancers, Tissue Array, BC4 (SuperBioChips Laboratories, Seoul, Korea) for immunohistochemical analysis. Immunohistochemical staining of CD8 or CD4 was done as described previously.[28]

***In vivo* tumor prevention model.** BMDC (5×10^5 cells/body) loaded with or without SPARC-$4_{225-234}$ peptide or phosphate-buffered saline (PBS) were transferred i.p. into BALB/c mice ($n = 8$, each group) twice on days −14 and −7, and N2C cells (3×10^4/body) were challenged s.c. into the shaved back region on day 0. The tumor sizes were determined biweekly using a caliper square, and the tumor volume (mm^3) was calculated as long diameter × squared short diameter.

Statistical analysis. We analyzed all data with the StatView statistical program for Macintosh (SAS Institute, Cary, NC, USA) and evaluated the statistical significance with an unpaired Student's *t*-test. $P < 0.05$ was considered significant. The percentage of overall survival rate was calculated by the Kaplan–Meier method, and statistical significance was evaluated with the Wilcoxon rank sum test.

Results

Expression of *SPARC* mRNA in mouse cancer cell lines and normal tissues. We examined the expression level of *SPARC* mRNA using reverse transcription polymerase chain reaction (RT-PCR). The mouse fibroblast cell line NIH/3T3 and various mouse cancer cell lines, including melanoma (B16, B16F1 and B16F10), fibrosarcoma (MCA) and lung cancer (3LL) originated from C57BL/6 mice, and sarcoma (BALB/3T3 and MethA), colon cancer (colon26) and breast cancer (N2C) originated from BALB/c mice, showed strong expression of *SPARC* (Fig. 1A). C57BL/6 mouse leukemia/lymphoma cell line, EL4, and BALB/c mouse lymphoma cell line, A20 and RL male 1, did not express *SPARC*. Although *SPARC* mRNA was expressed in the ovary, spinal cord, brain and uterus, the expression levels observed in these tissues were lower than those observed in cancer cell lines (Fig. 1B). Moreover, normal tissues including ovary, spinal cord, brain and uterus did not express SPARC at protein level investigated by the immunohistochemical analysis (Fig. 1C).

Identification of the SPARC-derived and H2-K^d-restricted CTL epitopes in BALB/c mice. Structural motifs of peptides bound to human HLA-A24 (*A*2402*) and mouse H2-K^d are similar. The amino acid sequences of human and mouse SPARC have a 92% homology.[4] Thereby, we searched for SPARC-derived and H2-K^d- or HLA-A24 (*A*2402*)-restricted peptides of which amino acid sequences were completely shared between human and mouse SPARC, and prepared four different synthetic peptides (Table 1). $CD4^-$ spleen cells isolated from BALB/c mice immunized twice with BMDC pulsed with mixture of these four peptides were stimulated *in vitro* with BMDC pulsed with each peptide for 5–6 days. Subsequently, we collected these $CD4^-$ T cells from the culture and we found that $CD4^-$ T cells stimulated with the SPARC-$1_{143-151}$ (DYIGPCKYI) or SPARC-$4_{225-234}$ (MYIFPVHWQF) peptides produced a large amount of IFN-γ in a peptide-specific manner in ELISPOT assays (Fig. 2A). Moreover, we tested cytotoxic activities of these cells directed against target cells by standard 6-h ^{51}Cr release assays. CTL induced by SPARC-$1_{143-151}$ or SPARC-$4_{225-234}$ peptides showed specific cytotoxicity against T2K^d (H2-K^d+, TAP negative) cells pulsed with each SPARC peptide but not against T2K^d cells unpulsed with SPARC peptide (Fig. 2B). In addition, those CTL had cytotoxic activities directed against MethA ($SPARC^+$, H-2^d) but not against RL male 1 ($SPARC^-$, H-2^d). These findings suggest that these SPARC-$1_{143-151}$ and SPARC-$4_{225-234}$ peptides had the capacity to induce the H2-K^d-restricted peptide-reactive CTL and that the CTL killed the tumor naturally expressing both SPARC and H2-K^d.

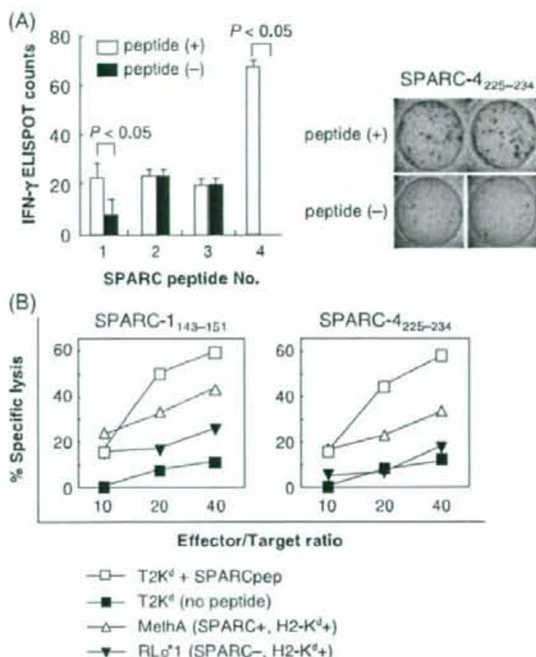

Fig. 2. Identification of the SPARC-derived and H2-K^d-restricted cytotoxic T lymphocyte (CTL) epitopes in BALB/c mice. We immunized the BALB/c mice with syngeneic bone marrow-derived dendritic cells (BMDC) (5×10^5/mouse) pulsed with the mixture of candidate peptides *in vivo* once a week for 2 weeks. Seven days after the last immunization, $CD4^-$ spleen cells isolated from immunized mice (2×10^6/well) were stimulated with BMDC (2×10^5/well) pulsed with each peptide *in vitro*. (A) Six days after the stimulation *in vitro*, the CTL-producing γ-interferon (IFN-γ) in response to the peptide-pulsed BMDC were detected by an enzyme-linked immunospot (ELISPOT) assay. (B) Five days after the stimulation *in vitro* under the same conditions, cytotoxic activities of these cells directed against indicated target cells was tested with standard ^{51}Cr release assays. We found that $CD4^-$ spleen cells stimulated with the SPARC-$1_{143-151}$ or SPARC-$4_{225-234}$ peptide produced a large amount of IFN-γ, and had cytotoxic activities directed against both H2-K^d and SPARC-expressing MethA tumor cell line or T2K^d pulsed with each peptide, but not to SPARC-negative RL male 1 and peptide-unpulsed T2K^d. These assays were done twice with similar results.

Immunization of SPARC-$4_{225-234}$ peptide did not induce the autoimmune diseases in BALB/c mice. To investigate whether the immunization of mice with the SPARC-derived H2-K^d-restricted peptide causes autoimmune diseases, the immunohistochemical staining of several important organs with anti-CD4 and anti-CD8 monoclonal antibody was performed in BALB/c mice immunized with BMDC pulsed with a mixture of four SPARC peptides once a week for 2 weeks. Tissue specimens of these mice were removed and analyzed 7 days after the second dendritic cell (DC) vaccination. As shown in Fig. 3, we could not find any pathological changes, such as lymphocyte infiltration or tissue destruction, in brain, heart, lung, liver, kidney, uterus, ovary and spinal cord of BALB/c mice. Although SPARC was expressed in spinal cord and brain by RT-PCR, the BALB/c mice immunized with BMDC pulsed with SPARC-$4_{225-234}$ peptide did not show any neurological disorders such as paralysis or abnormal behavior. No sign of autoimmune diseases

doi: 10.1111/j.1349-7006.2008.01016.x
© 2008 Japanese Cancer Association