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

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

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

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

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

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

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

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

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

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

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Conflict of interest

The authors have no conflicts of interest to declare.

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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 *ABCB1* (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-alfa) 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. *ABCB1*, *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

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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

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: *HMGB1*, *HMGB2*, *ERCC1*, *XPA*, *XPD*, *MSH2*, *MLH1*, *PMS2*, *APEX1*, *MGMT*, *BRCA1*, and *GLO1*; cell-cycle regulators: *RB1*, *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:

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

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

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-alfa) 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)
ABCB1						
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 (n = 322): 0.16 (0.05–0.59)						
ABCC1 (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)
ABCC2 (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)
ABCG2 (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)
MVP (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

[a] Complete response rate (%)

[b] In 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)
TYMS (thymidylate synthetase)						
Foekens[24] (2001, Netherlands)	5-FU- based	TRA	Low expression	13	8	12.0
			High expression	108	50	(1.51–95.5)
TUBB (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)
TUBB4 (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)
TOP2A (topoisomerase II-alfa)						
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-F-U 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 (n = 323): 2.73 (1.027–7.27)						
GSTP1 (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)
BRCA1 (Breast cancer 1)						
Egawa[32] (2003, Japan)	CPA, EPI	Real-time PCR	Low	25	32	4.01
			High	26	65	(1.25–12.9)
CCND1 (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)

[a] Complete 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[b,34] (1997, Finland)	CPA, EPI, 5-FU	IHC	Low expression	89	33	2.07
			High expression	14	50	(0.66–6.45)
Rozan[35] (1998, France)	CPA, DOX, 5-FU	IHC	Low expression	131	21	1.62
			High expression	36	31	(0.71–3.69)
Jarvinen[36] (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[28] (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[40] (2005, UK)	Anthracycline-based	IHC	Low expression	84	71	1.87
			High expression	34	82	(0.69–5.08)
Prisack[30] (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[c,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
[a] Pathological complete response rate
[b,c] 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[c,34] (1997, Finland)	CPA, EPI, 5-FU	IHC	Normal	86	37	0.52
			Mutated	17	24	(0.16–1.73)
Frassoldati[47] (1997, Italy)	CPA, DOX or CPA, MTX, 5-FU	IHC	Normal	26	42	0.68
			Mutated	3	33	(0.05–8.50)
Bonetti[d,48] (1998, Italy)	CPA, MTX, 5-FU or Anthracycline-based	IHC	Normal	21	30	0.94
			Mutated	22	27	(0.25–3.56)
Rozan[35] (1998, France)	CPA, DOX, 5-FU	IHC	Normal	97	22	1.25
			Mutated	70	26	(0.61–2.58)
Jarvinen[36] (1998, Finland)	EPI	IHC	Normal	37	57	0.61
			Mutated	18	44	(0.20–1.90)
Colleoni[49] (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[50] (2000, Austria)	CPA, EPI, 5-FU	IHC	Normal	20	85	0.01
			Mutated	15	7	(0.00–0.13)
Kandioler-Eckersberger[50] (2000, Austria)	PTX	IHC	Normal	20	35	3.71
			Mutated	12	67	(0.82–16.8)
Bonnefoi[33] (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[c,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[54] (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
[a] Pathological complete response rate
[b] Good pathological response rate
[c,d] In 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[47] (1997, Italy)	CPA, DOX or CPA, MTX, 5-FU	IHC	Low	19	47	0.48
			High	10	30	(0.09–2.42)
Bonetti[c,48] (1998, Italy)	CPA, MTX, 5-FU or Anthracycline-based	IHC	Low	32	44	0.19
			High	23	13	(0.05–0.78)
Colleoni[49] (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)						

[a] Pathological complete response rate
[b] Good pathological response rate
[c] In 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)
BCL2L1 (Bcl2-like 1, Bcl-xL)						
Sjostrom[56] (2002, Finland)	DTX or MTX, 5-FU (second-line)	IHC	Low	59	36	1.32
			High	64	42	(0.64–2.73)
BAX (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[56] (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*	0.04
			High	69	6*	(0.01–0.20)
TNFRSF6 (tumor necrosis factor receptor superfamily, member 6, FAS, CD95)						
Sjostrom[56] (2002, Finland)	DTX or MTX, 5-FU (second-line)	IHC	Low	53	42	0.83
			High	70	37	(0.40–1.73)

*Pathological 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 overexpression 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, *ABCB1*, *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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ORIGINAL REPORT

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 standard 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 samples[17] 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/m^2 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, MC).

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 (±standard deviation) of the entire 60,888 peaks of the 47 triplicate runs was as high as 0.978 (±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 antirabbit or antimouse 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 nondenaturing (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 gemcitabine[15] (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)	P
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, $\times 10^3/\mu L$			.0002
Mean	7.4	4.8	
SD	2.8	1.4	
Absolute neutrophil count, $\times 10^3/\mu L$			.0002
Mean	5.3	3.0	
SD	2.4	1.1	
Platelet, $\times 10^4/\mu L$			< .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.

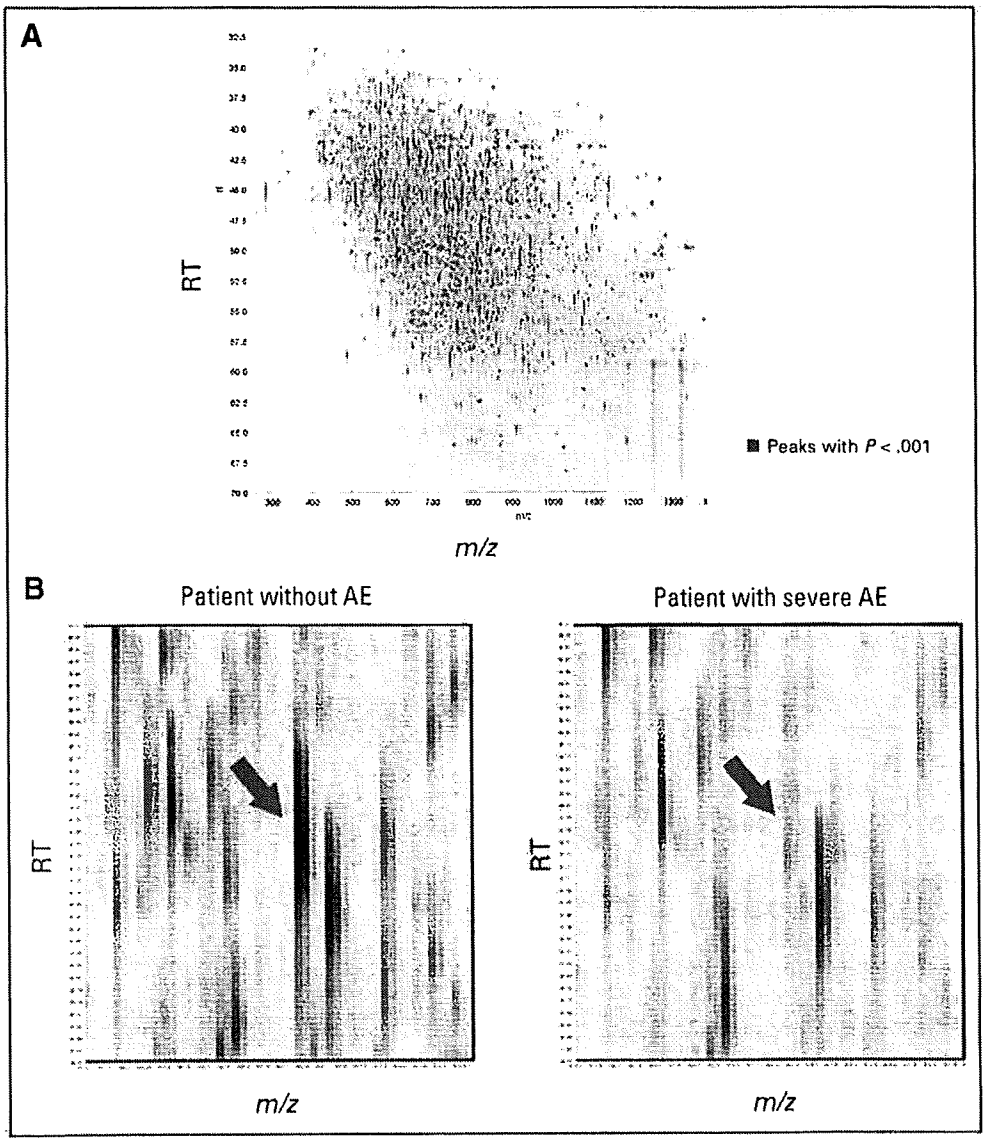

Fig 1. (A) Two-dimensional display of the entire (> 60,000) mass spectrometry (MS) peaks. The 757 MS peaks whose mean intensity differed significantly between patients with severe adverse events (AEs) and patients without AEs ($P < .001$, Welch t test) are highlighted in red. (B) MS peak with the smallest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

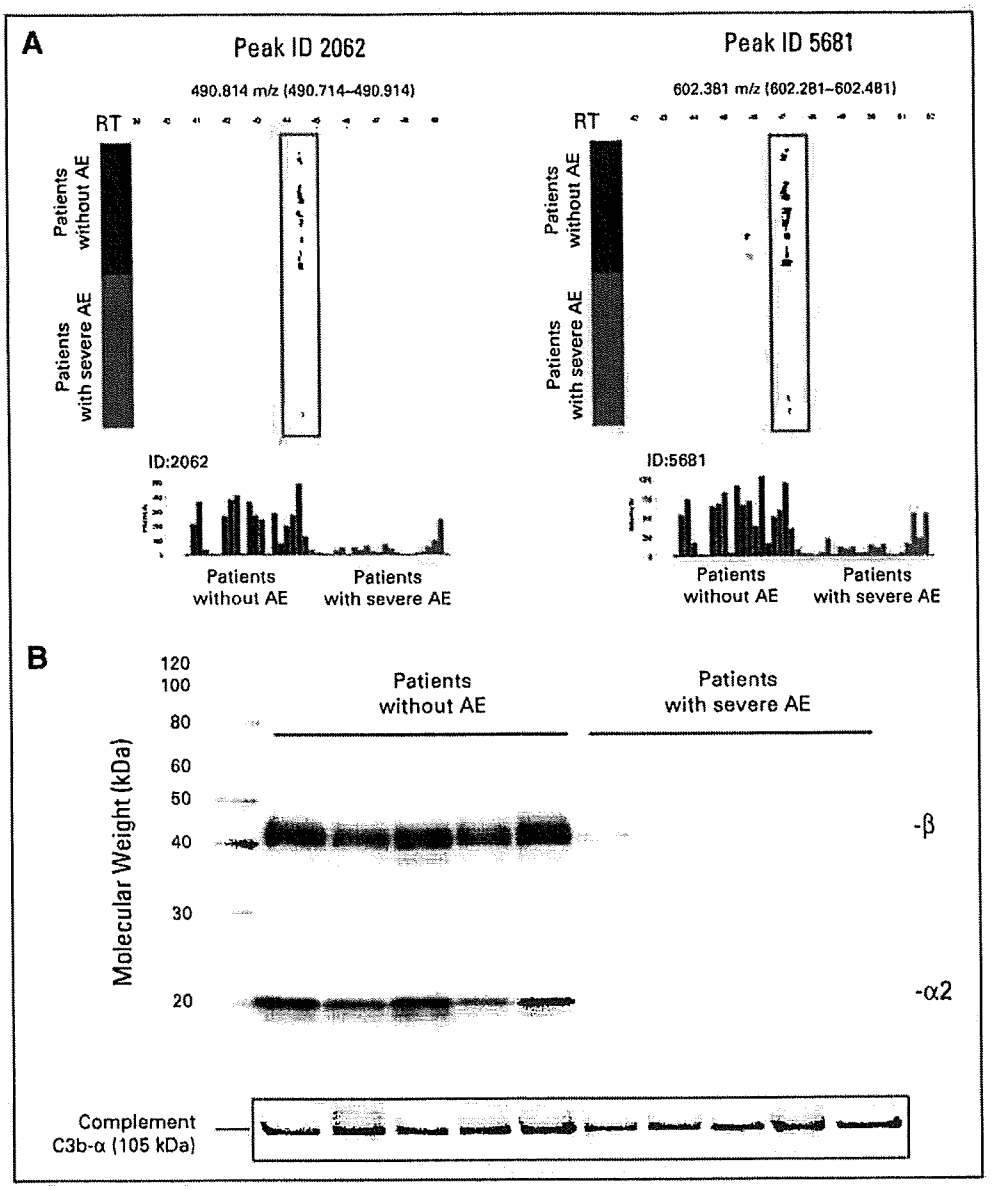

Fig 2. (A) Representative haptoglobin-derived mass spectrometry (MS) peaks in 47 triplicate liquid chromatography (LC)/MS runs (22 without adverse events [AEs], blue; and 25 with severe AEs, red) aligned along the retention time (RT) of LC (top). Columns represent the mean intensity of triplicates (bottom). (B) Detection of β- and α2-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).

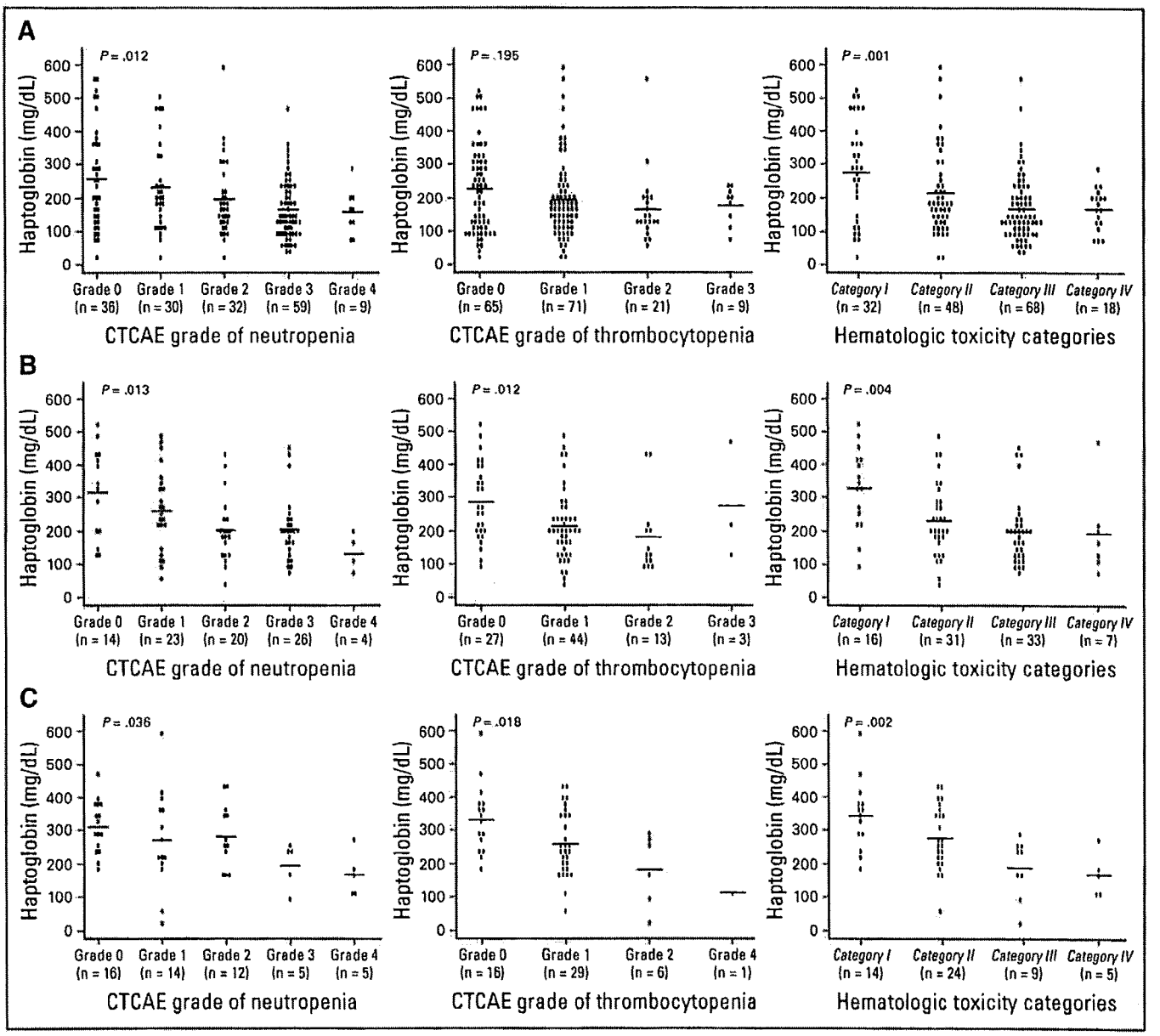

Fig 3. Plasma/serum haptoglobin levels according to the Common Terminology Criteria of Adverse Events (CTCAE; version 3.0). Grades of neutropenia (left), thrombocytopenia (middle), and hematologic toxicity categories (right) in the (A) modeling (M0), (B) validation-1 (V1), and (C) validation-2 (V2) cohorts. Horizontal lines represent the average levels of haptoglobin.

There was no significant difference in age distribution, Eastern Cooperative Oncology Group performance status, liver function, renal function, or prior chemoradiotherapy between the groups (Table 1 and data not shown), indicating that the occurrence of AEs does not merely reflect the general poor condition of patients but is based on certain biologic differences among individuals. We found that individuals who experienced severe AEs after administration of gemcitabine showed decreased baseline levels of plasma haptoglobin (Figs 1B and 2A), and this result was validated in three large cohorts using a different methodology (Fig 3 and Appendix Tables A1 to A3). Haptoglobin is an abundant plasma protein that usually cannot be measured by direct MS. However, constant depletion using an IgY-12 High Capacity Spin Column[32] allowed us to accentuate the differences in haptoglobin levels.

The molecular mechanisms that regulate the plasma haptoglobin level under physiologic and pathologic conditions are largely unknown. Haptoglobin is produced mainly in the liver, taken up by neutrophils, and stored within their cytoplasmic granules. Haptoglobin is released in response to a variety of stimuli, such as infection, trauma, and malignancy,[33] and modulates inflammatory responses. Tumor necrosis factor α induces the release of haptoglobin from neutrophils in vitro.[34] Interestingly, tumor necrosis factor α and its soluble receptors have been reported to be associated with an increased risk of hematologic toxicities.[12,35,36]

Table 2. Contribution of Parameters to Prediction of Hematologic Toxicities Associated With Gemcitabine

Factor	Odds Ratio*	95% CI	P
Haptoglobin level	0.71	0.53 to 0.97	.031†
Phenotype of haptoglobin (v Hp 2-2)			
Hp 2-1	0.61	0.31 to 1.21	.159
Hp 1-1	2.16	0.70 to 6.69	.180
Absolute neutrophil count	0.72	0.61 to 0.86	.0003†
Platelet count	0.63	0.39 to 1.01	056
Body-surface area	3.86	0.63 to 23.76	.145

NOTE. A forward stepwise selection based on Akaike's Information Criterion was used to select parameters for multivariate analysis.
*Odds ratios are per 100 mg/dL increase for haptoglobin level, per 1,000/μL increase for absolute neutrophil count, per $10 \times 10^4/\mu L$ increase for platelet, and per 1.00 m^2 increase for body-surface area.
†$P < .05$.

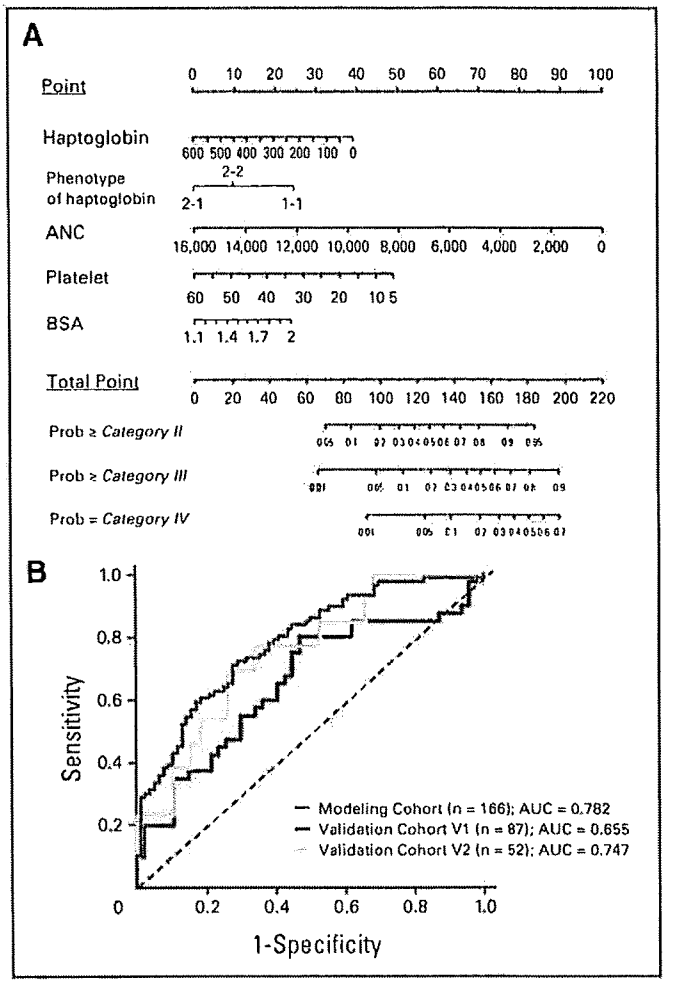

Fig 4. (A) Nomogram to estimate the risk of hematologic toxicities more severe than category II (top), category III (middle), and category IV (bottom). Please see Appendix Figure A4 and its legend for usage. (B) Receiver operating characteristic (ROC) analysis of nomogram for the prediction of category III and IV hematologic toxicities in the modeling (gray), validation-1 (V1; blue), and validation-2 (V2; gold) cohorts. ANC, absolute neutrophil count; BSA, body-surface area; AUC, area under the curve.

To derive clinical applicability from these basic findings, we constructed a model (nomogram) that estimates the possibility of occurrence of hematologic AE before administration of gemcitabine (Fig 4A and Appendix Fig A4). The significance of the model was further confirmed in two independent validation cohorts (Fig 4B). Although its accuracy was far from perfect, the model seems to be practically sufficient for identifying individuals who are likely to suffer from hematologic toxicities after administration of gemcitabine. Various cytotoxic or molecular targeting agents have been tested in combination with gemcitabine in phase III trials, but no apparent additional therapeutic benefit has been demonstrated.[5,6,9,10] The application of this model to patient selection may improve the outcome of such trials. We are now trying to identify new biomarkers that can predict the efficacy of gemcitabine treatment using a similar strategy.

The phenotypes of haptoglobin have been reported to be associated with different hemoglobin-binding, antioxidative, and prostaglandin synthesis–initiating activities.[33] Although haptoglobin phenotype was not significantly associated with hematologic toxicities (Table 1 and Appendix Tables A1 to A3), the average levels of haptoglobin differed among individuals with different phenotypes (Appendix Fig A3), as described previously.[33] For this reason, haptoglobin phenotype was selected in the prediction model by AIC analysis (Table 2). BSA has been repeatedly selected as one of the multivariate parameters for predicting the AEs of anticancer therapies in other studies,[14,37] suggesting a potential lack of accuracy in calculating individually optimized drug dose based solely on BSA, as pointed out previously.[38,39]

In conclusion, we have revealed that a decreased level of haptoglobin is the second most significant factor predicting hematologic toxicities associated with gemcitabine monotherapy after ANC (Table 2). Measurement of haptoglobin is now established as a laboratory test and could be readily incorporated into routine oncologic practice. However, the predictive significance of haptoglobin was revealed only in a retrospective population from a single institution and must, therefore, be validated in an independent prospective multi-institutional study. It was not determined in this study whether haptoglobin could be a predictive biomarker for the AEs of other chemotherapeutic agents. To improve the accuracy of prediction, the discovery of new biomarkers with higher specificity and sensitivity will be necessary. While bearing all these limitations in mind, the present findings may provide novel insights not only into the molecular mechanisms by which gemcitabine causes hematologic toxicities, but also into new avenues for the development of new chemotherapeutic agents with lower toxicity.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Although all authors completed the disclosure declaration, the following author(s) indicated a financial or other interest that is relevant to the subject matter under consideration in this article. Certain relationships marked with a "U" are those for which no compensation was received; those relationships marked with a "C" were compensated. For a detailed description of the disclosure categories, or for more information about ASCO's conflict of interest policy, please refer to the Author Disclosure Declaration and the Disclosures of Potential Conflicts of Interest section in Information for Contributors.

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Phase III trial of docetaxel plus gemcitabine versus docetaxel in second-line treatment for non-small-cell lung cancer: results of a Japan Clinical Oncology Group trial (JCOG0104)

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Background: This trial evaluated whether a combination of docetaxel and gemcitabine provides better survival than docetaxel alone in patients with previously treated non-small-cell lung cancer (NSCLC).

Patients and methods: Eligibility included pathologically or cytologically proven NSCLC, failure of one platinum-based regimen, performance status of zero or one, 20–75 years old, and adequate organ function. Patients received docetaxel 60 mg/m^2 (day 1) or docetaxel 60 mg/m^2 (day 8) and gemcitabine 800 mg/m^2 (days 1 and 8), both administered every 21 days until disease progression.

Results: Sixty-five patients participated in each arm. This trial was terminated early due to an unexpected high incidence of interstitial lung disease (ILD) and three treatment-related deaths due to ILD in the combination arm. Docetaxel plus gemcitabine compared with docetaxel-alone patients experienced similar grade and incidence of toxicity, except for ILD. No baseline factor was identified for predicting ILD. Median survival times were 10.3 and 10.1 months (one-sided $P = 0.36$) for docetaxel plus gemcitabine and docetaxel arms, respectively.

Conclusion: Docetaxel alone is still the standard second-line treatment for NSCLC. The incidence of ILD is higher for docetaxel combined with gemcitabine than for docetaxel alone in patients with previously treated NSCLC.

Key words: docetaxel, gemcitabine, non-small-cell lung cancer, platinum-refractory, second-line chemotherapy

introduction

Lung cancer is the most common cancer worldwide, with an estimated 1.2 million new cases globally (12.3% of all cancers) and 1.1 million deaths (17.8% of all cancer deaths) in 2000 [1]. The estimated global incidence of non-small-cell lung cancer (NSCLC) in 2000 was ~1 million, which accounted for ~80% of all cases of lung cancer [1]. Treatment of advanced NSCLC is palliative; the aim is to prolong survival without leading to deterioration in quality of life [2]. The recommended first-line treatment of advanced NSCLC currently involves up to four cycles of platinum-based combination chemotherapy, with no single combination recommended over others [3]. Although this treatment improves survival rates, a substantial proportion of patients do progress and should be offered second-line treatment. With unsurpassed efficacy compared with other chemotherapeutic regimens or best supportive care [4, 5], docetaxel alone is the current standard as second-line chemotherapy for advanced NSCLC. The recommended regimen of docetaxel 75 mg/m^2 given i.v. every 3 weeks as second-line therapy has been associated with median survival times of 5.7–7.5 months [4, 5] and is also associated with better quality-of-life outcomes compared with best supportive care [2]. Docetaxel monotherapy for recurrent NSCLC after platinum-based chemotherapy has several limitations, however, including low response rates (7–11%), brief duration of disease control, and minimal survival advantage [4, 5].

Gemcitabine is also active against recurrent NSCLC after platinum-based chemotherapy [6]. Gemcitabine 1000 mg/m^2 once a week for 3 weeks every 28 days produced a 19% response rate in a phase II trial, and it shows significant activity mainly

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in patients previously responsive to chemotherapy [6]. Single-agent gemcitabine has a low toxicity profile and is well tolerated [6].

Docetaxel and gemcitabine have distinct mechanisms of action and nonoverlapping toxic effects except for neutropenia. Many studies of the combination of docetaxel and gemcitabine have been conducted in first- and second-line settings [7–16]. The following doses and schedule have been adopted in most studies: docetaxel 80–100 mg/m^2 on day 1 or 8 and gemcitabine 800–1000 mg/m^2 on days 1 and 8 or on days 1, 8, and 15. Furthermore, most studies required use of prophylactic granulocyte colony-stimulating factor (G-CSF) support.

In Japan, however, the recommended dose of docetaxel is 60 mg/m^2 every 3 weeks [17, 18]. Several studies to confirm the dose and schedule of this combination without prophylactic G-CSF support have been conducted in Japan [19–21]. Two studies recommended docetaxel 60 mg/m^2 on day 8 and gemcitabine 800 mg/m^2 on days 1 and 8, and another study recommended docetaxel 50 mg/m^2 on day 8 and gemcitabine 1000 mg/m^2 on days 1 and 8, without prophylactic G-CSF support, every 3 weeks. These studies demonstrated the consistent promising efficacy of this combination regimen. An objective response was observed in 28%–40% of patients, with a median survival time of 11.1–11.9 months and a 1-year survival rate of 41%–47%.

We conducted a multicenter, randomized, phase III trial to evaluate whether the combination regimen of docetaxel and gemcitabine provides better survival than docetaxel alone in patients with previously treated NSCLC.

patients and methods

patient selection

Eligible patients were 20–75 years of age, with histologically or cytologically confirmed stage IIIB (with malignant pleural effusion or contralateral hilar lymph node metastases) or stage IV NSCLC who had failed one platinum-based chemotherapy regimen previously. Patients who had received gemcitabine or docetaxel were excluded. Additional inclusion criteria included a Eastern Cooperative Oncology Group performance status of zero to one, and adequate organ function as indicated by white blood cell count ≥4000/μl, absolute neutrophil count ≥2000/μl, hemoglobin ≥9.5 g/dl, platelets ≥100 000/μl, aspartate aminotransferase (AST)/alanine amonotransferase (ALT) ≤2.5 times the upper limit of normal, total bilirubin ≤1.5 mg/dl, serum creatinine ≤1.2 mg/dl, and PaO_2 in arterial blood ≥70 torr. Asymptomatic brain metastases were allowed provided that they had been irradiated and were clinically and radiologically stable. Prior thoracic radiotherapy was allowed provided that treatment was completed at least 12 weeks before enrollment. Patients were excluded from the study if they had radiologically and clinically apparent interstitial pneumonitis or pulmonary fibrosis. All patients provided written informed consent, and the study protocol was approved by Japan Clinical Oncology Group (JCOG) Clinical Trial Review Committee and the institutional review board of each participating institution.

treatment plan and dose modifications

Eligible patients were centrally registered at JCOG Data Center and were randomly assigned to either docetaxel 60 mg/m^2 as a 60-min i.v. infusion on day 1 or docetaxel 60 mg/m^2 as a 60-min i.v. infusion on day 8 plus gemcitabine 800 mg/m^2 as a 30-min i.v. infusion on days 1 and 8, using a minimization method with institutions and response to prior chemotherapy (progressive disease or not) as balancing factors. Patients receiving docetaxel were administered standard dexamethasone premedication (8 mg orally at the day before, on the day, and the day after docetaxel administration) as previously reported [7] and 50 mg of diphenhidramine 30 min before docetaxel administration. Recombinant human G-CSF was not given prophylactically. Chemotherapy cycles were repeated every 3 weeks until disease progression. Docetaxel was given before gemcitabine in the docetaxel plus gemcitabine regimen.

Dose adjustments were based mainly on hematologic parameters. The doses of docetaxel and gemcitabine were reduced by 10 and 200 mg/m^2, respectively, in subsequent cycles if chemotherapy-induced febrile neutropenia, grade 4 anemia, grade 4 thrombocytopenia, grade 4 leukopenia, or grade 4 neutropenia lasting for >3 days occurred in the absence of fever. Dose reductions were maintained for all subsequent cycles. Patients requiring more than one dose reduction were off-protocol treatment.

baseline and follow-up assessments

Pretreatment evaluation included a complete medical history and physical examination, a complete blood count (CBC) test with differential and platelet count, standard biochemical profile, electrocardiogram, chest radiographs, computed tomographic scans of the chest, abdomen, and brain, magnetic resonance imaging, and a whole-body bone scan. During treatment, a CBC and biochemical tests were carried out weekly. A detailed medical history was taken and a complete physical examination with clinical assessment was carried out weekly to assess disease symptoms and treatment toxicity, and chest radiographs were done every treatment cycle. Toxicity was evaluated according to the National Cancer Institute Cancer—Common Toxicity Criteria Version 2 [22].

All patients were assessed for response by computed tomography scans after every two cycles of chemotherapy. Response Evaluation Criteria in Solid Tumors (RECIST) were used for the evaluation of response [23].

The progression-free survival (PFS) was calculated from the day of randomization until the day of the first evidence of disease progression or death. If the patient had no progression, PFS was censored at the day when no clinical progression was confirmed. Overall survival (OS) was measured from the day of randomization to death.

Disease-related symptoms were evaluated and scored at baseline and 6 weeks after the start of treatment with the seven-item Lung Cancer Subscale (LCS) of the Functional Assessment of Cancer Therapy-Lung version 4 [24], which were translated from English to Japanese. The questionnaire entries were listed as follows: 'I have been short of breath', 'I am losing weight', 'My thinking is clear', 'I have been coughing', 'I have a good appetite', 'I feel tightness in my chest', and 'Breathing is easy for me'. Patients scored using a five-point Likert scale (0–4) by themselves. The maximum attainable score of the LCS was 28, where the patient was considered to be asymptomatic.

statistical analysis

The primary endpoint was OS; secondary endpoints were PFS, the overall response rate, disease-related symptoms, and toxicity profile. Based on previous trials evaluating the docetaxel [4, 5] and docetaxel plus gemcitabine [19–21] regimens, the present study was designed to detect a 12% difference of 1-year survival rate. To attain an 80% power at a one-sided significance level of 0.05, assuming 1-year survival of docetaxel arm as 35% with 1 year of follow-up after 2 years of accrual, 284 patients (142 per each arm) were required. Analyses were to be carried out with all randomized patients. Both the OS and PFS were estimated with the Kaplan–Meier method. The comparisons of OS and PFS between arms were assessed by the stratified log-rank test with a factor used at randomization, response to prior chemotherapy. Two interim analyses were planned after half of the patients were registered and the end of registration.