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

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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), mitoxantrone (Norum *et al*, 1998), mitomycin C (Kaira *et al*, 2005) and ³²P-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 ≥ 3000 per mm^3 , platelet count $\geq 75\,000$ per mm^3 , haemoglobin ≥ 9.0 g dl^{-1} and no renal or hepatic failure; however, laboratory abnormalities related to cardiac tamponade were allowed). Patients with chemotherapy-naïve 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

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

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.

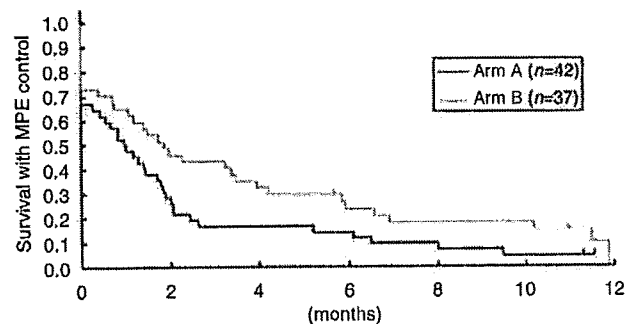


Figure 1 Effusion failure-free survival (EFFS). The median EFFS was 30 days in arm A and 57 days in arm B, with a hazard ratio of 0.64 (95% confidence interval: 0.40–1.03), with arm B significantly favouring this parameter (one-sided $P=0.030$ by log-rank test).

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.

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

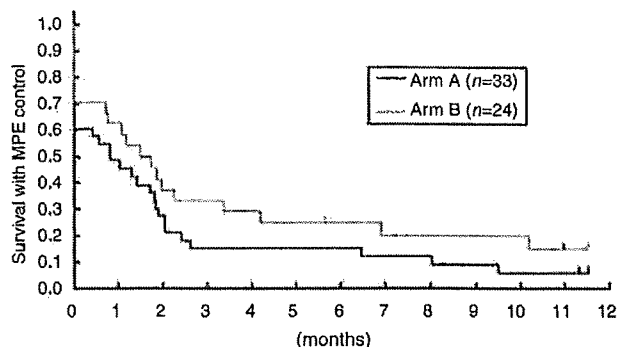


Figure 2 Effusion failure-free survival (EFFF) in effusion cytology-positive patients. In the effusion cytology-positive patient subset, arm B favoured EFFF. The hazard ratio was 0.69 (95% confidence interval: 0.39–1.21).

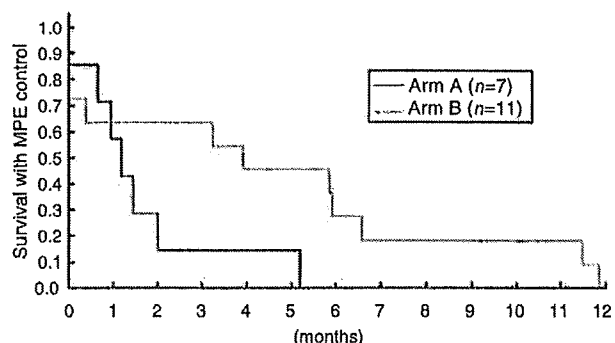


Figure 3 Effusion failure-free survival (EFFF) in effusion cytology-negative or -indeterminate patients. In the effusion cytology-negative or -indeterminate patient subset, arm B favoured EFFF. The hazard ratio was 0.39 (95% confidence interval: 0.12–1.21).

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

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

Subgroup analysis

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

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 EFFF (Figure 4). The effect of ipc BLM was observed irrespective of the drainage method employed; arm B tended to favour EFFF both in patients with surgical drainage (hazard ratio 0.62, 95% confidence interval: 0.30–1.29) and in those with non-surgical drainage (hazard ratio 0.56, 95% confidence interval: 0.29–1.05).

Symptom palliation

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

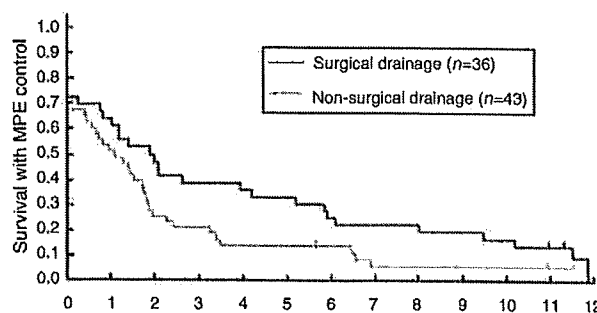


Figure 4 Effusion failure-free survival (EFFF) and drainage method. Patients with surgical drainage tended to have longer EFFF (median EFFF: 2.0 vs 1.1 month).

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. ^aThe scores at 1 month were compared with those at enrolment.

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

DISCUSSION

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

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

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

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

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

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

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

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

The third limitation of our study was that we did not control for the method of primary pericardial drainage, and each institution chose it in accordance with its daily practice. We do not believe that our results were much biased by the drainage methods, as each participating institution basically adhered to one method of

its choice, and the ipc BLM arm tended to favour EFFS in both subgroups with surgical and non-surgical drainage. However, control for the drainage method or indication (emergent vs elective) for drainage might be necessary in future trials, as they might well affect the patient outcomes. In fact, we did observe that, although not a randomised comparison and thus it should be interpreted with caution, patients who underwent surgical drainage tended to have a better MPE control.

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

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

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

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

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Conflict of interest

The authors have no conflicts of interest to declare. Registered in www.clinicaltrials.gov, ClinicalTrials.gov number, NCT00132613 and in UMIN-CTR [www.umin.ac.jp/ctr/], identification number, C00000030.

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Appendix

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REVIEW ARTICLE

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A literature review of molecular markers predictive of clinical response to cytotoxic chemotherapy in patients with breast cancer

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Abstract

Background. We aimed to identify, through a review of the literature, candidate genes for a prospective predictive chemosensitivity test in patients with breast cancer.

Methods. Papers demonstrating an association between gene alterations in tumor tissue and clinical chemosensitivity in breast cancer patients were selected by Medline searches. We calculated odds ratios (ORs) and their 95% confidence intervals (CIs) of response rates for patients who had tumors with or without gene alteration. Combined ORs and CIs were estimated using the DerSimonian-Laird method.

Results. A total of 18 genes were evaluated for association with clinical chemosensitivity in 6378 patients registered in 69 studies. The median (range) number of patients in each study was 73 (29–319). Overexpression of *ABCBI* (P-glycoprotein) was associated with poor responses to first-line chemotherapy (combined OR [CI], 0.16 [0.05–0.59]; $n = 322$). Overexpression and amplification of *TOP2A* (topoisomerase II- α) were more frequently observed in patients who responded to first-line chemotherapy (combined OR [CI], 2.73 [1.02–7.27]; $n = 323$). Overexpression of *ERBB2*

(c-erbB2) was associated with favorable responses in patients treated with both first-line anthracycline-based chemotherapy and second-line taxane-based chemotherapy (combined ORs [CIs], 1.60 [1.19–2.17]; $n = 1807$ and 2.24 [1.06–4.74]; $n = 259$, respectively). *BCL2* overexpression was associated with resistance to first-line chemotherapy (combined OR [CI], 0.44 [0.21–0.91]; $n = 816$).

Conclusion. *ABCBI*, *TOP2A*, *ERBB2*, and *BCL2* were good candidates for future clinical trials of predictive chemosensitivity tests in patients with breast cancer.

Key words Chemotherapy · Sensitivity · Drug resistance · Breast cancer · Gene alterations

Introduction

Breast cancer remains a major medical problem in women in spite of dramatic advances in the past three decades in the understanding of the biologic and clinical nature of the disease. About 1% to 5% of patients with breast cancer have distant metastasis at the time of initial diagnosis and 20% to 30% of patients develop systemic recurrence after surgery for local disease.¹ Chemotherapy for these patients, however, has limited efficacy, such that clinical objective response rates to standard chemotherapy regimens are 20%–40% at most, and such that patients with distant metastases rarely live long.¹ In addition, 40% to 80% of patients with breast cancer who undergo surgical resection receive adjuvant chemotherapy without its efficacy ever being monitored.

Tumor response to chemotherapy varies from one patient to another. Thus, it would be extremely useful to know ahead of time which patients have tumors that would respond to chemotherapeutic agents and also which tumors would be resistant to such therapy. For this purpose, cell culture-based chemosensitivity tests have been developed for more than 20 years, but they are not widely accepted because of technical problems, including the large amount of surgical material required, a low success rate for primary

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culture, the time-consuming nature of the technique, and a poor correlation with the clinical response.^{2,3} To overcome these obstacles, DNA, RNA, and protein-based chemosensitivity tests have been tried, but it remains unknown which gene alteration is well predictive of the clinical drug response. In our previous studies, 80 in vitro chemosensitivity-associated genes were identified in the medical literature,⁴ and the association between alterations of these genes and clinical drug responses in lung cancer patients was described.⁵ The purpose of this study was to find candidate genes to develop clinically useful chemosensitivity tests for patients with breast cancer.

Materials and methods

We identified 80 in vitro chemosensitivity-associated genes that met the following definition in the medical literature: (1) their alteration could be identified in human drug-induced resistant solid tumor cell lines; (2) their transfection induced drug resistance; or (3) their downregulation increased drug sensitivity. The genes included transporters: *ABCA2*, *ABCB1*, *ABCB11*, *ABCC1*, *ABCC2*, *ABCC3*, *ABCC4*, *ABCC5*, *ABCG2*, *MVP*, *ATP7A*, *ATP7B*, *SLC29A1*, *SLC28A1*, and *SLC19A1*; drug targets: *TUBB*, *TUBB4*, *TUBA*, *TYMS*, *TOP1*, *TOP2A*, *TOP2B*, and DHFR; target-associated proteins: *MAP4*, *MAP7*, *STMN1*, *KIF5B*, *HSPA5*, *PSMD14*, and *FPGS*; intracellular detoxifiers: *GSTP1*, *GPX*, *GCLC*, *GGT2*, *MT*, *RRM2*, and *AKR1B1*; DNA damage recognition and repair proteins: *HMGBl*, *HMGBl2*, *ERCC1*, *XPA*, *XPD*, *MSH2*, *MLH1*, *PMS2*, *APEX1*, *MGMT*, *BRCA1*, and *GLO1*; cell-cycle regulators: *RBI*, *GML*, *CDKN1A*, *CCND1*, *CDKN2A*, and *CDKN1B*; mitogenic signal regulators: *ERBB2*, *EGFR*, *KRAS2*, *HRAS*, and *RAF1*; survival signal regulators: *AKT1* and *AKT2*; integrins: *ITGB1*; transcription factors: *JUN*, *FOS*, *MYC*, and *NFKB1*; and apoptosis regulators: *TP53*, *MDM2*, *TP73*, *BCL2*, *BCL2L1*, *MCL1*, *BAX*, *BIRC4*, *BIRC5*, *TNFRSF6*, *CASP3*, *CASP8*, and *HSPB1*.⁴ Papers describing an association between the alteration of the gene and clinical drug response in patients with breast cancer were identified by extensive Medline searches using the name of the gene as a key word. Papers in which the association was evaluated in 25 or more patients were included in this study.

We calculated odds ratios (ORs) and their 95% confidence intervals (CIs) of response rates for patients who had tumors with or without gene alteration. Combined ORs and CIs were estimated using the DerSimonian-Laird method, as previously described.⁵ The formula used for the combined OR and that for 95% CI were as follows:

$$\text{Combined OR} = \exp\{\sum(\text{weight}_i \cdot \ln\text{OR}_i) / \sum \text{weight}_i\}$$

$$95\% \text{ CI of combined OR} = \exp\{\ln \text{combined OR} \pm 1.96 (1/\sum \text{weight}_i)^{1/2}\}$$

where weight_i is the weight for each study determined by variance of the study, and OR is the OR of each study.

Results

Clinical drug responses were evaluated in 18 genes from 69 studies, which included a median of 73 patients (range, 29–319 patients) per study to give a total of 6378 patients. The methods used to identify the gene alteration were immunohistochemical protein expression analysis ($n = 52$), protein activity analysis using tritium-release assay ($n = 1$), polymerase chain reaction (PCR)-based mRNA expression analysis ($n = 8$), PCR-based mutation analysis ($n = 3$), and gene amplification analysis using fluorescence in situ hybridization or chromogenic in situ hybridization ($n = 5$). The gene alteration was associated with the clinical response in 25 of the 69 (36%) studies.

High expression of *ABCB1* was associated with a poor response to first-line chemotherapy in three of five studies, and the combined OR (CI) in a total of 322 patients was 0.16 (0.05–0.59). Other transporter expressions were not associated with chemotherapy responses (Table 1). Study results showing associations between drug target alterations and clinical responses were promising. The alteration of *TYMS* (thymidylate synthetase), *TUBB* (beta-tubulin class I), and *TUBB4* (beta-tubulin class III) was associated with chemosensitivity, although there was only one study for each gene. The overexpression and amplification of *TOP2A* (topoisomerase II- α) were more frequently observed in patients who responded to first-line chemotherapy in four out of five studies with a combined OR (CI) of 2.73 (1.02–7.274) in a total of 323 patients (Table 2). The high expression of the DNA repair gene *BRCA1* (Breast cancer 1) was associated with chemosensitivity in one study (Table 3). The overexpression of *ERBB2* (c-erbB2, Her2, or neu) was associated with favorable responses in patients treated with first-line anthracycline-based chemotherapy, and the combined OR (CI) was 1.60 (1.19–2.17) in a total of 1807 patients (Table 4). This was also true among patients treated with second-line chemotherapy containing taxanes (combined OR [CI], 2.24 [1.06–4.74]; $n = 259$; Table 5). *TP53* mutations were not associated with clinical drug responses (combined OR [CI], 1.09 [0.73–1.62]; $n = 1588$; Table 6), whereas *BCL2* overexpression was associated with resistance to first-line chemotherapy (combined OR [CI], 0.44 [0.21–0.91]; $n = 816$; Tables 7 and 8).

Discussion

Association between a gene alteration and clinical chemosensitivity was evaluated in 18 of the 80 in vitro chemosensitivity-associated genes in patients with breast cancer. Among them, *ABCB1*, *TOP2A*, *ERBB2*, and *BCL2* were good candidates for further studies.

ABCB1 has been extensively studied as a major cellular mechanism of multidrug resistance,⁶ but there has been no firm evidence that the expression of this transporter in tumor cells has been associated with a poor response to cytotoxic chemotherapy in patients with breast cancer. A

Table 1. Expression of transporter proteins and clinical response to first-line chemotherapy

Author (year, country)	Drugs	Method	Expression	No. of pts	RR (%)	Odds ratio (95% CI)
<i>ABCB1</i>						
Ro ¹⁹ (1990, USA)	CPA, DOX, VCR	IHC	Low	20	95	0.08
			High	20	60	(0.01–0.71)
Veneroni ²⁰ (1994, Italy)	DOX ± VCR	IHC	Low	21	86	0.02
			High	18	11	(0.0–0.14)
Chevillard ²¹ (1996, France)	CPA, DOX, 5-FU	IHC	Low	36	50	0.75
			High	7	43	(0.15–3.84)
Bottini ²² (2000, Italy)	CPA, MTX, 5-FU, or EPI	IHC	Low	99	28 ^a	0.51
			High	42	17 ^a	(0.20–1.27)
Burger ^{23,b} (2003, Netherlands)	CPA, MTX, 5-FU, or CPA, DOX or EPI, 5-FU	RT-PCR	Low	47	68	0.09
			High	12	17	(0.02–0.48)
Combined odds ratio (95% CI) for ABCB1 (<i>n</i> = 322): 0.16 (0.05–0.59)						
<i>ABCC1</i> (Multidrug resistance-associated protein 1; MRP1)						
Burger ^{23,b} (2003, Netherlands)	CPA, MTX, 5-FU, or CPA, DOX or EPI, 5-FU	RT-PCR	Low	30	60	0.82
			High	29	55	(0.29–2.31)
<i>ABCC2</i> (Multidrug resistance-associated protein 1; MRP2)						
Burger ^{23,b} (2003, Netherlands)	CPA, MTX, 5-FU, or CPA, DOX or EPI, 5-FU	RT-PCR	Low	28	64	0.48
			High	28	46	(0.16–1.41)
<i>ABCG2</i> (Breast cancer resistance protein; BCRP)						
Burger ^{23,b} (2003, Netherlands)	CPA, MTX, 5-FU, or CPA, DOX or EPI, 5-FU	RT-PCR	Low	42	64	0.39
			High	17	41	(0.12–1.23)
<i>MVP</i> (major vault protein, lung resistance-related protein)						
Burger ^{23,b} (2003, Netherlands)	CPA, MTX, 5-FU, or CPA, DOX or EPI, 5-FU	RT-PCR	Low	37	65	0.45
			High	22	45	(0.15–1.33)

RR, response rate. Drugs: CPA, cyclophosphamide; DOX, doxorubicin; EPI, epirubicin; 5-FU, 5-fluorouracil; MTX, methotrexate; VCR, vincristine. Methods: IHC, immunohistochemical analysis; RT-PCR, reverse transcriptase-polymerase chain reaction

^aComplete response rate (%)

^bIn this study 20% of patients had received adjuvant chemotherapy

Table 2. Drug targets, intracellular detoxifier, and clinical response to first-line chemotherapy

Author (year, country)	Drugs	Method	Alteration	No. of pts	RR (%)	Odds ratio (95% CI)
<i>TYMS</i> (thymidylate synthetase)						
Foekens ²⁴ (2001, Netherlands)	5-FU- based	TRA	Low expression	13	8	12.0
			High expression	108	50	(1.51–95.5)
<i>TUBB</i> (beta-tubulin class I)						
Hasegawa ²⁵ (2003, Japan)	DTX	Real-time PCR	Low expression	19	63	0.25
			High expression	20	30	(0.07–0.95)
<i>TUBB4</i> (beta-tubulin class III)						
Hasegawa ²⁵ (2003, Japan)	DTX	Real-time PCR	Low expression	19	68	0.15
			High expression	20	25	(0.04–0.62)
<i>TOP2A</i> (topoisomerase II- α)						
Jarvinen ²⁶ (1998, Finland)	EPI	IHC	Low expression	31	58	0.61
			High expression	24	46	(0.21–1.79)
Coon ²⁷ (2002, USA)	Anthracycline-based	IHC	Low expression	26	77	2.40
			High expression	9	89	(0.25–23.2)
MacGrogan ²⁸ (2003, France)	EPI, MTX, VCR	IHC	Low expression	68	32	2.88
			High expression	57	58	(1.38–5.97)
Martin-Richard ²⁹ (2004, Spain)	CPA, DOX, 5-FU or CPA, EPI, 5-FU	IHC	Low expression	25	24	5.28
			High expression	16	63	(1.35–20.7)
Park ³⁰ (2003, Korea)	DOX	CISH	Normal	48	54	15.2
			Amplified	19	95	(1.88–123)
Combined odds ratio (95% CI) for TOP2A (<i>n</i> = 323): 2.73 (1.027–7.27)						
<i>GSTP1</i> (glutathione S-transferase pi)						
Wright ³¹ (1992, UK)	MIT	IHC	Low expression	30	37	1.22
			High expression	29	41	(0.43–3.48)

Drugs: DTX, docetaxel; MTX, methotrexate; MIT, mitoxantrone; CISH, chromogenic in situ hybridization; TRA, tritium-release assay

previous meta-analysis, summarizing the data of 115 patients published between 1990 and 1996, showed only a marginal association between *ABCB1* expression in tumor tissue before treatment and failure of response (relative risk, 1.47;

95% CI, 0.94–2.29; *P* = 0.088).⁷ The present study included recent studies with a total of 322 patients, and showed that the expression of *ABCB1* was significantly associated with a poor drug response. Key anticancer agents in the

Table 3. DNA repair gene, cell-cycle regulator and clinical response to first-line chemotherapy

Author (year, country)	Drugs	Method	Expression	No. of pts	RR (%)	Odds ratio (95% CI)
<i>BRCA1</i> (Breast cancer 1)						
Egawa ³² (2003, Japan)	CPA, EPI	Real-time PCR	Low	25	32	4.01
			High	26	65	(1.25–12.9)
<i>CCND1</i> (cyclin D1)						
Bonnefoi ³³ (2003, Switzerland)	CPA, EPI ± 5-FU	IHC	Low	126	22 ^a	2.02
			High	52	37 ^a	(1.00–4.07)

^aComplete response rate (%)**Table 4.** *ERBB2* (erythroblastic leukemia viral oncogene homolog 2, c-erbB2) expression and clinical response to first-line anthracycline-based chemotherapy

Author (year, country)	Drugs	Method	Alteration	No. of pts	RR (%)	Odds ratio (95% CI)
Niskanen ^{h 34} (1997, Finland)	CPA, EPI, 5-FU	IHC	Low expression	89	33	2.07
			High expression	14	50	(0.66–6.45)
Rozañ ³⁴ (1998, France)	CPA, DOX, 5-FU	IHC	Low expression	131	21	1.62
			High expression	36	31	(0.71–3.69)
Jarvinen ³⁰ (1998, Finland)	EPI	IHC	Low expression	36	64	0.26
			High expression	19	32	(0.08–0.85)
Vincent-Salomon ³⁶ (2000, France)	CPA, DOX, 5-FU	IHC	Low expression	36	78	0.57
			High expression	18	67	(0.16–2.01)
Geisler ³⁷ (2001, Norway)	DOX	IHC	Low expression	72	37	1.17
			High expression	17	41	(0.40–3.43)
Coon ³⁷ (2002, USA)	Anthracycline-based	IHC	Low expression	20	70	2.79
			High expression	15	87	(0.47–16.4)
MacGrogan ³⁸ (2003, France)	EPI, MTX, VCR	IHC	Low expression	102	40	1.82
			High expression	20	55	(0.69–4.78)
Bonnefoi ³³ (2003, Switzerland)	CPA, EPI ± 5-FU	IHC	Low expression	132	24 ^a	1.61
			High expression	47	34 ^a	(0.78–3.32)
Zhang ³⁸ (2003, USA)	CPA, DOX, 5-FU	IHC	Low expression	69	78	3.61
			High expression	28	93	(0.77–17.0)
Martin-Richard ³⁹ (2004, Spain)	CPA, DOX, 5-FU or CPA, EPI, 5-FU	IHC	Low expression	30	37	1.44
			High expression	11	45	(0.35–5.84)
Burcombe ³⁹ (2005, UK)	Anthracycline-based	IHC	Low expression	84	71	1.87
			High expression	34	82	(0.69–5.08)
Prisack ⁴⁰ (2005, Germany)	CPA, EPI	IHC	Low expression	257	10 ^a	2.13
			High expression	62	19 ^a	(1.01–4.51)
Manna Edel ⁴¹ (2006, Brazil)	Anthracycline-based	IHC	Low expression	86	63	1.11
			High expression	23	65	(0.42–2.91)
Park ³⁰ (2003, Korea)	DOX	CISH	Normal	36	47	7.54
			Amplified	31	87	(2.19–26.0)
Konecny ^{e 42} (2004, USA)	CPA, EPI	FISH	Normal	88	33	1.80
			Amplified	49	46	(0.88–3.68)
Bozzetti ⁴³ (2006, Belgium)	Anthracycline-based	FISH	Normal	86	62	1.63
			Amplified	29	72	(0.65–4.11)

Combined odds ratio (95% CI) for *ERBB2* (anthracyclines; $n = 1807$): 1.60 (1.19–2.17)

FISH, fluorescence in situ hybridization

^aPathological complete response rate^{bc}In these studies, 15% and 40%, respectively, of patients had received adjuvant chemotherapy**Table 5.** *ERBB2* (erythroblastic leukemia viral oncogene homolog 2, c-erbB2) expression and clinical response to second-line taxanes

Author (year, country)	Drugs	Method	Alteration	No. of pts	RR (%)	Odds ratio (95% CI)
Taxanes						
Baselga ⁴⁴ (1997, USA)	DTX or PTX	IHC	Low expression	76	65	3.40
			High expression	46	36	(1.58–7.33)
Sjostrom ⁴⁵ (2002, Finland)	DTX	IHC	Low expression	36	53	1.02
			High expression	30	53	(0.39–2.70)
Di Leo ⁴⁶ (2004, Europe)	DTX	FISH	Normal	50	40	3.00
			Amplified	21	67	(1.03–8.74)

Combined odds ratio (95% CI) for *ERBB2* (taxanes, $n = 259$): 2.24 (1.06–4.74)

DTX, docetaxel; PTX, paclitaxel

Table 6. Tumor protein *TP53* (p53) mutation and clinical response to first-line chemotherapy

Author (year, country)	Drugs	Method	Mutation	No. of pts	RR (%)	Odds ratio (95% CI)
Niskanen ¹⁴ (1997, Finland)	CPA, EPI, 5-FU	IHC	Normal	86	37	0.52
			Mutated	17	24	(0.16–1.73)
Frassoldati ¹⁷ (1997, Italy)	CPA, DOX or CPA, MTX, 5-FU	IHC	Normal	26	42	0.68
			Mutated	3	33	(0.05–8.50)
Bonetti ¹⁸ (1998, Italy)	CPA, MTX, 5-FU or Anthracycline-based	IHC	Normal	21	30	0.94
			Mutated	22	27	(0.25–3.56)
Roza ¹⁵ (1998, France)	CPA, DOX, 5-FU	IHC	Normal	97	22	1.25
			Mutated	70	26	(0.61–2.58)
Jarvinen ²⁰ (1998, Finland)	EPI	IHC	Normal	37	57	0.61
			Mutated	18	44	(0.20–1.90)
Colleoni ¹⁹ (1999, Italy)	CPA, DOX or VNR, 5-FU	IHC	Normal	59	53	5.42
			Mutated	14	86	(1.11–26.4)
Bottini ²² (2000, Italy)	CPA, MTX, 5-FU or EPI	IHC	Normal	111	72	1.16
			Mutated	32	75	(0.47–2.86)
Kandioler-Eckersberger ⁵¹ (2000, Austria)	CPA, EPI, 5-FU	IHC	Normal	20	85	0.01
			Mutated	15	7	(0.00–0.13)
Kandioler-Eckersberger ⁵¹ (2000, Austria)	PTX	IHC	Normal	20	35	3.71
			Mutated	12	67	(0.82–16.8)
Bonnefoi ³¹ (2003, Switzerland)	CPA, EPI ± 5-FU	IHC	Normal	126	29 ^a	0.73
			Mutated	53	23 ^a	(0.35–1.55)
MacGrogan ²⁸ (2003, France)	EPI, MTX, VCR	IHC	Normal	89	40	2.38
			Mutated	34	62	(1.06–5.35)
Rahko ⁵¹ (2003, Finland)	Anthracycline-based	IHC	Normal	15	33	0.73
			Mutated	15	27	(0.15–3.49)
Ogston ⁵² (2004, UK)	CPA, DOX, VCR	IHC	Normal	65	52 ^b	1.25
			Mutated	38	59 ^b	(0.56–2.81)
Prisack ⁴⁰ (2005, Germany)	CPA, EPI	IHC	Normal	269	11 ^a	2.12
			Mutated	38	21 ^a	(0.89–5.06)
Berns ⁵³ (2000, Netherlands)	CPA, DOX, 5-FU or CPA, MTX, 5-FU	sequencing	Normal	16	63	0.34
			Mutated	25	36	(0.09–1.24)
Geisler ³⁷ (2001, Norway)	DOX	TTGE, sequencing	Normal	64	36	1.31
			Mutated	26	42	(0.52–3.32)
Geisler ⁵¹ (2003, Norway)	MMC, 5-FU	TTGE, sequencing	Normal	17	41	0.55
			Mutated	18	28	(0.13–2.26)

Combined odds ratio (95% CI) for *TP53* ($n = 1588$): 1.09 (0.73–1.62)

Drugs: MMC, mitomycin C; VNR, vinorelbine. Method: TTGE, temporal temperature gel electrophoresis

^aPathological complete response rate

^bGood pathological response rate

^cIn these studies, 15% and 30%, respectively, of patients had received adjuvant chemotherapy

Table 7. *BCL2* (B-cell CLL/lymphoma 2) and clinical response to first-line chemotherapy

Author (year, country)	Drugs	Method	Expression	No. of pts	RR (%)	Odds ratio (95% CI)
Frassoldati ¹⁷ (1997, Italy)	CPA, DOX or CPA, MTX, 5-FU	IHC	Low	19	47	0.48
			High	10	30	(0.09–2.42)
Bonetti ¹⁸ (1998, Italy)	CPA, MTX, 5-FU or Anthracycline-based	IHC	Low	32	44	0.19
			High	23	13	(0.05–0.78)
Colleoni ¹⁹ (1999, Italy)	CPA, DOX or VNR, 5-FU	IHC	Low	27	52	1.58
			High	46	63	(0.60–4.15)
Bottini ²² (2000, Italy)	CPA, MTX, 5-FU or EPI	IHC	Low	48	71	1.15
			High	95	74	(0.53–2.49)
Geisler ³⁷ (2001, Norway)	DOX	IHC	Low	46	37	1.12
			High	43	40	(0.47–2.62)
Ogston ⁵² (2004, UK)	CPA, DOX, VCR	IHC	Low	55	71 ^b	0.22
			High	48	25 ^b	(0.10–0.52)
Buchholz ⁵⁵ (2005, USA)	CPA, DOX, 5-FU	IHC	Low	33	27 ^a	0.11
			High	49	4 ^a	(0.02–0.57)
Prisack ⁴⁰ (2005, Germany)	CPA, EPI	IHC	Low	118	25 ^a	0.16
			High	124	5 ^a	(0.06–0.42)

Combined odds ratio (95% CI) for *BCL2* ($n = 816$): 0.44 (0.21–0.91)

^aPathological complete response rate

^bGood pathological response rate

^cIn this study, 30% of patients had received adjuvant chemotherapy

Table 8. Other apoptosis regulators and clinical response to chemotherapy

Author (year, country)	Drugs	Method	Expression	No. of pts	RR (%)	Odds ratio (95% CI)
<i>BCL2L1</i> (Bcl2-like 1, Bcl-xL) Sjostrom ³⁶ (2002, Finland)	DTX or MTX, 5-FU (second-line)	IHC	Low	59	36	1.32
			High	64	42	(0.64–2.73)
<i>BAX</i> (Bcl2-associated X protein) Krajewski ⁵⁷ (1995, Finland)	CPA, EPI, 5-FU (first-line)	IHC	Low	39	21	2.84
			High	65	43	(1.13–7.13)
Sjostrom ³⁶ (2002, Finland)	DTX or MTX, 5-FU (second-line)	IHC	Low	59	39	1.03
			High	53	39	(0.48–2.20)
Buchholz ⁵⁵ (2005, USA)	CPA, DOX, 5-FU (first-line)	IHC	Low	12	58 ^a	0.04
			High	69	6 ^a	(0.01–0.20)
<i>TNFRSF6</i> (tumor necrosis factor receptor superfamily, member 6, FAS, CD95) Sjostrom ³⁶ (2002, Finland)	DTX or MTX, 5-FU (second-line)	IHC	Low	53	42	0.83
			High	70	37	(0.40–1.73)

^aPathological complete response rate

treatment of breast cancer, such as anthracyclines, vinca alkaloids, and taxanes, are substrates of ABCB1 protein, and its expression must therefore be an important determinant for chemosensitivity. The association between the expression and clinical drug responses of other transporters is also worth evaluating, although no statistically significant association has been obtained due to the too-small sample size.

Qualitative and quantitative alterations of the drug's target are another important mechanism involved in classical drug resistance. DNA topoisomerase II enzymes pass one double-stranded DNA segment through a transient, enzyme-mediated break in another strand to relax a highly twisted superhelical DNA.⁸ One isoform of these enzymes, TOP2A, is the target of most active anticancer agents, including anthracyclines, because its expression levels are tightly linked to the proliferative state of the cell, and are higher in tumor tissue than in adjacent normal tissue.⁸ Although there have been many attempts to correlate TOP2A status with anthracycline efficacy in breast cancer patients, the results have been controversial.⁹ The present study showed that TOP2A gene amplification and protein overexpression were associated with a higher response rate in a total of 323 patients. TYMS and beta-tubulins are also important targets for fluoropyrimidines and taxanes, respectively. Further studies are needed before the association can be definitively established between alteration of these gene expressions and clinical chemotherapy responses.

ERBB2 is a member of the human epidermal growth factor receptor family, which plays an important role in regulating cell growth, survival, adhesion, migration, and differentiation, by forming heterodimers within the family. The ERBB2 receptor is the most potent oncoprotein, and amplification and overexpression of *ERBB2*, noted in about 30% of breast cancers, are associated with a poor prognosis.^{10,11} The predictive value of *ERBB2* overexpression for poor responses to endocrine therapy and trastuzumab therapy has been well documented, but the association between *ERBB2* status and chemosensitivity remains controversial.^{11,12} This issue has been evaluated mainly in the adjuvant setting after surgery, and the association between

ERBB2 status and difference in progression-free survival can therefore be attributable to the overall prognosis as well as the efficacy of chemotherapy. The *ERBB2* status and responses to chemotherapy in patients with locally advanced or the metastatic breast cancer have been evaluated in small studies. Few studies, however, showed any significant difference in the response rates between *ERBB2*-normal and *ERBB2*-overexpressed patients.¹² The present study showed that patients with overexpression or amplification of *ERBB2* responded significantly better to anthracycline-based chemotherapy than patients with a normal *ERBB2* status. This was explained by the correlation between the expressions of the *ERBB2* and *TOP2A* genes; high expression of the *TOP2A* gene was detected in 30%–60% of breast cancer tissue with *ERBB2* overexpression, while it was detected in only 5%–10% of breast cancer tissue without *ERBB2* overexpression. The mechanism of this correlation remains unclear. The *ERBB2* and *TOP2A* genes were previously thought to be coamplified, because both the genes are located on chromosome 17q12-21. Recent studies, however, showed that when these genes were amplified, they were located in different amplicons. In other studies, the number of copies of the *ERBB2* and *TOP2A* genes were not identical.¹³ The present study also showed that the overexpression or amplification of *ERBB2* was significantly associated with better responses to taxanes. Other genetic events on the 17q12-21 and other chromosomal regions that occur when *ERBB2* is amplified may be involved in its mechanisms.¹⁴

TP53 preserves genome integrity as the "guardian of the genome" in response to various cellular stresses by invoking cell-cycle arrest and allowing the repair system to eliminate mutations, or by inducing apoptosis when the correct DNA repair is not accomplished.¹⁵ Because most chemotherapeutic agents induce apoptosis through either DNA damage or microtubule disruption, the TP53 status may affect the sensitivity of tumor cells against these agents. Animal and in vitro studies, however, failed to show general trends of associations between TP53 status and drug sensitivity.^{15,16} The present study also showed inconsistent results in clinical studies. This is probably because only TP53 gene mutations and mutated TP53 protein accumulation have been

examined, but many mechanisms regulating TP53 protein activity have never been evaluated, which include post-translational modification and interaction with other upstream and downstream molecules.¹⁵

The Bcl-2 family of proteins plays a central role in regulating apoptosis by balancing expression between pro- and anti-apoptotic family members. Cytotoxic stimuli that promote apoptosis, including DNA damage or microtubule disruption by chemotherapy, can be prevented by *BCL2* expression. An in vitro study consistently showed that over-expression of *BCL2* increased the resistance of MCF-7 cells to doxorubicin, and this resistance was positively correlated with *BCL2* expression levels of individual MCF/BCL2 clones.¹⁷ In clinical studies, however, the association between the expression of *BCL2* and chemosensitivity was not conclusive, mostly due to the small sample size of each study. The present study showed that patients with *BCL2*-positive breast cancer were twice as likely to be resistant to chemotherapy.

The methodological limitations of studies on the association between gene alterations and clinical drug sensitivity are summarized as follows: (1) all the studies were retrospective subgroup analyses; (2) the endpoint of these studies was the response rate in the metastatic or neoadjuvant setting, which is not as objective an endpoint as survival; (3) the sample size of these studies was relatively small; and (4) the majority of the studies assessed the alterations by immunohistochemistry using monoclonal antibodies, but no international standard criteria of positivity and negativity have been defined.¹⁸ In addition, the present study had major problems, such as large heterogeneity among studies; publication bias; and a selection bias, in that studies with incomplete information were excluded from this study. In spite of these limitations, the exploratory analyses in this study will help select genes for future confirmatory studies of molecular markers associated with the clinical response to cytotoxic chemotherapy.

In conclusion, *ABCBI*, *TOP2A*, *ERBB2*, and *BCL2* were good candidates for future clinical trials of predictive chemosensitivity tests in patients with breast cancer.

Conflict of Interest

The authors indicate no potential conflicts of interest.

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Identification of a Predictive Biomarker for Hematologic Toxicities of Gemcitabine

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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®).

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A B S T R A C T

Purpose

Gemcitabine monotherapy is the current standard for patients with advanced pancreatic cancer, but the occurrence of severe neutropenia and thrombocytopenia can sometimes be life threatening. This study aimed to discover a new diagnostic method for predicting the hematologic toxicities of gemcitabine.

Patients and Methods

Using quantitative mass spectrometry (MS), we compared the baseline plasma proteomes of 25 patients who had developed severe hematologic adverse events (grade 3 to 4 neutropenia and/or grade 2 to 4 thrombocytopenia) within the first two cycles of gemcitabine with those of 22 patients who had not (grade 0).

Results

We identified 757 peptide peaks whose intensities were significantly different ($P < .001$, Welch t test) among a total of 60,888. The MS peak with the highest statistical significance ($P = .0000282$) was revealed to be derived from haptoglobin by tandem MS. A scoring system (nomogram) based on the values of haptoglobin, haptoglobin phenotype, neutrophil count, platelet count, and body-surface area was constructed to estimate the risk of hematologic adverse events (grade 3 to 4 neutropenia and/or grade 2 to 4 thrombocytopenia) with an area under curve value of 0.782 in a cohort of 166 patients with pancreatic cancer. Predictive ability of the system was confirmed in two independent validation cohorts consisting of 87 and 52 patients with area under the curve values of 0.655 and 0.747, respectively.

Conclusion

Although the precise mechanism responsible for the correlation of haptoglobin with the future onset of hematologic toxicities remains to be clarified, our prediction model seems to have high practical utility for tailoring the treatment of patients receiving gemcitabine.

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INTRODUCTION

Pancreatic adenocarcinoma is one of the most aggressive and lethal cancers.¹ It is the fifth leading cause of cancer-related mortality in Japan and the fourth leading cause in the United States, accounting for an estimated more than 23,000 annual deaths in Japan and more than 33,000 deaths in the United States.^{2,3} The median survival time of patients with advanced pancreatic cancer had remained at only 3 to 4 months until the introduction of the nucleoside anticancer drug gemcitabine (2',2'-difluorodeoxycytidine). Gemcitabine monotherapy extended the overall survival of pancreatic cancer patients up to 6 months, along with significant clinical benefits such as pain relief and improvement of performance status,⁴⁻⁶ and is now accepted as a stan-

dard first-line treatment for unresectable advanced pancreatic cancer.⁷ However, hematologic toxicity is the dose-limiting factor of gemcitabine therapy.⁸ Although severe nonhematologic toxicity is infrequent,⁴⁻⁶ 20% to 30% of patients receiving gemcitabine experience grade 3 to 4 neutropenia (National Cancer Institute [NCI] Common Toxicity Criteria, version 2.0), and approximately 10% experience grade 3 to 4 thrombocytopenia.^{5,6,9,10} These levels of severe hematologic adverse events (AEs) can be potentially life threatening.

Several attempts have been made to predict the occurrence of AE associated with chemotherapy. Old age, poor performance status, and reduced initial blood cell counts have been reported to be the risk factors of hematotoxicities.^{11,12} To further improve prediction accuracy, combinations of these

risk factors have also been proposed,¹¹⁻¹⁴ but no reliable predictor has been established for gemcitabine-induced hematologic AEs. We previously identified a significant correlation of a nonsynonymous single nucleotide polymorphism of the cytidine deaminase (*CDA*) gene with altered pharmacokinetics of gemcitabine, but its prediction accuracy for hematologic AE was not satisfactory.^{15,16}

Recent advanced proteomic technologies have been increasingly applied to studies of clinical samples¹⁷ to identify biomarkers that could facilitate the tailoring of cancer treatments. Protein expression is not always correlated with mRNA expression,¹⁸ and it is anticipated that alterations in the protein content of clinical samples more directly reflect the biologic and pathologic status of patients. Matrix-assisted laser desorption/ionization mass spectrometry (MS) is becoming a method of choice for profiling of clinical samples as a result of its high sensitivity and throughput. In fact, previous studies have successfully identified biomarkers that could predict the outcome of cancer patients and the efficacy of molecular-targeting drugs.^{19,20} However, only low molecular weight proteins can be analyzed by matrix-assisted laser desorption/ionization MS, and thus, a method allowing more comprehensive protein profiling is desirable.

Shotgun proteomics is an emerging concept in which whole proteins are enzymatically digested into a large array of small peptide fragments having uniform physical and chemical characteristics and then analyzed directly by MS. We previously developed a new platform, namely two-dimensional image converted analysis of liquid chromatography and mass spectrometry (2DICAL), to give a quantitative dimension to shotgun proteomics.²¹ To identify new biomarkers that might be useful for prediction of gemcitabine-induced neutropenia and thrombocytopenia in patients with pancreatic cancer, we compared the plasma protein profiles of two extreme populations of patients who had shown different responses to the same gemcitabine treatment by 2DICAL. Here we report the identification of plasma/serum haptoglobin as a biomarker of hematologic toxicities associated with gemcitabine treatment.

PATIENTS AND METHODS

Patients

Plasma or serum samples were collected from three cohorts (modeling [M0], validation-1 [V1], and validation-2 [V2] cohorts) totaling 305 patients. All the patients had locally advanced or metastatic (stage IVA or IVB),²² histologically or cytologically proven pancreatic ductal adenocarcinoma and received at least two cycles of gemcitabine monotherapy (1,000 mg/m² intravenously over 30 minutes on days 1, 8, and 15 of a 28-day cycle). Demographic and laboratory data for the patients before administration of gemcitabine are listed in Appendix Tables A1 to A3 (online only). The severity of early hematologic AEs that appeared within the first two cycles of the gemcitabine treatment was graded according to NCI Common Terminology Criteria for Adverse Events (CTCAE; version 3.0).

Cohort M0 comprised 166 patients who had been enrolled onto our previous study at the National Cancer Center (NCC) Hospital (Tokyo, Japan) and Hospital East (Kashiwa, Japan) between September 2002 and July 2004.^{15,16} Cohort V1 comprised 87 patients who had been treated consecutively at the NCC Hospital between August 2005 and June 2007, and cohort V2 comprised 52 patients treated at the NCC Hospital consecutively between August 2004 and July 2005.

Sample Preparation

Blood was drawn before the administration of gemcitabine. Plasma (cohorts M0 and V1) or serum (cohort V2) was separated by centrifugation at

4°C and frozen at -70°C (cohort M0) or -20°C (cohorts V1 and V2) until analysis. Macroscopically hemolyzed samples were excluded from the current analysis. The protocol of this retrospective study was reviewed and approved by the institutional ethics committee boards of the NCC (Tokyo, Japan) and the National Institute of Health Sciences (Tokyo, Japan).

Liquid Chromatography/MS

Samples were passed through an IgY-12 High Capacity Spin Column (Beckman Coulter, Fullerton, CA) in accordance with the manufacturer's instructions to reduce the amounts of the 12 most abundant plasma proteins. The flow-through portion was digested with sequencing-grade modified trypsin (Promega, Madison, WI) and analyzed in triplicate using a nano-flow high-performance liquid chromatograph (NanoFrontier nLC; Hitachi High-Technologies, Tokyo, Japan) connected to an electrospray ionization quadrupole time-of-flight (ESI-Q-TOF) mass spectrometer (Q-ToF Ultima; Waters, Milford, MA).

MS peaks were detected, normalized, and quantified using the in-house 2DICAL software package, as described previously.²¹ A serial identification (ID) number was applied to each of the MS peaks detected (1 to 60,888). The stability of liquid chromatography/MS was monitored by calculating the correlation coefficient of every triplicate measurement. The mean correlation coefficient (\pm standard deviation) of the entire 60,888 peaks of the 47 triplicate runs was as high as 0.978 (\pm 0.017).

Tandem MS

Peak lists were generated using the Mass Navigator software package (version 1.2; Mitsui Knowledge Industry, Tokyo, Japan) and searched against the SwissProt database (downloaded from <http://www.expasy.ch/sprot/sprot-top.html> on October 18, 2007) using the Mascot software package (version 2.2.1; Matrix Science, London, United Kingdom). The score threshold was set to $P < .05$ based on the size of the database used in the search.

Western Blot Analysis

Primary antibodies used were rabbit polyclonal antibody against human haptoglobin (Dako, Glostrup, Denmark) and mouse monoclonal antibody against human complement C3b- α (Progen, Heidelberg, Germany). Ten microliters of partitioned sample were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted onto a polyvinylidene difluoride membrane. The membrane was then incubated with the primary antibody and subsequently with relevant horseradish peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin G as described previously.^{23,24} Blots were developed using an enhanced chemiluminescence (ECL) detection system (GE Healthcare, Buckinghamshire, United Kingdom).

Quantification and Subtyping of Haptoglobin

The concentration of plasma or serum haptoglobin was measured using an automated immunonephelometry BN-II system (Siemens Healthcare Diagnostics, Tokyo, Japan). The phenotype of haptoglobin α -chain was determined by nonreducing (native) SDS-PAGE.²⁵

Categorization of Hematologic Toxicities

Overall severity of hematologic toxicities after gemcitabine treatment was classified into categories I to IV based on the worst CTCAE grades of neutropenia and thrombocytopenia (Appendix Fig A1, online only), as follows: category I, grade 0 to 1 neutropenia and grade 0 thrombocytopenia; category II, grade 2 neutropenia or grade 1 thrombocytopenia; category III, grade 3 neutropenia or grade 2 thrombocytopenia; and category IV, grade 4 neutropenia or grade 3 to 4 thrombocytopenia.

Statistical Analysis

Statistical significance of intergroup differences was assessed using the Welch *t* test, χ^2 test, Wilcoxon test, or Kruskal-Wallis test, as appropriate. Multivariate regression analysis was performed using ordinal logistic regression modeling. Factors included in the prediction model were selected with a forward stepwise selection procedure using Akaike's Information Criterion (AIC). To correct biased sample sizes of categories, each observation was weighted according to the sample size of its category in the fitting process. The significance of differences between models with and without haptoglobin was assessed with the likelihood ratio test. Statistical analyses were performed using

an open-source statistical language R (version 2.7.0; <http://www.r-project.org/>) with the optional module design package.

RESULTS

Plasma Proteins Associated With Hematologic AEs

To identify a biomarker that can predict the occurrence of hematologic AEs associated with gemcitabine treatment, we compared the baseline plasma proteome between 25 patients who developed severe AEs (grade 3 to 4 neutropenia and/or grade 2 to 4 thrombocytopenia) and 22 patients who did not (grade 0) using 2DICAL. These levels of hematologic AEs have been used as criteria for dose reduction or postponement of gemcitabine-based treatments.²⁶⁻²⁸ There was no significant difference in age, sex, Eastern Cooperative Oncology Group performance status, routine biochemical laboratory data, or pharmacokinetics of gemcitabine¹⁵ (Table 1 and data not shown) between the two extreme groups of patients who were selected from cohort M0, but the patients who experienced severe AEs had significantly lower baseline peripheral-blood leukocyte, neutrophil, and platelet counts than patients without AEs (Table 1).

Among a total of 60,888 independent MS peaks detected within the range of 250 to 1,600 m/z and within the time range 20 to 70 minutes, we found that the mean intensity of triplicates differed significantly in 757 peaks ($P < .001$, Welch *t* test). Figure 1A is a representative two-dimensional view of all the MS peaks displayed with m/z along the x-axis and the retention time of LC along the y-axis. The 757 MS peaks whose expression differed significantly between patients with severe AEs and patients without AEs are highlighted in red.

One hundred fifteen MS/MS spectra acquired from 200 peaks with the smallest *P* values were matched to 41 proteins in the database (Mascot score of > 15 ; Appendix Tables A4 and A5, online only). Notably, MS peaks including one that was decreased in patients with severe AEs with the highest statistical significance ($P = .0000282$; Fig 1B) most recurrently (six times) matched the amino acid sequences of the haptoglobin (HP) gene product (Appendix Fig A2, online only). Figure 2A shows the distribution of two representative haptoglobin-derived MS peaks (ID 2062 [at 491 m/z and 44.5 minutes] and ID 5681 [at 602 m/z and 47 minutes]) in patients with severe AEs and without AEs. The differential expression and identification of haptoglobin were confirmed by denaturing SDS-PAGE and immunoblotting (Fig 2B).

Correlation of Haptoglobin With the Degree of Hematologic Toxicities

The levels of haptoglobin in plasma or serum samples obtained from 305 patients with advanced pancreatic cancer before gemcitabine treatment were measured by immunonephelometry and compared with the occurrence and severity of hematologic AEs. Consistent with 2DICAL analysis, the plasma levels of haptoglobin were significantly lower in the 25 patients with severe AEs than in the 22 patients without AEs ($P = .0002$, Wilcoxon test; Table 1).

The plasma level of haptoglobin showed a significant correlation with the NCI-CTCAE grade of neutropenia ($P = .012$, Kruskal-Wallis test) and hematologic toxicity categories ($P = .001$) in the 166 patients of cohort M0 (Fig 3A and Appendix Table A1). The correlation of haptoglobin levels with the grades of neutropenia and thrombocytopenia as well as the toxicity categories was consistently observed in the

Table 1. Clinical and Laboratory Data of Patients Without AEs and With Severe AEs

Factor	Patients Without AEs (n = 22)	Patients With Severe AEs (n = 25)	<i>P</i>
Haptoglobin, mg/dL			.0002
Mean	286	155	
SD	130	59	
Haptoglobin phenotype, No of patients			.705*
Hp 2-2	12	14	
Hp 2-1	8	7	
Hp 1-1	2	4	
Sex, No. of patients			.344*
Male	12	17	
Female	10	8	
Age, years			.616
Mean	64	63	
SD	8	8	
ECOG performance status, No. of patients			.862*
0	12	13	
1	10	12	
2	0	0	
Body-surface area, m ²			.733
Mean	1.51	1.53	
SD	0.20	0.18	
Prior therapy, No. of patients			.867*
None	19	22	
Chemoradiotherapy using FU for LAPC	3	3	
Leucocyte, ×10 ³ /μL			.0002
Mean	7.4	4.8	
SD	2.8	1.4	
Absolute neutrophil count, ×10 ³ /μL			.0002
Mean	5.3	3.0	
SD	2.4	1.1	
Platelet, ×10 ⁴ /μL			< .0001
Mean	28	17	
SD	11	6	
Hemoglobin, g/dL			.806
Mean	12.1	11.9	
SD	1.4	1.4	
Albumin, g/dL			.131
Mean	3.6	3.7	
SD	0.4	0.3	
Creatinine, mg/dL			.931
Mean	0.72	0.70	
SD	0.25	0.17	
AST, U/L			.430
Mean	37	29	
SD	26	13	
ALT, U/L			.624
Mean	43	32	
SD	37	24	
ALP, U/L			.815
Mean	593	459	
SD	591	283	
Pharmacokinetic parameters of gemcitabine			
C _{max} , μg/mL			.594
Mean	24.02	23.21	
SD	7.18	6.68	
AUC, h · μg/mL			.462
Mean	9.95	10.74	
SD	2.36	3.03	

NOTE: Kruskal-Wallis test was applied to assess differences of values. Abbreviations: AE, adverse event; SD, standard deviation; ECOG, Eastern Cooperative Oncology Group; FU, fluorouracil; LAPC, locally advanced pancreatic cancer; ALP, alkaline phosphatase; C_{max}, peak concentration; AUC, area under the curve. *Calculated using the χ^2 test.

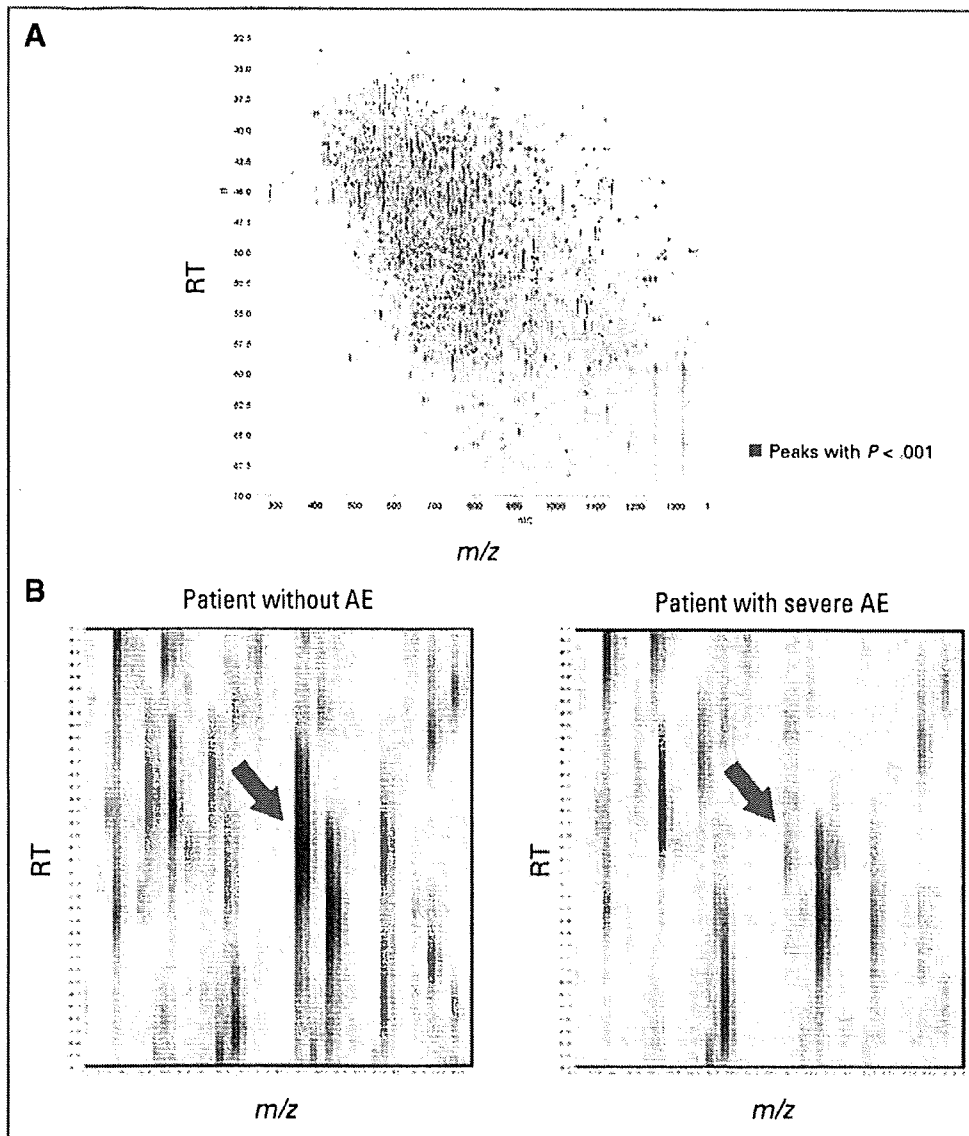


Fig 1. (A) Two-dimensional display of the entire (> 60,000) mass spectrometry (MS) peaks. The 757 MS peaks whose mean intensity differed significantly between patients with severe adverse events (AEs) and patients without AEs ($P < .001$, Welch t test) are highlighted in red. (B) MS peak with the smallest P value ($P = .0000282$; red arrows) in representative patients with severe AEs (right) and without AEs (left). RT, retention time.

two independent validation cohorts V1 (Fig 3B and Appendix Table A2) and V2 (Fig 3C and Appendix Table A3). The correlations between the levels of haptoglobin and the toxicity categories showed the highest statistical significance in all three cohorts (Figs 3A to 3C). The toxicity categories are criteria that we devised to evaluate the clinical severity of overall hematologic toxicities with emphasis on thrombocytopenia (Appendix Fig A1) from a practical viewpoint.²⁶⁻²⁸ The management of neutropenia is largely uncomplicated because of the availability of granulocyte colony-stimulating factor.

Haptoglobin Phenotype and Hematologic Toxicities

Haptoglobin is a plasma protein that binds free hemoglobin and inhibits its oxidative activity. The human *HP* gene has two common polymorphic alleles (*H1* and *H2*), yielding individuals with the following three distinct phenotypes in the α -chain of haptoglobin protein: Hp 1-1, Hp 2-1, and Hp 2-2. The *H2* genotype has been reported to be associated with an increased risk of myocardial infarction and juvenile diabetes.²⁹ Although the frequency of the three phenotypes did not

differ significantly with the severity of hematologic toxicities ($P > .360$, χ^2 test; Table 1 and Appendix Tables A1 to A3), the levels of haptoglobin were lower in individuals with the Hp 2-2 phenotype than in those with the Hp 2-1 or Hp 1-1 phenotype (Appendix Fig A3, online only).

Construction and Validation of a Model Predicting Hematologic Toxicities

In the M0 cohort ($n = 166$), 68 patients (41%) experienced category III hematologic toxicities, and 18 patients (11%) experienced category IV hematologic toxicities. Such levels of AE often necessitate the postponement of chemotherapy, and therefore, their prediction before drug administration is desirable. Because none of the parameters, including haptoglobin, was able to predict AEs satisfactorily when used individually (data not shown), we attempted to construct a multivariate predictive model to estimate the relative risk of suffering from hematologic toxicities of category III or worse. We searched for these parameters using a forward stepwise selection procedure by AIC

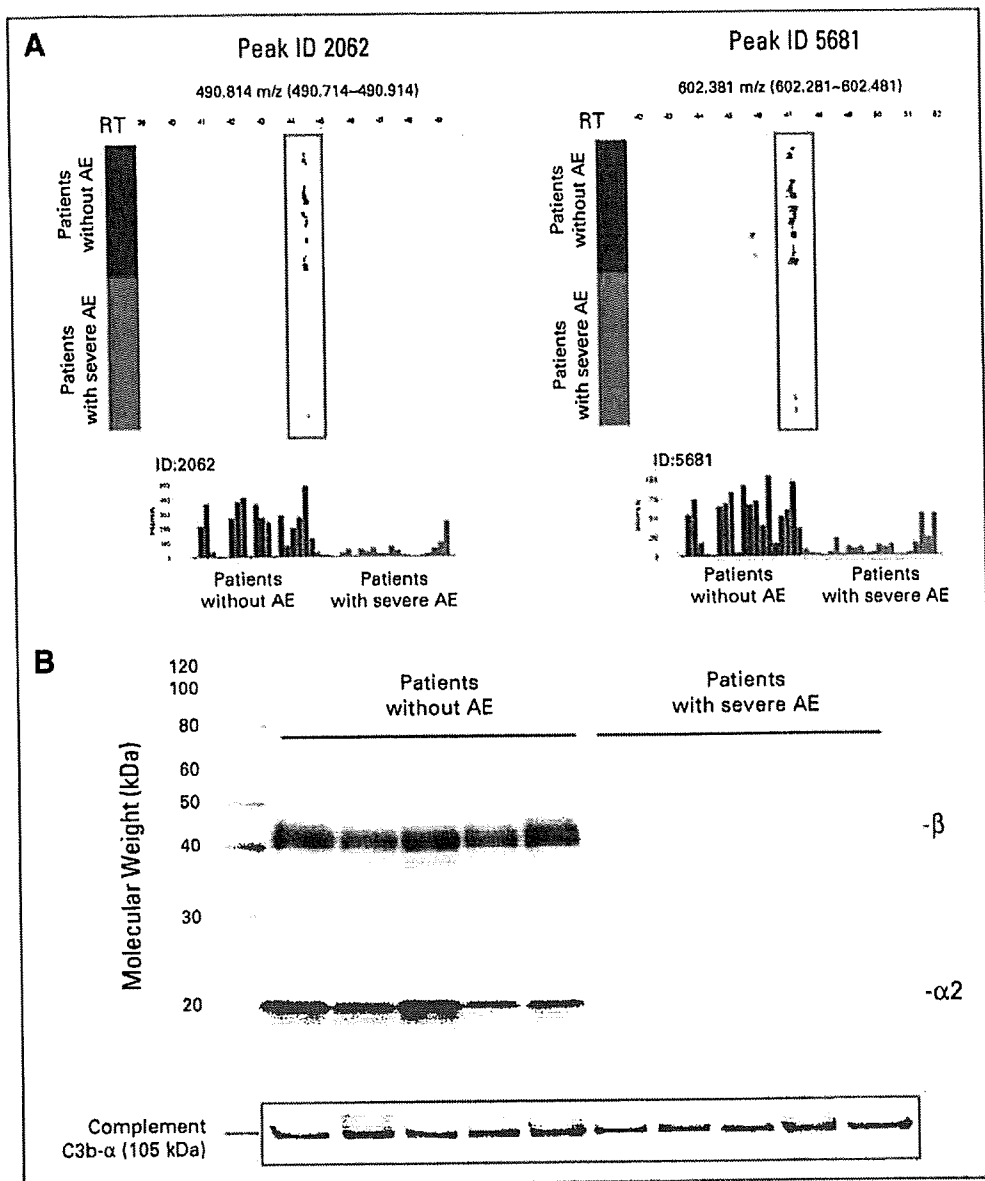


Fig 2. (A) Representative haptoglobin-derived mass spectrometry (MS) peaks in 47 triplicate liquid chromatography (LC)/MS runs (22 without adverse events [AEs], blue; and 25 with severe AEs, red) aligned along the retention time (RT) of LC (top). Columns represent the mean intensity of triplicates (bottom). (B) Detection of β - and α_2 -chains of haptoglobin and complement C3b- α (loading control) by immunoblotting.

from all of the clinical and laboratory data listed in Appendix Table A1 (available for 162 patients) and found that a combination of plasma haptoglobin level, haptoglobin phenotype, absolute neutrophil count (ANC), platelet count, and body-surface area (BSA) provided the lowest AIC value. The prediction model using this combination of parameters was significantly compromised when haptoglobin level and phenotype were excluded ($\chi^2 = 11.49$, $df = 3$, $P = .009$, likelihood ratio test). We estimated the independent contribution of each parameter to this prediction model and found that the baseline haptoglobin level was the second most important contributor to the model (Table 2).

On the basis of the results of multivariate logistic regression analysis, we constructed a nomogram in which the values of the five parameters (haptoglobin level, haptoglobin phenotype, ANC, platelet count, and BSA) are integrated into a single score (total point) to estimate the relative risk of having hematologic toxicities more severe than category II, category III, or category IV (Fig 4A). The area under

the curve value for the prediction of categories III to IV was calculated to be 0.782 (95% CI, 0.711 to 0.843) in cohort M0 (Fig 4B). Predictive ability was confirmed in two independent validation cohorts, V1 and V2, that were not used for construction of the nomogram, with area under the curve values of 0.655 (95% CI, 0.546 to 0.754) and 0.747 (95% CI, 0.606 to 0.858), respectively (Fig 4B).

DISCUSSION

The early onset of severe AE necessitates dose reduction or postponement of treatment, leading to failure of chemotherapy.^{30,31} In particular, the current gemcitabine monotherapy against advanced pancreatic cancer is mainly aimed at disease palliation, and thus, avoidance of life-threatening AEs is necessary. In this study, we first compared the plasma proteome of two groups of patients who showed distinct responses to the same protocol of gemcitabine therapy (Fig 1).