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Expression of breast cancer resistance protein is associated with a poor clinical outcome in patients with small-cell lung cancer

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ABSTRACT

Background: ATP-binding cassette (ABC) transporter and DNA excision repair proteins play a pivotal role in the mechanisms of drug resistance. The aim of this study was to investigate the expression of ABC transporter and DNA excision repair proteins, and to elucidate the clinical significance of their expression in biopsy specimens from patients with small-cell lung cancer (SCLC).
Methods: We investigated expression of the ABC transporter proteins, P-glycoprotein (Pgp), multidrug resistance associated-protein 1 (MRP1), MRP2, MRP3, and breast cancer resistance protein (BCRP), and the DNA excision repair proteins, excision repair cross-complementation group 1 (ERCC1) protein and breast cancer susceptibility gene 1 (BRCA1) protein, in tumor biopsy specimens obtained before chemotherapy from 130 SCLC patients who later received platinum-based combination chemotherapy, and investigated the relationship between their expression and both response and survival.
Results: No significant associations were found between expression of Pgp, MRP1, MRP2, MRP3, ERCC1, or BRCA1 and either response or survival. However, there was a significant association between BCRP expression and both response ($p = 0.026$) and progression-free survival (PFS; $p = 0.0103$).
Conclusions: BCRP expression was significantly predictive of both response and progression-free survival (PFS) in SCLC patients receiving chemotherapy. These findings suggest that BCRP may play a crucial role in drug resistance mechanisms, and that it may serve as an ideal molecular target for the treatment of SCLC.

1. Introduction

Lung cancer is the leading cause of cancer-related deaths in many industrialized countries. Although the proportion of patients with small-cell lung cancer (SCLC) has been decreasing, it still accounts for approximately 15% of all cases of lung cancer. SCLC is one of the most chemo-sensitive solid tumors, but the vast majority of patients eventually experience a relapse, and as a result the median survival time is 14–20 months for limited disease (LD) and 7–10 months for extensive disease (ED) [1].

Intrinsic or acquired drug resistance is considered to be a major factor limiting the effectiveness of chemotherapy. Drug resistance by tumors occurs not only to a single cytotoxic agent, but in the form of cross-resistance to other cytotoxic agents, called multidrug resistance (MDR). One of the major mechanisms of MDR is increased ability of tumor cells to actively efflux drugs, which leads to a decrease in intracellular drug accumulation, and the mechanism is mediated by ATP-dependent drug efflux pumps that are known as ATP-binding cassette (ABC) transporters [2,3]. To date, at least 48 human ABC transporters have been identified, and they have been divided into seven subfamilies, ABC-A through ABC-G. Five of them, P-glycoprotein (Pgp), multidrug resistance associated-protein 1 (MRP1), MRP2, MRP3, and breast cancer resistance protein (BCRP), have been most intensively investigated, and *in vitro* studies have demonstrated associations between their expression and resistance to cytotoxic drugs commonly used in the treatment of SCLC, including etoposide, irinotecan, and topotecan [4].

Another important mechanism of drug resistance is increased repair of DNA damage mediated by the DNA excision repair gene. Resistance to platinum is associated with increased removal of platinum-DNA adducts, and DNA excision repair plays a pivotal role in this process [5]. Nucleotide excision repair (NER) is a major mechanism for repairing platinum-DNA adducts, and it is

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Table 1
Panel of primary antibodies.

Antibody	Clone	Pretreatment	Dilution	City/nation	Source
Pgp (mono)	JSB-1	Autoclave	1:20	Newcastle/United Kingdom	Novocastra
MRP1 (mono)	MRPm6	Autoclave	1:50	Uden/Netherlands	Sanbio
MRP2 (mono)	M2III-6	Autoclave	1:20	Uden/Netherlands	Sanbio
MRP3 (mono)	DTX1	Autoclave	1:100	Newcastle/United Kingdom	Novocastra
BCRP (mono)	BXP21	Autoclave	1:20	Uden/Netherlands	Sanbio
ERCC1 (mono)	8F1	Autoclave	1:100	Warm Springs/United States	Lab vision
BRCA1 (mono)	MS110	Microwave	1:100	San Diego/United States	Carbiochem

now known that there are two pathways in NER: transcription-coupled NER (TC-NER) and global genomic NER (GG-NER) [5]. Among NER proteins, excision repair cross-complementation group 1 (ERCC1) protein, which is involved in the GG-NER pathway, has been most intensively investigated. Expression of ERCC1 has recently been shown to be a significant negative predictive factor for survival of non-small cell lung cancer (NSCLC) patients receiving cisplatin-based adjuvant chemotherapy [6]. On the other hand, the results of an *in vitro* study have suggested the superiority of TC-NER pathway, in which breast cancer susceptibility gene 1 (BRCA1) protein is involved, to GG-NER pathway in predicting platinum resistance [7]. Since platinum agents are considered to be key drugs in the treatment of SCLC as well as NSCLC [8–10], it is of great interest to determine whether there is an association between the expression of DNA excision repair genes and the effectiveness of platinum-based chemotherapy in SCLC patients.

In this retrospective study we investigated the immunohistochemical expression of the ABC transporter proteins, Pgp, MRP1, MRP2, MRP3, and BCRP, and the DNA excision repair proteins, ERCC1 protein and BRCA1 protein, in tumor biopsy specimens obtained before chemotherapy from 130 SCLC patients who later received platinum-based combination chemotherapy, and we investigated the relationship between their expression and the patients' clinical outcome.

2. Materials and methods

2.1. Subjects

A total of 626 patients were diagnosed with SCLC at the National Cancer Center Hospital East between July 1992 and December 2005, and 578 of them received platinum-based combination chemotherapy as an initial treatment. After excluding the 246 patients who received thoracic radiotherapy and 2 patients who received surgery in order to eliminate the effects of treatment other than chemotherapy, the 191 patients of the remaining 330 patients diagnosed only cytologically, and therefore with no specimens available for analysis, and the nine patients whose specimens were unsuitable for immunohistochemistry. In this study, we analyzed biopsy specimens from 130 patients consisting of 104 responders and 26 non-responders. Institutional Review Board-approved informed consent was obtained from all patients.

2.2. Clinical evaluation

The classification system proposed by the Veterans' Administration Lung Study Group was used to stage SCLC as limited disease (LD) or extensive disease (ED) [11]. LD is defined as disease confined to one hemithorax that can be encompassed within a single radiation field, and ED is defined as disease that extends beyond these confines. Performance status (PS) was determined based on the Eastern Cooperative Oncology Group (ECOG) scale. Patient response was evaluated by using the Response Evaluation Criteria in Solid Tumors (RECIST) [12].

2.3. Immunohistochemistry

Tissue blocks were cut into 4-μm sections and mounted on silane-coated slides (Matsunami, Tokyo, Japan). The slides were then deparaffinized in xylene and dehydrated in a graded alcohol series. For antigen retrieval, the slides for Pgp, MRP1, MRP2, BCRP, ERCC1, and BRCA1 were immersed in 10 mM citric buffer solution (pH 6.0) at 120 °C for 20 min and the slides for MRP3 were immersed in 1 mM EDTA retrieval fluid (pH 8.0) at 95 °C for 20 min. The slides were then allowed to cool for 1 h at room temperature and washed in PBS. Nonspecific binding was blocked by incubation with 2% BSA plus 0.1% NaN_3 for 30 min, and after draining off the blocking solution, the slides were incubated overnight at 4 °C with the primary antibodies listed in Table 1. Endogenous peroxidase was then blocked with 0.3% H_2O_2 in methanol for 10 min, and after washing three times in PBS, the slides were incubated for 60 min with a labeled polymer En Vision+, peroxidase Mouse (DAKO, Glostrup, Denmark). The chromogen used was 2% 3,3′-diaminobenzidine in 50 mM Tris buffer (pH 7.6) containing 0.3% hydrogen, and the slides were counterstained with hematoxylin. Normal human liver tissue was used as a positive control for Pgp, MRP2, MRP3, and BCRP, normal human lung tissue for MRP1, normal human tonsil tissue for ERCC1, and breast cancer tissue human for BRCA1. Negative controls for each antibody were prepared by using non-immune serum instead of the primary antibodies. Membranous or cytoplasmic staining was evaluated for ABC transporter proteins [13], while nuclear staining was evaluated for DNA excision repair proteins [6,14]. Staining of each antibody was considered positive if >10% of the tumor cells stained. All of the slides were examined and scored independently by two observers (Y.K. and G.I.) without knowledge of the patients' clinical data. When judgments differed between two observers, they discussed it until an agreement was reached.

2.4. Statistical analysis

The significance of the relationship between immunohistochemical expression and clinical variables or response to chemotherapy was evaluated by using the χ^2 test or Fisher's exact test, as appropriate. The logistic regression model was used for multivariate analysis of response. Progression-free survival (PFS) was used as a clinical marker for duration of response to chemotherapy. Overall survival (OS) was measured from the start of chemotherapy to the date of death from any cause or the date patients were last known to be alive. Survival rates were calculated by the Kaplan–Meier method, and the statistical significance of any differences in PFS and OS were evaluated by a log-rank test. The Cox proportional hazards model was used for multivariate analysis of survival. p values less than 0.05 were considered significant. All statistical analyses were performed using

Table 2
Patient characteristics ($n = 130$).

Characteristics	No. of patients (%)
Age	
Median	67
Range	28–83
Gender	
Male	108 (83)
Female	22 (17)
Disease extent	
LD	18 (14)
ED	112 (86)
Performance status	
0	2 (2)
1	93 (71)
2	25 (19)
3	8 (6)
4	2 (2)
Chemotherapy regimen	
CE	36 (28)
PE	35 (27)
PI	25 (19)
CODE	18 (14)
CAV/PE	7 (5)
PEI	7 (5)
PT	2 (2)

LD, limited disease; ED, extensive disease; CE, Carboplatin + Etoposide; PE, Cisplatin + Etoposide; PI, Cisplatin + Irinotecan; CODE, Cisplatin + Vincristine + Doxorubicin + Etoposide; CAV/PE, Cyclophosphamide + Doxorubicin + Vincristine/Cisplatin + Etoposide; PEI, Cisplatin + Etoposide + Irinotecan; PT, Cisplatin + Topotecan.

the statistical program StatView, Version 5.0 (Abacus Concepts, Berkley, CA).

3. Results

3.1. Patient characteristics

The patient characteristics are summarized in Table 2. The median age of the patients was 67 years (range: 28–83 years). More than 80% of the patients were male, and more than 80% had ED. Despite excluding patients who had received thoracic radiotherapy or surgery, our study included 18 LD patients. The major reasons for omitting thoracic radiotherapy in these LD patients were the presence of a malignant pleural effusion (9 patients) and interstitial pneumonia (5 patients). PS was generally good; approximately 70% of the patients were PS 0 or 1. All patients received chemotherapy containing etoposide, irinotecan, or topotecan. The details of administered chemotherapy are shown in Table 3.

3.2. Expression of ABC transporter and DNA excision repair proteins in SCLC

The immunostaining of ABC transporter proteins was both membranous and cytoplasmic, whereas the immunostaining of the DNA excision repair proteins was mostly restricted to the nucleus. Forty-two (33%) of the 130 tumors were Pgp-positive, 29 (22%) were MRP1-positive, 25 (19%) were MRP2-positive, 9 (7%) were MRP3-positive, 48 (37%) were BCRP-positive, 36 (27%) were ERCC1-positive, and 109 (83%) were BRCA1-positive. The relationships between expression of the ABC transporter and DNA excision repair proteins and the clinical variables are shown in Table 4. BCRP expression was significantly greater in the PS 2–4 cases than in the PS 0-1 cases ($p = 0.0223$). There were no significant correlations between expression of Pgp, MRP1, MRP2, MRP3, ERCC1, or BRCA1 and the clinical variables.

3.3. Association between expression of ABC transporter and DNA excision repair proteins and clinical outcome

The relationships between clinical variables and response to chemotherapy and survival are shown in Table 5. Response rate was not associated with any clinical variables, but PFS ($p = 0.0199$) and OS ($p = 0.0159$) were significantly associated with PS. Table 6 shows the associations between expression of ABC transporter and DNA excision repair proteins and response to chemotherapy and survival. BCRP expression was significantly predictive of response to chemotherapy ($p = 0.026$), and MRP2 expression was marginally predictive ($p = 0.0515$).

The median follow-up time was 8.3 years, and 119 patients had been dead until the time of analysis. The results for survival showed that BCRP expression was significantly associated with PFS ($p = 0.0103$), but not with OS ($p = 0.1427$). No significant associations were observed between expression of Pgp, MRP1, MRP3, ERCC1, or

Table 3
Details of administered chemotherapy.

Regimen	Dosage of each agent		Schedule		Median number of treatment cycles (range)
CE	Carboplatin	AUC 6	Day 1	q3w	4 (1–4)
	Etoposide	100 mg/m²	Days 1–3		
PE	Cisplatin	60 mg/m²	Day 1	q3w	4 (1–4)
	Etoposide	100 mg/m²	Days 1–3		
PI	Cisplatin	60 mg/m²	Day 1	q4w	4 (1–4)
	Irinotecan	60 mg/m²	Days 1, 8, 15		
CODE	Cisplatin	25 mg/m²	Day 1 (1, 2, 3, 4, 5, 6, 7, 8, 9 weeks)	Weekly	9 (2–9)
	Vincristine	1 mg/m²	Day 1 (2, 4, 6, 8 weeks)		
	Doxorubicin	40 mg/m²	Day 1 (1, 3, 5, 7 weeks)		
	Etoposide	80 mg/m²	Day 1–3 (1, 3, 5, 7 weeks)		
CAV/PE	Cyclophosphamide	800 mg/m²	Day 1	Alternatively	6 (3–6)
	Doxorubicin	50 mg/m²	Day 1		
	Vincristine	1.4 mg/m²	Day 1		
	Cisplatin	80 mg/m²	Day 1		
	Etoposide	100 mg/m²	Day 1, 3, 5		
PEI	Cisplatin	25 mg/m²	Day 1 (1, 2, 3, 4, 5, 6, 7, 8, 9 weeks)	Weekly	4 (2–9)
	Etoposide	60 mg/m²	Days 1–3 (1, 3, 5, 7 weeks)		
	Irinotecan	90 mg/m²	Day 1 (2, 4, 6, 8 weeks)		
PT	Cisplatin	60 mg/m²	Day 5	q3w	4.5 (4–5)
	Topotecan	1 mg/m²	Days 1–5		

AUC, area under the curve.

Table 4
Relationship between clinical variables and expression of ABC transporter and DNA excision repair proteins.

	n	Pgp-positive (%)	MRP1-positive (%)	MRP2-positive (%)	MRP3-positive (%)	BCRP-positive (%)	ERCC1-positive (%)	BRCA1-positive (%)
Total	130	42 (33)	29 (22)	25 (19)	9 (7)	48 (37)	36 (27)	109 (83)
Age								
<70	83	29 (35)	16 (19)	15 (18)	5 (6)	29 (35)	24 (29)	70 (84)
≥70	47	13 (28)	13 (28)	10 (21)	4 (9)	19 (40)	12 (26)	39 (83)
Gender								
Male	108	36 (33)	23 (21)	19 (18)	9 (8)	41 (38)	30 (28)	93 (86)
Female	22	6 (27)	6 (27)	6 (27)	0 (0)	7 (32)	6 (27)	16 (73)
Disease extent								
LD	18	8 (44)	3 (17)	6 (33)	3 (17)	8 (44)	4 (22)	16 (89)
ED	112	34 (30)	26 (23)	19 (17)	6 (5)	40 (36)	32 (29)	93 (83)
PS								
0–1	95	33 (35)	20 (21)	21 (22)	8 (8)	29 (31)[a]	27 (28)	80 (84)
2–4	35	9 (26)	9 (26)	4 (11)	1 (3)	19 (54)	9 (26)	29 (83)

ABC, ATP-binding cassette; Pgp, P-glycoprotein; MRP, multidrug resistance protein; BCRP, breast cancer resistance protein; ERCC, excision repair cross-complementation group; BRCA, breast cancer susceptibility gene; LD, limited disease; ED, extensive disease; PS, performance status.
[a] $p = 0.0223$.

Table 5
Summary of relationship between clinical variables and response to chemotherapy and survival.

	n	Response rate (%)	p	PFS (mo)	p	MST (mo)	p
Total	130	79		5.2		9.0	
Age							
<70	83	80	>0.9999	5.1	0.1296	9.4	0.3493
≥70	47	81		5.4		10.9	
Gender							
Male	108	81	0.7715	5.1	0.5496	9.4	0.6528
Female	22	77		5.7		13.2	
Disease extent							
LD	18	67	0.2277	5.6	0.4838	9.4	0.8856
ED	112	82		5.2		10.4	
PS							
0–1	95	82	0.4584	5.5	0.0199*	10.8	0.0159*
2–4	35	74		4.2		8.1	

LD, limited disease; ED, extensive disease; PS, performance status; PFS, progression-free survival; MST, median survival time.
* $p < 0.05$.

Table 6
Association between expression of ABC transporter and DNA excision repair proteins and response to chemotherapy and survival ($n = 130$).

	n	Response rate (%)	p	PFS (mo)	p	MST (mo)	p
Pgp							
Positive	42	83	0.6730	5.5	0.7257	10.5	0.3006
Negative	88	78		5.1		9.9	
MRP1							
Positive	29	90	0.1902	5.3	0.8141	11.0	0.2249
Negative	101	77		5.2		9.4	
MRP2							
Positive	25	64	0.0515	5.6	0.5832	12.6	0.1261
Negative	105	84		5.2		9.3	
MRP3							
Positive	9	78	>0.9999	5.2	0.3181	11.9	0.1326
Negative	121	80		5.3		9.4	
BCRP							
Positive	48	69	0.0260*	4.0	0.0103*	9.1	0.1427
Negative	82	87		5.6		10.6	
ERCC1							
Positive	36	89	0.1452	5.4	0.5383	11.9	0.6250
Negative	94	77		4.3		9.3	
BRCA1							
Positive	109	79	0.5666	5.3	0.8404	10.5	0.4611
Negative	21	86		4.7		8.1	

ABC, ATP-binding cassette; Pgp, P-glycoprotein; MRP, multidrug resistance protein; BCRP, breast cancer resistance protein; ERCC, excision repair cross-complementation group; BRCA, breast cancer susceptibility gene; PFS, progression-free survival; MST, median survival time.
* $p < 0.05$.

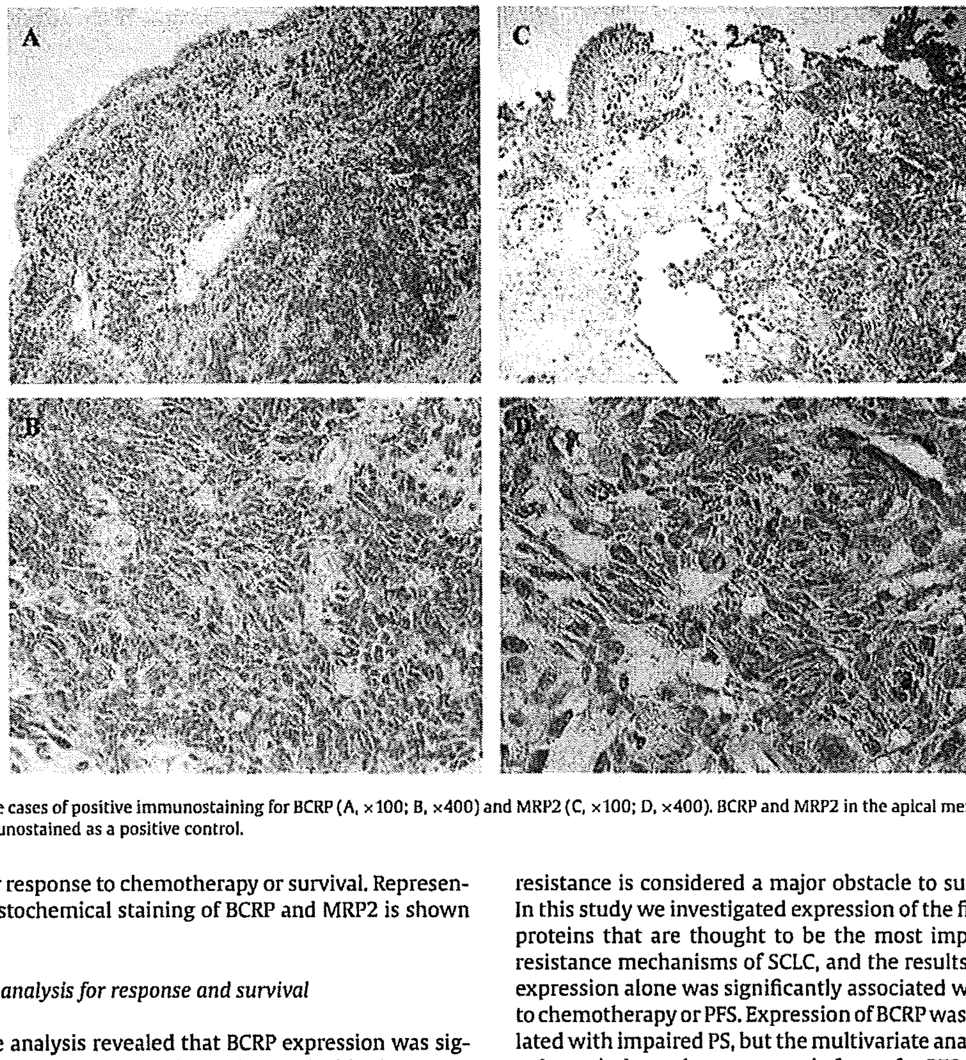

Fig. 1. Representative cases of positive immunostaining for BCRP (A, ×100; B, ×400) and MRP2 (C, ×100; D, ×400). BCRP and MRP2 in the apical membrane of the bronchial layer have been immunostained as a positive control.

BRCA1 and either response to chemotherapy or survival. Representative immunohistochemical staining of BCRP and MRP2 is shown in Fig. 1.

3.4. *Multivariate analysis for response and survival*

A multivariate analysis revealed that BCRP expression was significantly predictive of response to chemotherapy (Table 7). PFS was significantly associated with both PS ($p = 0.0299$) and BCRP expression ($p = 0.0138$), whereas OS was significantly associated with PS alone ($p = 0.0295$; Table 8). The PFS and OS curves according to BCRP expression are shown in Fig. 2.

4. Discussion

Although initial chemotherapy succeeds in 80–90% of SCLC patients, most patients eventually experience a relapse and their survival time is quite limited. Unfortunately, little progress in the chemotherapy of SCLC has been made during the past 30 years [15]. If drug resistance could be overcome, it would no doubt lead to an improved prognosis of this challenging disease, because drug resistance is considered a major obstacle to successful treatment. In this study we investigated expression of the five ABC transporter proteins that are thought to be the most important in the drug resistance mechanisms of SCLC, and the results showed that BCRP expression alone was significantly associated with either response to chemotherapy or PFS. Expression of BCRP was significantly correlated with impaired PS, but the multivariate analysis revealed BCRP to be an independent prognostic factor for PFS.

BCRP, which is classified as ABCG2 and known as the mitoxantrone resistance gene (MXR) or ABC transporter in placenta (ABC-P), is expressed in a variety of normal tissues, with the highest levels having been found in the placenta, and lower levels in the liver, small intestine, brain, and ducts and lobules of the breast [2,16]. BCRP was initially isolated from doxorubicin-resistant breast

Table 7
Multivariate analysis for response ($n = 130$).

Variables	Category	Risk ratio	95% CI	p
Age	<70 vs. ≥70	0.701	0.263–1.869	0.4776
Gender	Female vs. Male	0.857	0.258–2.848	0.8014
Disease extent	LD vs. ED	1.81	0.545–6.018	0.3329
PS	0–1 vs. 2–4	1.315	0.471–3.676	0.6013
MRP2	(−) vs. (+)	2.238	0.779–6.429	0.1346
BCRP	(−) vs. (+)	2.804	1.103–7.128	0.0303*

* $p < 0.05$.

Table 8
Multivariate analysis for survival ($n = 130$).

Variables	Category	Risk ratio	95% CI	p
A. Progression-free survival				
Age	<70 vs. ≥70	0.691	0.464–1.028	0.0682
Gender	Female vs. Male	1.062	0.650–1.733	0.8105
Disease extent	LD vs. ED	0.87	0.501–1.512	0.6251
PS	0–1 vs. 2–4	1.592	1.046–2.424	0.0299*
BCRP	(−) vs. (+)	1.614	1.102–2.363	0.0138*
B. Overall survival				
Age	<70 vs. ≥70	0.832	0.565–1.224	0.3496
Gender	Female vs. Male	1.067	0.658–1.729	0.7936
Disease extent	LD vs. ED	1.131	0.673–1.901	0.6430
PS	0–1 vs. 2–4	1.588	1.047–2.407	0.0295*
BCRP	(−) vs. (+)	1.235	0.831–1.833	0.2962

LD, limited disease; ED, extensive disease; PS, performance status; BCRP, breast cancer resistance protein.
* $p < 0.05$.

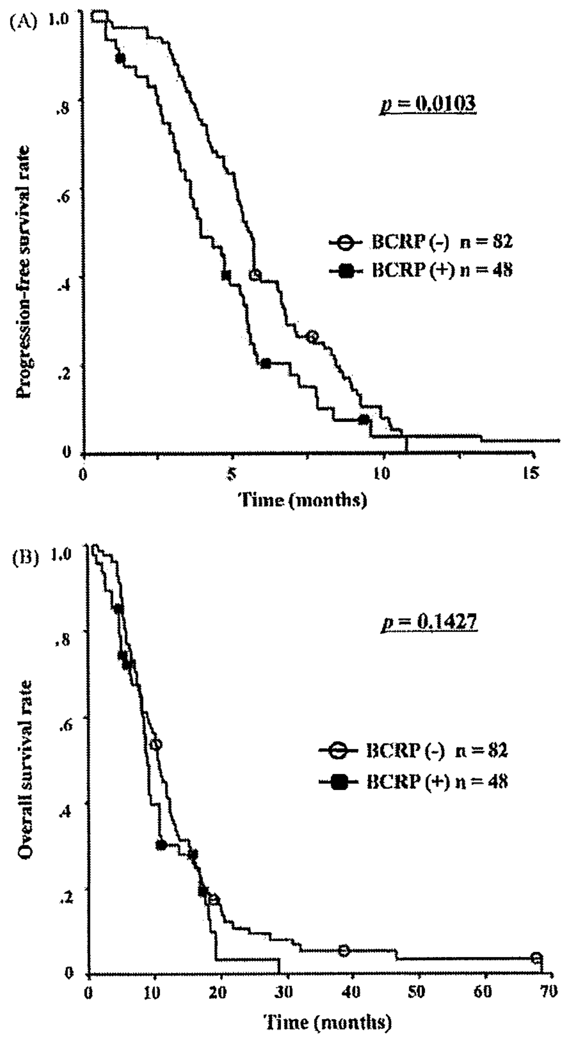

Fig. 2. Progression-free survival curves (A) and overall survival curves (B) for 130 SCLC patients, according to breast cancer resistance protein (BCRP) expression.

cancer cell line MCF-7, and its overexpression was found to promote resistance to topoisomerase I inhibitors, including irinotecan and topotecan [17]. We previously reported the finding that BCRP expression is a significant predictor of survival in advanced NSCLC [18], but to our knowledge no data have been reported regarding BCRP expression in SCLC.

No significant association was found between the expression of other ABC transporter proteins and clinical outcome in the present study. Some studies have shown a relationship between expression of Pgp or MRP1 and response or survival [19–23], however, their clinical usefulness as therapeutic targets is still obscure. In fact, two randomized phase III studies that incorporated modulators of Pgp and one phase II study of VX-710, an inhibitor of both Pgp and MRP1, failed to show any survival benefit in SCLC patients [24–26].

In this study we also investigated the expression of the DNA excision repair proteins ERCC1 and BRCA1 in SCLC, but neither of them was related to response or survival. Expression of DNA excision repair proteins has hardly ever been investigated in SCLC, and to our knowledge there has been only one study in regard to it. In that study high expression of ERCC1 was associated with poor survival, but when the cases were grouped according to stage, a significant decrease in survival was observed only in the LD patients, and the correlation between ERCC1 expression and response was not mentioned [27]. By contrast, expression of DNA excision repair proteins, especially ERCC1, has been intensively investigated in NSCLC recently, and expression of ERCC1 has been demonstrated to be related to platinum resistance in several studies [6,28,29]. We analyzed the ERCC1 expression also using the criterion by Olaussen et al. [6], but the results were similar and our conclusions did not change (data not shown). BRCA1 expression was also demonstrated to be significantly associated with chemoresistance in one study [30]. However, in other studies no significant association was observed between expression of ERCC1 or BRCA1 and either response or survival [14,31]. Their clinical significance in lung cancer including SCLC has yet to be determined, and further studies are awaited.

The concept of "cancer stem cells", a very small fraction of the whole cell population repeating self-renewal continues to supply cancer-constitute cells, has recently gained wide acceptance. Although the origin of cancer stem cells has not yet been elucidated, the idea that malignant transformation of a normal stem cell has been proposed [32]. Side population (SP) cells, defined by Hoechst 33342 dye exclusion in flow cytometry, are considered to be an enriched source of normal stem cells [33]. In addition, BCRP has been shown to be a molecular determinant of the SP phenotype, and it can be used as a marker for stem cell selection [34]. In a recent study, SP cells isolated from lung cancer displayed elevated expression of BCRP and showed resistance to multiple chemotherapeutic agents [35]. These findings indicate that it may be possible to use BCRP as a marker of cancer stem cells in certain types of lung cancer.

In conclusion, the results of the present study indicated that immunohistochemical expression of BCRP is significantly associated with response and PFS in SCLC patients treated with platinum-based chemotherapy. Our results should be tested in LD patients who received thoracic radiotherapy, and it is also desirable that our results will be validated in other methods, such as mRNA expression analysis. Although confirmatory studies are needed, BCRP may be an ideal therapeutic target for SCLC. A variety of BCRP inhibitors have already been identified [36–39]. Clinical trials of combination of these agents with conventional chemotherapy might be acceptable in SCLC.

Conflict of interest statement

None declared.

Acknowledgements

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Short Communication

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

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ABSTRACT:

The anticancer prodrug, irinotecan, is converted to its active form 7-ethyl-10-hydroxycamptothecin (SN-38) by carboxylesterases, and SN-38 is inactivated by UDP-glucuronosyltransferase (UGT)1A1-mediated glucuronidation. UGT1A9 also mediates this reaction. In a recent study, it was reported that the *UGT1A9* IVS1+399 (I399)C>T polymorphism is associated with increased SN-38 glucuronidation both in vitro and in vivo. However, its role in UGT1A9 expression levels and activity is controversial. Thus, we evaluated the role of I399C>T in SN-38 glucuronidation using 177 Japanese cancer patients administered irinotecan. I399C>T was detected at a 0.636 allele frequency. This polymorphism was in strong linkage disequilibrium (LD) with *UGT1A9*1b* (−126_−118T_9>T_{10}, |D'| = 0.99) and *UGT1A1*6* (211G>A, 0.86), in moderate LD with *UGT1A1*60* (−3279T>G, 0.55), but weakly associated with *UGT1A1*28* (−54_−39A(TA)$_6$TAA>A(TA)$_7$TAA, 0.25). Haplotype analysis showed that 98% of the I399C alleles were linked with low-activity haplotypes, either *UGT1A1*6, *28*, or **60*. On the other hand, 85% of the T alleles were linked with the *UGT1A1* wild-type haplotype **1*. Although I399T-dependent increases in SN-38 glucuronide/SN-38 area under concentration-time curve (AUC) ratio (an in vivo marker for UGT1A activity) and decreases in SN-38 AUC/dose were apparent (P < 0.0001), these effects were no longer observed after stratified patients by *UGT1A1*6, *28*, or **60* haplotype. Thus, at least in Japanese populations, influence of I399C>T on SN-38 glucuronidation is attributable to its close association with either *UGT1A1*6, *28*, or **60*.

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

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

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

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

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

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

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

Materials and Methods

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

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

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

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

Results

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

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

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

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

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

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

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

Discussion

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

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

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

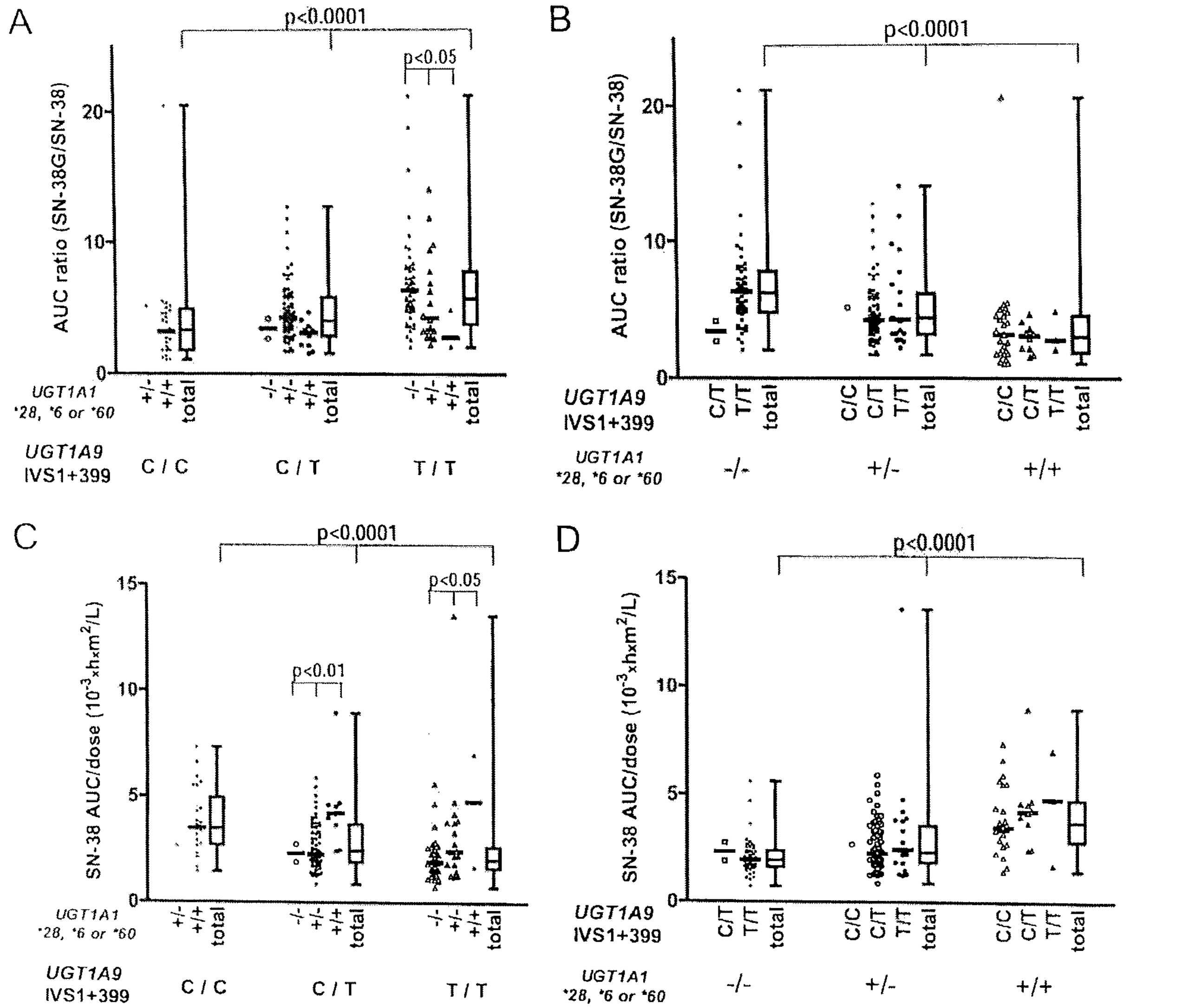

FIG. 2. Association analysis of *UGT1A9* IVS1+399 (I399)C>T with SN-38G/SN-38 AUC ratio (A and B) and SN-38 AUC/dose (C and D). A and C, I399 C/C, C/T, and T/T patients were further divided by the presence of *UGT1A1**28, *6, or *60 haplotypes: −/−, no *UGT1A1**28, *6, or *60; −/+, heterozygotes for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

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

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

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

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A randomised trial of intrapericardial bleomycin for malignant pericardial effusion with lung cancer (JCOG9811)

Clinical Studies

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Safety and efficacy of intrapericardial (ipc) instillation of bleomycin (BLM) following pericardial drainage in patients with malignant pericardial effusion (MPE) remain unclear. Patients with pathologically documented lung cancer, who had undergone pericardial drainage for MPE within 72 h of enrolment, were randomised to either arm A (observation alone after drainage) or arm B (ipc BLM at 15 mg, followed by additional ipc BLM 10 mg every 48 h). The drainage tube was removed when daily drainage was 20 ml or less. The primary end point was survival with MPE control (effusion failure-free survival, EFFS) at 2 months. Eighty patients were enrolled, and 79 were eligible. Effusion failure-free survival at 2 months was 29% in arm A and 46% in arm B (one-sided $P = 0.086$ by Fisher's exact test). Arm B tended to favour EFFS, with a hazard ratio of 0.64 (95% confidence interval: 0.40–1.03, one-sided $P = 0.030$ by log-rank test). No significant differences in the acute toxicities or complications were observed. The median survival was 79 days and 119 days in arm A and arm B, respectively. This medium-sized trial failed to show statistical significance in the primary end point. Although ipc BLM appeared safe and effective in the management of MPE, the therapeutic advantage seems modest.
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Malignant pericardial effusion (MPE) is a grave complication of malignant tumours. The frequency of pericardial involvement by malignancy has been estimated to be 10–21% at autopsy (Theologides, 1978; Klatt and Heitz, 1990).

Malignant pericardial effusions are often asymptomatic and detected incidentally by echocardiography or computed tomography. Symptomatic cases, however, often manifest cardiac tamponade, which can rapidly lead to cardiovascular collapse and death, unless promptly treated (Press and Livingston, 1987).

Lung cancer is the most frequent cause of MPE, and other common primary sites include breast cancer, oesophageal cancer, lymphoma and leukaemia (Abraham *et al*, 1990; Wilkes *et al*, 1995; Yonemori *et al*, 2007). The prognosis of MPE in lung cancer patients is particularly poor, with a reported median survival of 3 months or less (Okamoto *et al*, 1993; Gornik *et al*, 2005).

Although prompt diagnosis and pericardial drainage result in good palliation of symptoms, drainage alone is often inadequate to prevent re-accumulation of the fluid after the drainage tube is removed (Shepherd, 1997). There are numerous reports of pericardial sclerosis for MPE by the instillation of various agents, such as tetracycline/doxycycline (Shepherd *et al*, 1987; Maher *et al*, 1996), a streptococcal preparation (Imamura *et al*, 1991), bleomycin (BLM) (Vaitkus *et al*, 1994; Liu *et al*, 1996; Maruyama *et al*, 2007), thiotepa (Colleoni *et al*, 1998; Martinoni *et al*, 2004), cisplatin/carboplatin (Moriya *et al*, 2000; Tomkowski *et al*, 2004), 5-fluorouracil (Lerner-Tung *et al*, 1997), anthracyclines (Kawashima *et al*, 1999), vinblastine (Primrose *et al*, 1983), mitoxyantrone (Norum *et al*, 1998), mitomycin C (Kaira *et al*, 2005) and ^{32}P-colloid (Dempke and Firusian, 1999), after drainage. Platinum agents are actually not 'classic' sclerosants to induce inflammatory adhesion of the pericardial sac; they were apparently used as local chemotherapy. Whereas each study reports favourable outcomes in terms of MPE control and prevention of re-accumulation, almost all were performed as phase II trials, and no definite conclusions could be drawn (Press and Livingston, 1987; Vaitkus *et al*, 1994).

In one of the very few randomised trials conducted to date, Liu *et al* (1996) reported that BLM is the preferred agent for sclerosis, because of the lower morbidity associated with it. However, to the best of our knowledge, the efficacy and safety of pericardial sclerosis itself has never been evaluated by a prospective randomised trial.

This trial was aimed at evaluating the safety and efficacy of pericardial sclerosis induced by intrapericardial (ipc) BLM

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instillation, as compared with pericardial drainage alone, in lung cancer patients with MPE.

PATIENTS AND METHODS

Patient eligibility criteria

Patients with pathologically documented lung cancer, who had undergone pericardial drainage for clinical MPE (moderate to large accumulation of fluid), were eligible for study entry. Indications for the drainage were clinically determined; cases after emergent drainage and those after elective one were both included. Patient registration should be done within 72 h of drainage. The eligibility criteria were as follows: 75 years of age or less, expected life prognosis of 6 weeks or more with control of the MPE and minimum organ functions (leukocyte count$\geqslant$3000 per mm^3, platelet count$\geqslant$75 000 per mm^3, haemoglobin$\geqslant$9.0 g dl^{-1} and no renal or hepatic failure; however, laboratory abnormalities related to cardiac tamponade were allowed). Patients with chemotherapy-naive small cell cancer were excluded. Other exclusion criteria included apparently non-malignant effusion (e.g., purulent effusion), recurrent MPE, myocardial infarction or unstable angina within the previous 3 months, constrictive pericarditis, active interstitial pneumonia, severe infection and disseminated intravascular coagulation. Those with an unstable clinical condition attributable to other severe complications, such as superior vena cava syndrome, central airway obstruction or uncontrollable massive pleural effusion, were also excluded.

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

Treatment plan

The study protocol did not limit the method used for the pericardial drainage. Both percutaneous tube pericardiostomy (non-surgical method), in which a drainage catheter is inserted using the Seldinger technique, and subxiphoid pericardiostomy (surgical method), in which a drainage tube is placed surgically, were allowed; each participating institution, however, basically adhered to one method, which they used in routine practice. The drainage method used was recorded on the case report form.

After registration with telephone or facsimile, the patients were randomly assigned to one of the two treatment arms with block randomisation stratified by the institution. In arm A, no additional intervention was performed and the patient was observed clinically after the pericardial drainage. In arm B, 15 mg of BLM dissolved in 20 ml of normal saline was instilled through the drainage catheter into the pericardial space immediately after the patient registration. The catheter was then clamped and reopened after 2 h, allowing resumption of the drainage. Additional doses of BLM at 10 mg were instilled similarly every 48 h, unless the criteria for tube removal, as described below, were met.

The drainage tube was removed, in both arm A and arm B, when the drainage volume per 24 h was 20 ml or less. If the criterion was met during the 24 h preceding randomisation in a patient allocated to arm A, the tube was immediately removed.

Patient evaluation and follow-up

Primary control of the MPE was considered to be achieved when the drainage tube could be successfully removed within 7 days of randomisation. When the criterion for tube removal, that is 20 ml per 24 h, could not be met by 7 days, the case was judged to show primary failure of the protocol therapy: treatment after off-protocol was not limited by the study protocol. When the drainage tube had to be removed because of obstruction, but re-drainage was clinically unnecessary, it was judged to have been successfully removed with primary control of MPE.

Monitoring for recurrence of the MPE in those who showed primary control was conducted by echocardiography at 1, 2, 4, 6 and 12 months. When the estimated fluid volume in the recurrent effusion exceeded 100 ml, the case was labelled as showing MPE re-accumulation and recurrence. Re-drainage was performed as clinically indicated.

The adverse effects of the therapy were evaluated according to the Japan Clinical Oncology Group Toxicity Criteria (Tobinai et al, 1993), modified from the National Cancer Institute Common Toxicity Criteria version 1.

The primary end point of the study was effusion failure-free survival (EFFS) rate at 2 months; EFFS was patient survival without MPE recurrence as defined above, in patients showing primary control. It was calculated as the period from the date of pericardial drainage to the date of MPE recurrence or the patient's death. For those patients with primary failure, MPE recurrence was considered to have occurred at the date of drainage, with an EFFS of zero. Effusion failure-free survival was judged regardless of the other disease status.

The secondary end points included the primary MPE control rate, time to drainage tube removal, EFFS, treatment-related morbidity, proportion of late pericardial or cardiac complication, overall survival (OS) and symptom scores.

Study-specific four-item symptom scores were completed by patients at the time of randomisation (i.e., after pericardial drainage) and at 1 month after the enrolment. The scores were to be interviewed by the health professionals other than the attending physicians. The items consisted of cough, pain, anorexia and shortness of breath. The scoring was conducted as follows: as not at all present (0), a little (1), moderate (2) and very much (3). The score for each item and the sum of the total score for all the four items were compared between the baseline and the follow-up assessments, and judged to be improved (lower scores in the follow-up assessments), stable (no change of scores) or worsened (higher scores, or the patient could not fill out the questionnaire, in the follow-up assessments).

Statistical considerations

From the historical data, the EFFS rate at 2 months in arm A was assumed to be 30% and that in arm B was presumed to be 60%. The study was designed to provide 80% power with 5% one-sided α. The required sample size was calculated as 80 patients, 40 in each arm, for comparing independent proportions.

The OS, time to tube removal and EFFS of both arms were calculated by the Kaplan-Meier method and compared by log-rank tests. The primary MPE control rate, symptom scores, complication rates and EFFS at each of the follow-up points were compared using Fisher's exact test. All analyses were performed with the SAS software version 9.1 (SAS Institute, Cary, NC, USA).

RESULTS

Patient characteristics and treatment delivery

From August 1999 to January 2006, 80 patients from 14 institutions were enrolled and randomised, 42 to arm A and 38 to arm B. One patient in arm B was found to be ineligible because of late registry, 2 weeks after the pericardial drainage. All 80 patients were analysed for their characteristics and chemotherapy morbidity, and the 79 eligible patients were analysed for efficacy and survival.

Table 1 lists the characteristics of the patients, which were generally well balanced between the arms, except for the effusion cytology: there were numerically more patients with

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Table 1 Patient characteristics

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

ipc BLM = intrapericardial bleomycin instillation.

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

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

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

Morbidity and early deaths

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

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

Table 2 Morbidity of the protocol therapy

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

ipc BLM = intrapericardial bleomycin instillation.

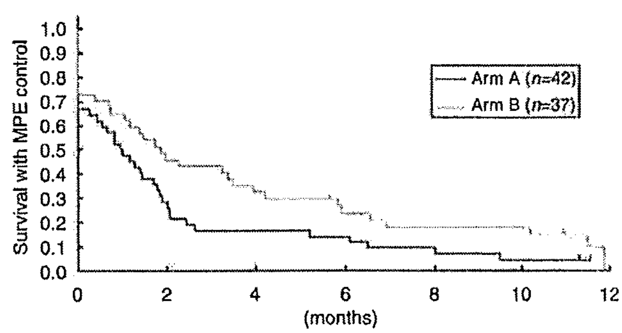

Figure 1 Effusion failure-free survival (EFFS). The median EFFS was 30 days in arm A and 57 days in arm B, with a hazard ratio of 0.64 (95% confidence interval: 0.40–1.03), with arm B significantly favouring this parameter (one-sided $P = 0.030$ by log-rank test).

Efficacy end points

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

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

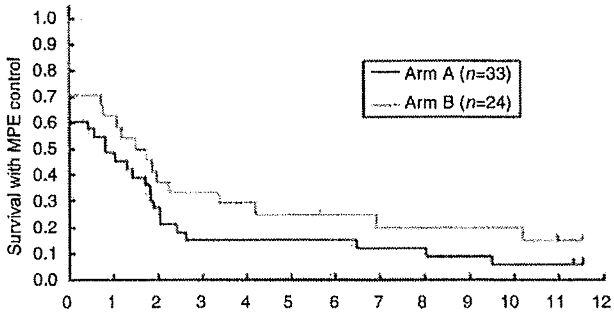

Figure 2 Effusion failure-free survival (EFFS) in effusion cytology-positive patients. In the effusion cytology-positive patient subset, arm B favoured EFFS. The hazard ratio was 0.69 (95% confidence interval: 0.39–1.21).

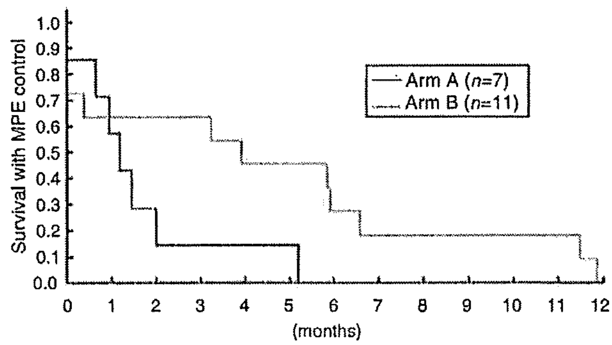

Figure 3 Effusion failure-free survival (EFFS) in effusion cytology-negative or -indeterminate patients. In the effusion cytology-negative or -indeterminate patient subset, arm B favoured EFFS. The hazard ratio was 0.39 (95% confidence interval: 0.12–1.21).

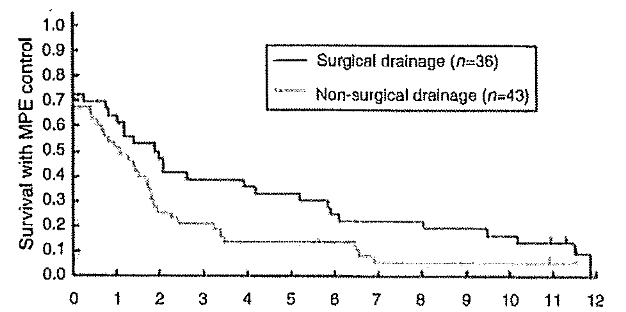

Figure 4 Effusion failure-free survival (EFFS) and drainage method. Patients with surgical drainage tended to have longer EFFS (median EFFS: 2.0 *vs* 1.1 month).

Table 3 Symptom palliation

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

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

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

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

Subgroup analysis

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

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

Symptom palliation

The baseline symptom scores were taken for all of the 79 eligible patients, at enrolment (after drainage). At the 1-month follow-up, approximately half of the patients (55% in arm A and 51% in arm B) had stable or improved overall scores. There were no significant differences between the arms for any of the symptom scores (Table 3).

DISCUSSION

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

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

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