and younger age is a recognized favorable prognostic factor. However, considering that the patients were in advanced stages and had a median follow-up of 128 months, these results were encouraging.

Shipp et al. developed a model, IPI, for predicting outcome in patients with aggressive lymphoma on the basis of patient characteristics before treatment, in which most patients received the CHOP regimen (29). The aa-IPI criteria, based on tumor stage, LDH level, and PS, were used to identify four risk groups: L, LI, HI, and H risk, with predicted 5-year survival rates of 83%, 69%, 46%, and 32%, respectively. Expressing our data according to aa-IPI, the OS at 10 years was 81%, and 74% for patients in the L/LI- and HI/H-risk groups, respectively. These findings suggest that the TCC-NHL91 regimen showed a significant benefit in the patients in the HI/H-risk groups, and overcame poor prognostic factors.

Dose reduction was not performed in 55 patients, while it was performed in three patients over 60 and one patient with hepatic dysfunction. Fourty-six patients received seven or eight cycles of therapy (Table 3), but 13 patients, including 10 of 15 patients over 60 years old, and 3 of 44 patients at ≤60 years old, received six or fewer cycles of therapy. Many patients over aged 60 could not tolerate this regimen. The TCC-NHL-91 regimen is not recommended for elderly patients.

The TCC-NHL-91 regimen was toxic and the predominant toxicity was hematological (Table 3). Grade 4 hematotoxicity (neutropenia) was observed in 100% of the patients in spite of G-CSF support. However, recovery of neutropenia was rapid and the median durations of neutropenia (<500/μl) in the first to the seventh cycles of therapy was 3–5 days, and no patient died of severe infection. In the eighth cycle of therapy, the median duration of neutropenia (<500/μl) was 7 days (1–15 days) and one patient died of pseudomonas sepsis. Although our approach adds considerably to the acute toxicity and expense of lymphoma treatment, significant improvement of OS and PFS justify its use.

Radiotherapy has been reported to improve the survival in patients with limited-stage lymphoma (30), but its use for consolidation therapy for primary bulky sites or residual mass against advanced aggressive lymphoma are still controversial, because the sites of relapse are variable in advanced lymphoma (31). In North America, radiotherapy has rarely been considered for advanced aggressive lymphoma (30).

We performed involved-field radiation in 43 patients. Only 2 of the 17 relapsed patients relapsed from the radiation sites. Wilder et al. reported that 86% of patients treated with chemotherapy alone developed a recurrence at the presenting site of the bulky mass whereas only 12% of the patients who were treated with chemotherapy followed by radiotherapy developed disease recurrence within the radiotherapy field (32). Our findings show striking similarity with Wilder et al.'s findings. Recent randomized trials suggest that adjuvant radiotherapy against primary bulky mass or residual mass may significantly improve the relapse-free survival and OS of patients with advanced aggressive lymphoma (33,34). Considering these clinical findings and our data, radiotherapy is considered to have made a significant contribution to the outcome of our study.

In our study, the patients with bulky disease showed better OS and PFS than those without bulky disease. The OS at 10 years was 83% (95% CI 72–95%) and 59% (95% CI 35–82%) in these groups, respectively ($p = 0.20$). The total number of treatment cycles was similar in the two groups. These results are in conflict with previous findings that bulky disease showed worse prognosis (13,32,35). However, the difference is not statistically significant.

CNS prophylaxis is not usually included in the treatment regimens because of the relatively low incidence of CNS relapse and the toxicity of the prophylactic therapy. However, several studies have suggested that CNS relapse is not always rare in patients with advanced intermediate-grade NHL: the cumulative risk of CNS relapse in these patients was 4–20% and systemic relapse occurred rapidly after CNS relapse, resulting in a median survival time after CNS relapse of only a few months (20–26). Risk factors for CNS relapse have include advanced stages, high LDH, more than one extranodal site, high IPI, and involvement of paranasal sinus, orbital cavity, testis, or bone marrow, etc. van Besien et al. reported that high LDH and >1 extranodal sites are most important factors for CNS relapse and these patients had a 17% probability of CNS recurrence at 1 year after diagnosis (24).

CNS prophylaxis could be important for improving the outcome of high-risk patients, but there is no consensus regarding indications for prophylaxis or a standard CNS chemoprophylaxis regimen (36). The CNS prophylaxis of our therapy including intrathecal MTX, intermediate-dose MTX, ifosfamide, and high-dose cytarabine was insufficient. Chua et al. reported that intrathecal chemotherapy alone was inadequate CNS prophylaxis in patients with intermediate-grade non-Hodgkin's lymphoma (37). Our data support their findings.

After August 1997, we initiated prophylactic cranial radiation (24 Gy) in new patients ($n = 14$) with high LDH and/or massive bone marrow involvement, because at that time, these seemed important risk factors for CNS relapse in patients with advanced lymphoma (20–23). These included nine patients with both elevated serum LDH and more than one extranodal site. After that, no CNS relapse was observed and no patients

Table 4. Comparison of Conventional Chemotherapy and Dose-Intensive Regimens With G-CSF Support Against Advanced Aggressive Lymphoma

Author	Regimen	Radiotherapy	Patient	No. of Patients	Median Age (Range)	aa-IPI (L+LI/HI+H) (%)	OS		Dose Delivery
							Years	% (All)	
Shipp (29)	CHOP etc.		aggressive lymphoma	1,274	<60	54/46	5	46/32	
Gordon et al. (40)	200% PRO-MACE-CytaBOM	–	advanced aggressive lymphoma	74	43 (19–65)	54/46	4	73*	56/74 patients received 6 or more cycles of therapy (total 8 cycle)
Gisselbrecht et al. (41)	ACVBP	–	advanced aggressive lymphoma	181	46 (15–60)	3/98	5	60†	
Wilson et al. (42)	Dose-adjusted EPOCH	–	diffuse large-B-cell lymphoma	50	46 (20–88)	48/52	5 + 2 months	73‡	39/50 patients received 6 or more cycles of therapy (total 8 cycle)
Blayney et al. (43)	Dose-intensified 2W-CHOP	–	advanced aggressive lymphoma	88	53 (19–77)	64/37§	5	60¶	81/88 received 6 cycles of therapy (total 6 cycle)
Present study	TCC-NHL-91	+	advanced intermediate-grade lymphoma	59	48 (17–69)	27/73	5	78	50/59 patients received 6 or more cycles of therapy (total 8 cycle)

*52% in H-risk group.
†52% in HI- and H-risk groups.
‡55% in HI-risk group, 100% in H-risk group.
§IPI but not aa-IPI.
¶66% in HI-risk group, 53% in H-risk group.

receiving cranial radiation developed neurocognitive deficits. Although the number of patients was too small to allow analysis, prophylactic low-dose cranial radiation in addition to intrathecal MTX, etc., might contribute to preventing CNS relapse and improving OS. Recently, prophylactic low dose of cranial radiation (24–30 Gy) was introduced for CR patients with small-cell lung cancer because it decreased the incidence of brain metastases and prolonged survival, and this can be achieved with a tolerable level of acute and delayed neurotoxicity using modern techniques (38,39). Further evaluation and prospective studies of prophylactic cra-

nial radiation appeared to be warranted for high-risk lymphoma patients with CNS relapse.

The use of dose-intensive chemotherapy with G-CSF support against advanced intermediate-grade NHL has been controversial. However, the results of several regimens included better outcomes (40–43) (Table 4). Although the patients' backgrounds in each study were slightly different, the 5-year OS achieved by this approach could be about 10–30% better than that by conventional chemotherapy. Interestingly, in these trials, the outcome of the patients in the HI- and H-risk groups was improved significantly and there was no significant difference in OS between the L-/LI- and HI/H-risk groups. The results of our study are in agreement with those of these previous studies.

The introduction of rituximab and its combination with CHOP or CHOP-like regimens resulted in a high remission rate and OS against aggressive B-cell lymphomas (7–9). Five-year OS for patients with DLBCL was improved significantly (10–20%) by the addition of rituximab to standard regimens, but the treatment outcome is still unsatisfactory for especially high-risk patients. The toxicity profile of rituximab is excellent and the absence of relevant hematotoxicity allows its combination with dose-intensive regimens. In addition, the lymphoma cell populations sensitive to rituximab may be different from those sensitive to conventional anti-lymphoma agents (44). The combined use of rituximab with TCC-NHL-91 may improve the OS and PFS in advanced intermediate grade B-cell lymphoma.

In conclusion, TCC-NHL-91 achieved high CR, OS, and PFS in patients with advanced intermediate-grade NHL. The outcome was not found to be dependent on aa-IPI. TCC-NHL-91, combined with appropriate radiotherapy, could be very effective for younger patients with advanced intermediate-grade lymphoma. However, as this study was a single-institution study with retrospective analysis, it is not possible to reach definite conclusions. Further evaluation and prospective studies of the TCC-NHL-91 appear to be warranted.

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ORIGINAL ARTICLE

Bortezomib overcomes cell adhesion-mediated drug resistance through downregulation of VLA-4 expression in multiple myeloma

K Noborio-Hatano[1,4], J Kikuchi[2,4], M Takatoku[1], R Shimizu[2], T Wada[2], M Ueda[1], M Nobuyoshi[1], I Oh[1], K Sato[1], T Suzuki[1], K Ozaki[1], M Mori[1], T Nagai[1], K Muroi[1], Y Kano[3], Y Furukawa[1,2] and K Ozawa[1]

[1]*Division of Hematology, Department of Internal Medicine, Jichi Medical University, Tochigi, Japan;* [2]*Division of Stem Cell Regulation, Center for Molecular Medicine, Jichi Medical University, Tochigi, Japan and* [3]*Division of Hematology, Department of Medical Oncology, Tochigi Cancer Center, Tochigi, Japan*

Multiple myeloma (MM) is incurable, mainly because of cell adhesion-mediated drug resistance (CAM-DR). In this study, we performed functional screening using short hairpin RNA (shRNA) to define the molecule(s) responsible for CAM-DR of MM. Using four *bona fide* myeloma cell lines (KHM-1B, KMS12-BM, RPMI8226 and U266) and primary myeloma cells, we identified CD29 (β1-integrin), CD44, CD49d (α4-integrin, a subunit of VLA-4), CD54 (intercellular adhesion molecule-1 (ICAM-1)), CD138 (syndecan-1) and CD184 (CXC chemokine receptor-4 (CXCR4)) as major adhesion molecules expressed on MM. shRNA-mediated knockdown of CD49d but not CD44, CD54, CD138 and CD184 significantly reversed CAM-DR of myeloma cells to bortezomib, vincristine, doxorubicin and dexamethasone. Experiments using blocking antibodies yielded almost identical results. Bortezomib was relatively resistant to CAM-DR because of its ability to specifically downregulate CD49d expression. This property was unique to bortezomib and was not observed in other anti-myeloma drugs. Pretreatment with bortezomib was able to ameliorate CAM-DR of myeloma cells to vincristine and dexamethasone. These results suggest that VLA-4 plays a critical role in CAM-DR of MM cells. The combination of bortezomib with conventional anti-myeloma drugs may be effective in overcoming CAM-DR of MM.
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Correspondence: Professor Y Furukawa, Division of Stem Cell Regulation, Center for Molecular Medicine, Jichi Medical University, 3311-1 Yakushiji, Shimotsuke, Tochigi 329-0498, Japan.
E-mail: furuyu@jichi.ac.jp
[4]These authors contributed equally to this work.
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Introduction

Despite recent advances in treatment strategies using dose-intensified regimens and new molecular-targeted compounds, multiple myeloma (MM) remains incurable (Kyle *et al.*, 2003). Most patients with MM eventually become resistant to the treatment and die of disease progression within 10 years. To improve the prognosis of myeloma patients, it is essential to overcome drug resistance (DR).

MM is characterized by the infiltration and growth of malignant plasma cells in the bone marrow (BM) microenvironment. MM cells localize within the BM through the interaction of adhesion receptors with their ligands on BM stromal cells and extracellular matrix proteins (Hideshima *et al.*, 2007). It has been demonstrated that MM cells in the BM microenvironment are much less sensitive to chemotherapeutic agents (Damiano *et al.*, 1999; Nefedova *et al.*, 2003). This type of DR has been termed cell adhesion-mediated DR (CAM-DR), which is believed to play a crucial role in both *de novo* and acquired DR in MM patients (Damiano *et al.*, 1999). Despite extensive investigations, the adhesion molecules critical for CAM-DR in MM have not been identified yet.

The proteasome inhibitor bortezomib (Velcade, formerly known as PS-341) has shown a clinical activity in patients with relapsed MM (Richardson *et al.*, 2003, 2005), and will be applied for the treatment of other hematologic malignancies and solid tumors in the near future (Fisher *et al.*, 2006; Davies *et al.*, 2007). Bortezomib is a reversible inhibitor of the 26S proteasome complex, which catalyses ubiquitin-dependent protein degradation. Inhibition of this complex ultimately leads to modulation of the abundance and functions of many intracellular proteins in bortezomib-treated cells (Hideshima *et al.*, 2001). Among them, the multifunctional transcription factor nuclear factor-kappa B (NF-κB) is considered the most relevant target in MM, because recent genome-wide approaches revealed that this factor is frequently activated in MM cells by mutations of the components of the NF-κB signaling cascade (Annunziata *et al.*, 2007; Keats *et al.*, 2007). Given the wide spectrum of transcriptional

targets of NF-κB including adhesion molecules and the IAP family of apoptosis inhibitors (Dolcet *et al*., 2005), it is reasonable to speculate that CAM-DR of MM is mediated by NF-κB and could be overcome by bortezomib. To date, however, such possibilities have not been investigated.

In this study, we first attempted to identify the adhesion molecules responsible for CAM-DR in MM. By functional screening using the lentiviral short hairpin/small interfering RNA (shRNA/siRNA) system, we identified VLA-4 as a critical molecule for the induction of CAM-DR in MM cells. Furthermore, we found a novel and unique property of bortezomib to overcome CAM-DR by downregulating the expression of CD49d, a subunit of VLA-4. These results suggest that bortezomib enhances the effects of conventional anti-myeloma agents by overcoming VLA-4-mediated CAM-DR, and bortezomib-based combination chemotherapy can improve the treatment outcome of patients with MM.

Results

Surface expression of adhesion molecules on MM cells

In an initial effort to identify the molecules responsible for CAM-DR, we screened for the expression of adhesion molecules on MM cells using flow cytometry. By referring to previous studies (Tatsumi *et al*., 1996; Cook *et al*., 1997), we selected the molecules to be checked as follows: CD11a (lymphocyte function-associated antigen-1 (LFA-1)), CD18 (β2-integrin), CD22, CD29 (β1-integrin), CD40, CD44 (homing-associated cell adhesion molecule (HCAM)), CD49d (α4-integrin, a subunit of VLA-4), CD49e (α5-integrin, a subunit of VLA-5), CD54 (intercellular adhesion molecule-1 (ICAM-1)), CD56 (neural cell adhesion molecule (NCAM)), CD138 (syndecan-1) and CD184 (CXC chemokine receptor-4 (CXCR4)). We examined the expression of these molecules in four *bona fide* human MM cell lines (KHM-1B, KMS12-BM, RPMI8226 and U266) and normal plasma cells from healthy volunteers. As shown in Figure 1a, MM cell lines readily expressed CD29, CD44, CD49d, CD54, CD138 and CD184, whereas CD22 was barely detectable. The expression of CD11a, CD18, CD40, CD49e and CD56 was highly variable among cell lines. Normal plasma cells expressed the same set of molecules as MM cell lines except CD22, but their expression levels were generally lower than those of MM cells. It is of note that RPMI8226 showed a slightly different pattern from other cell lines: it expressed CD29, CD44 and CD49d lower but CD49e higher. Overall, we identified CD29, CD44, CD49d, CD54, CD138 and CD184 as major adhesion molecules expressed on MM cell lines.

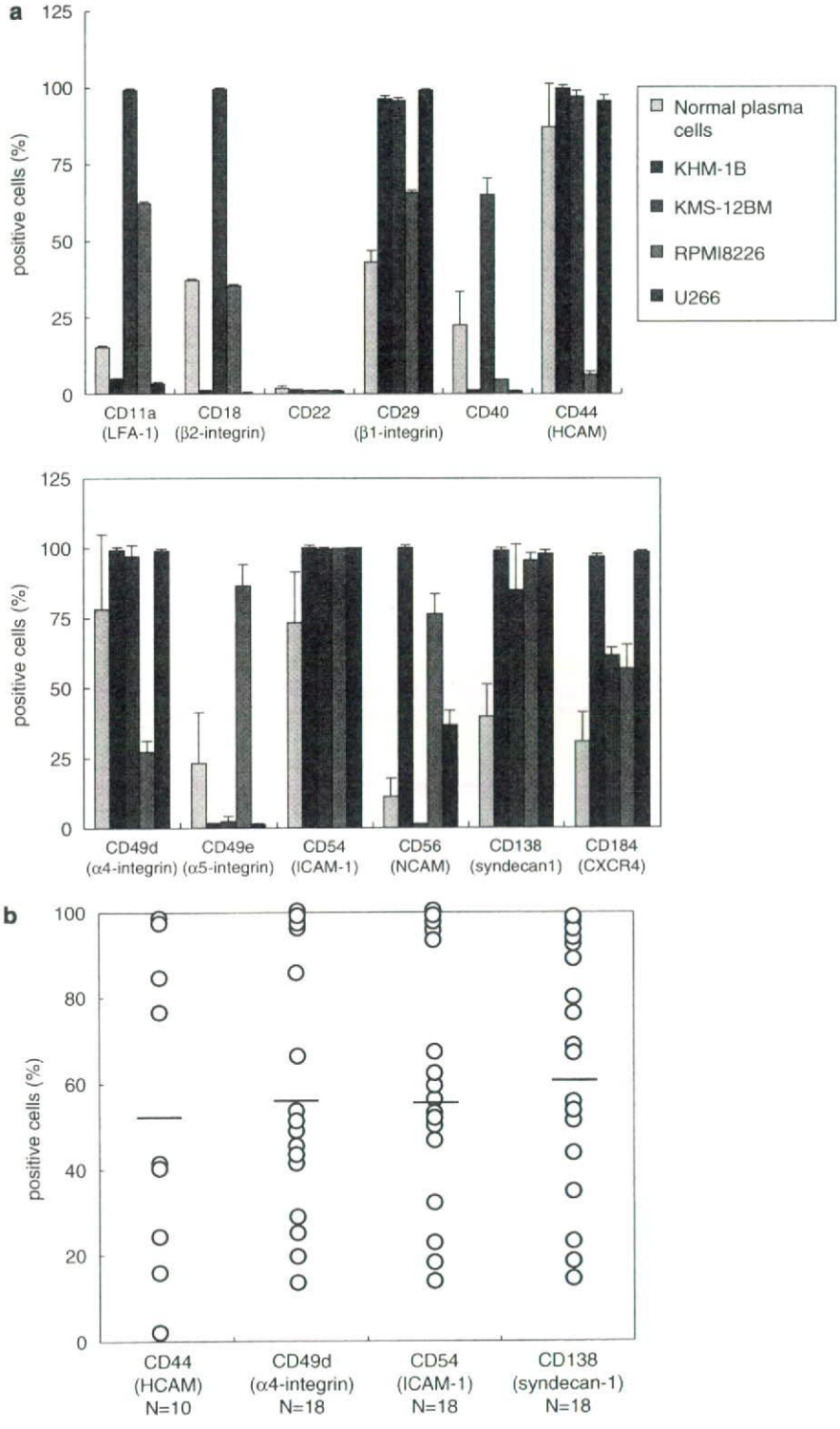
To further elucidate the expression pattern of adhesion molecules in MM, we screened for their expression on primary MM cells. As CD138 is commonly used as a specific marker for myeloma cells in BM specimens, we detected the expression of CD44, CD49d and CD54 in CD138-positive fractions in BM-mononuclear cells (MNCs) from 18 patients with MM by dual staining on flow cytometry. As shown in Figure 1b, CD44, CD49d and CD54 were moderately to markedly expressed in all patients involved in this study. The proportions of positive cells were 52.8 ± 37.7% for CD44, 57.0 ± 31.6% for CD49d and 56.8 ± 30.9% for CD54 in the CD138-positive fractions (CD138 positivity was 60.0 ± 31.0% in the entire fraction). This pattern closely resembled that of the cell lines. On the basis of these results, we focused on CD29 (β1-integrin), CD44 (HCAM), CD49d (α4-integrin), CD54 (ICAM-1), CD138 (syndecan-1) and CD184 (CXCR4) to determine the functional adhesion molecules in MM in further studies.

Establishment of the in vitro *culture system for the assessment of CAM-DR of MM cells*

To investigate the involvement of these adhesion molecules in CAM-DR of MM cells, we established a culture system recapitulating CAM-DR *in vitro*. As described in Materials and methods, green fluorescent protein (GFP)-expressing MM cells were added into culture dishes with (co-culture) or without (stroma free) a preseeded UBE6T-7 stromal cell line, and cultured for 2 days in the absence or presence of four anti-myeloma drugs. We determined the cytotoxic effects of the drugs on MM cells specifically by measuring annexin-V positivity in GFP-positive fractions on flow cytometry. Figure 2a shows the representative results of KMS-12BM cells treated with suboptimal doses of each drug determined in pilot experiments: bortezomib 5 nM, vincristine 1 nM, doxorubicin 100 nM and dexamethasone 50 nM (Supplementary Figure S1). All of them are lower than clinically achievable concentrations *in vivo* according to recent clinical trials (Fisher *et al*., 2006; Davies *et al*., 2007). These drugs were capable of

Figure 1 Surface expression of adhesion molecules on multiple myeloma (MM) cells. (**a**) We screened for surface expression of adhesion molecules on MM cells using four myeloma cell lines (KHM-1B, KMS-12BM, RPMI8226 and U266) and normal plasma cells. Cells were stained with phycoerythrin (PE)-conjugated antibodies against CD11a (LFA-1), CD18 (β2-integrin), CD22, CD29 (β1-integrin), CD40, CD44 (HCAM), CD49d (α4-integrin), CD49e (α5-integrin), CD54 (ICAM-1), CD56 (NCAM), CD138 (syndecan-1), and CD184 (CXCR4), and subjected to flow cytometry. To analyse normal plasma cells, BM-MNCs were triple-stained with allophycocyanine (APC)-conjugated anti-CD38, PE-Cy7-conjugated anti-CD45 and PE-conjugated antibodies against each adhesion molecule. Cells in the $CD38^{+}/CD45^{lo/neg}$ fraction were gated as normal plasma cells. The means ± s.d. (bars) of three independent experiments are shown. (**b**) The expression of adhesion molecules was detected in primary MM cells. BM-MNCs were double-stained with an FITC-conjugated anti-CD138 antibody and PE-conjugated antibodies against CD44, CD49d and CD54. Each circle represents the positivity (%) of CD44, CD49d, and CD54 in the CD138-positive fractions, and that of CD138 in the entire fraction of BM-MNCs of individual patients (N = sample numbers). Bars indicate the average values of each molecule.

inducing apoptosis in more than 20% of KMS-12BM cells under stroma-free condition. In addition, we stained cells with propidium iodide to estimate the contribution of other forms of cell death to the cytotoxicity of these drugs. The percentages of dead cells obtained with propidium iodide staining were almost equal to or slightly higher than those obtained with annexin-V staining, implying that the major form



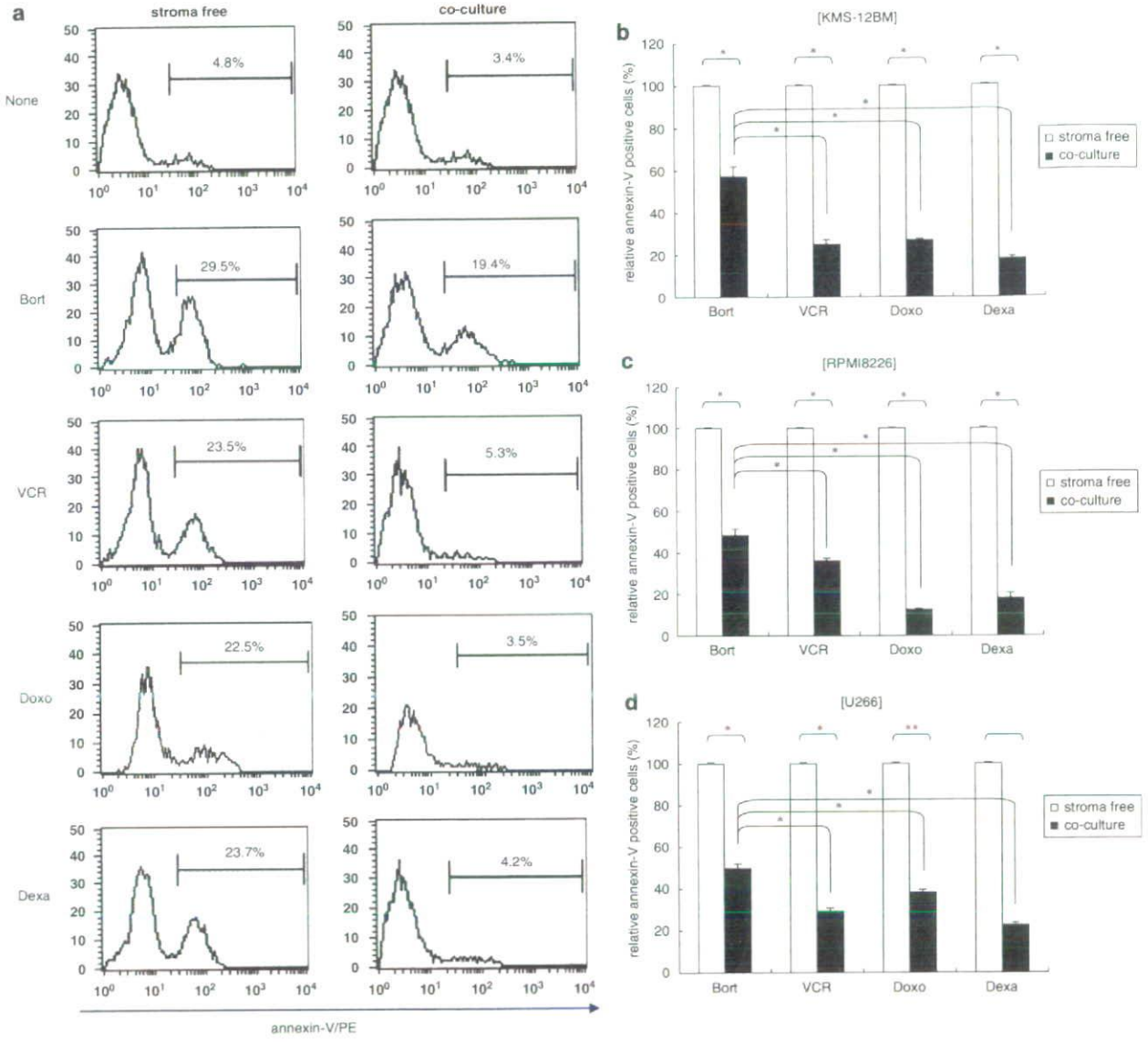


Figure 2 Establishment of the *in vitro* culture system for the assessment of cell adhesion-mediated drug resistance (CAM-DR) of multiple myeloma (MM) cells. (**a**) Green fluorescent protein (GFP)-transduced KMS-12BM cells were treated with either 5 nM bortezomib (Bort), 1 nM vincristine (VCR), 100 nM doxorubicin (Doxo) or 50 nM dexamethasone (Dexa) in the presence (co-culture) or absence (stroma free) of UBE6T-7 stromal cells for 48 h. Cell death/apoptosis was determined by reactivity with phycoerythrin (PE)-conjugated annexin-V (annexin-V/PE) in GFP-positive fractions on flow cytometry. Representative histogram plots are shown. Annexin-V positivity is indicated as a percentage in each histogram. (**b**) The *Y* axis shows the proportion of annexin-V-positive cells under co-culture condition with that under stroma-free condition setting at 100% in KMS-12BM cells treated with each drug. The means ± s.d. (bars) of three independent experiments are shown. The same experiments were carried out in RPMI8226 (**c**) and U266 (**d**). Drug concentrations were 2 nM for bortezomib, 1 nM for vincristine, 100 and 70 nM for doxorubicin, and 50 and 20 nM for dexamethasone in RPMI8226 and U266 cells, respectively. The *P*-values were calculated by Student's *t*-test. $*P<0.05$.

of cell death is apoptosis (Supplementary Figure S2A). The cytotoxic effects were markedly diminished under the co-culture condition, suggesting that CAM-DR was successfully reproduced in our system (Figure 2a; Supplementary Figure S2). DR was not acquired in KMS-12BM cells cultured with stroma cells in trans-wells, which preclude direct cell-to-cell interactions, indicating that direct contact is indispensable for CAM-DR of MM (data not shown). As shown in Figures 2b–d and Supplementary Table S1, CAM-DR was similarly observed in all three myeloma cell lines treated with all four drugs tested, although the extent of CAM-DR was relatively low for bortezomib (discussed later). Furthermore, CAM-DR was reproduced with different concentrations of the drugs (Supplementary Figure S2b). Using this system, we attempted to

determine which adhesion molecule(s) is important for CAM-DR in MM cells.

Reversal of CAM-DR by shRNA/siRNA- and blocking antibody-mediated knockdown of VLA-4 in MM cells

To investigate which adhesion molecule(s) is critical for the acquisition of CAM-DR in MM cells, we performed loss-of-function analyses for CD44 (HCAM), CD49d (α4-integrin), CD54 (ICAM-1), CD138 (syndecan-1) and CD184 (CXCR4) using the shRNA/siRNA lentivirus system (Kikuchi *et al.*, 2007). Because CD29 (β1-integrin) is heterodimerized with CD49d and functions as VLA-4 (α4β1-integrin) on MM cells, we could achieve loss of function of VLA-4 by solely targeting CD49d. As shown in Figure 3a, shRNA/siRNA expression vectors were constructed by inserting chemically synthesized oligonucleotides containing target sequences (Supplementary Table S2) into pLL3.7 vector, and their inhibitory activities were checked in KMS-12BM cells (data not shown). Constructs with the strongest activities were transfected into three MM cell lines along with sh controls, and a specific reduction of target expression was confirmed (Figure 3b; Supplementary Figure S3). Overall, we established 15 sublines in which the expression of individual adhesion molecules was markedly downregulated, and examined the levels of CAM-DR to four anti-myeloma drugs. To quantitatively assess the contribution of each molecule to CAM-DR, we defined the ratio of annexin-V reactivity of GFP-positive cells under the co-culture condition to that under the stroma-free condition as a reversal of CAM-DR. The reversal of CAM-DR to bortezomib was detectable in CD49d-knockdown sublines of all three cell lines, whereas no reversal was observed in sublines carrying shRNA/siRNA against other four adhesion molecules and inactive sh controls (Figure 3c; Supplementary Table S3). In addition, we performed the same experiments using vincristine and dexamethasone in KMS-12BM sublines. The CAM-DR to vincristine and dexamethasone was also reversed by knockdown of CD49d but not other four molecules (Figure 3d; Supplementary Table S4). It is of note that the reversal of CAM-DR to either vincristine or dexamethasone was at the almost equal level to that to bortezomib in CD49d-knockdown sublines (compare Figures 3c and d). In view of the fact that bortezomib is relatively resistant to CAM-DR (see Figure 2 and Supplementary Table S1), this implies that bortezomib modulates the expression of CD49d in MM cells (discussed later).

Furthermore, we confirmed the importance of CD49d in CAM-DR using adhesion-blocking antibodies instead of shRNA/siRNA introduction. We used specific antibodies against CD44, CD49d, CD54 and CD184 to revert CAM-DR, but were not able to test an anti-CD138 antibody because it is not commercially available. MM cells were pretreated with these antibodies, cultured with or without stromal cells in the presence of bortezomib, and subjected to flow cytometric analysis for annexin-V reactivity. As shown in Figure 4 and Supplementary Table S5, significant reversal of CAM-DR was achieved by treatment with anti-CD49d (α4-integrin) and anti-CD54 (ICAM-1) antibodies in KMS-12BM and U266 cells, albeit the effect of the former was much stronger. The effectiveness of anti-CD54 may stem from its effects on stromal cells, because previous studies revealed that CD54 was expressed on BM stromal cells (Corso *et al.*, 2005). In contrast, the other antibodies failed to revert CAM-DR in KMS-12BM and U266 cells. Unexpectedly, CAM-DR was not affected by any antibodies in RPMI8226 cells, probably due to the relatively low expression of CD49d. Although there was slight discrepancy between the results obtained with shRNA/siRNA and adhesion-blocking antibodies, our data clearly indicate that VLA-4, a heterodimer of CD49d and CD29, is the most important adhesion molecule for CAM-DR in MM cells.

Downregulation of CD49d expression by bortezomib

It is tempting to speculate that bortezomib modulates the expression of CD49d in MM cells from our two findings: the relative resistance of bortezomib to CAM-DR (see Figure 2) and equal reversal of CAM-DR to all three drugs on disruption of VLA-4 signaling (see Figure 3). In support of this view, Duechler *et al.* (2005) reported that bortezomib decreased the surface expression of CD23 in chronic lymphocytic leukemia. Therefore, we investigated the effect of bortezomib and other anti-myeloma drugs on the expression of adhesion molecules on MM cell lines. Figure 5a and Supplementary Figure S4 show the representative data of flow cytometric analysis of viable KMS-12BM cells before and after treatment with bortezomib. Untreated KMS-12BM cells strongly expressed CD29, CD44, CD49d, CD54, CD138 and CD184. Bortezomib did not affect the expression levels of CD29, CD44, CD54, CD138 and CD184, but decreased the expression of CD49d from 99.4 ± 0.1 to $34.5 \pm 0.9\%$ ($n=3$, $P<0.05$). Bortezomib-induced downregulation of CD49d expression was similarly observed in RPMI8226 and U266 cells: from 33.2 ± 0.6 to $12.4 \pm 1.9\%$ in RPMI8226 cells ($n=3$, $P<0.05$) and from 99.4 ± 0.04 to $74.3 \pm 4.1\%$ in U266 cells ($n=3$, $P<0.05$). Furthermore, we confirmed bortezomib-mediated reduction of CD49d expression by immunoblotting using whole-cell lysates (Figure 5b), semiquantitative reverse transcription (RT)–PCR (Figure 5c) and real-time quantitative RT–PCR (Figure 5d), suggesting that this phenomenon takes place at mRNA levels. In striking contrast, other anti-myeloma drugs, such as vincristine, doxorubicin and dexamethasone, did not affect the expression of CD49d (Figure 5e; Supplementary Figure S4) and other adhesion molecules (Supplementary Figure S5) in any cell lines examined. The specific reduction of CD49d expression by bortezomib may underlie the relative resistance of the drug to CAM-DR in MM cells.

Next, we investigated the mechanisms by which bortezomib suppresses the expression of CD49d mRNA. For this purpose, we directly inhibited NF-κB activity in KMS-12BM cells using SN-50 peptide, which interferes with nuclear translocation of p50 by binding to its nuclear localization sequence (Lin *et al.*, 1995). Surface expression of CD49d was not affected by the

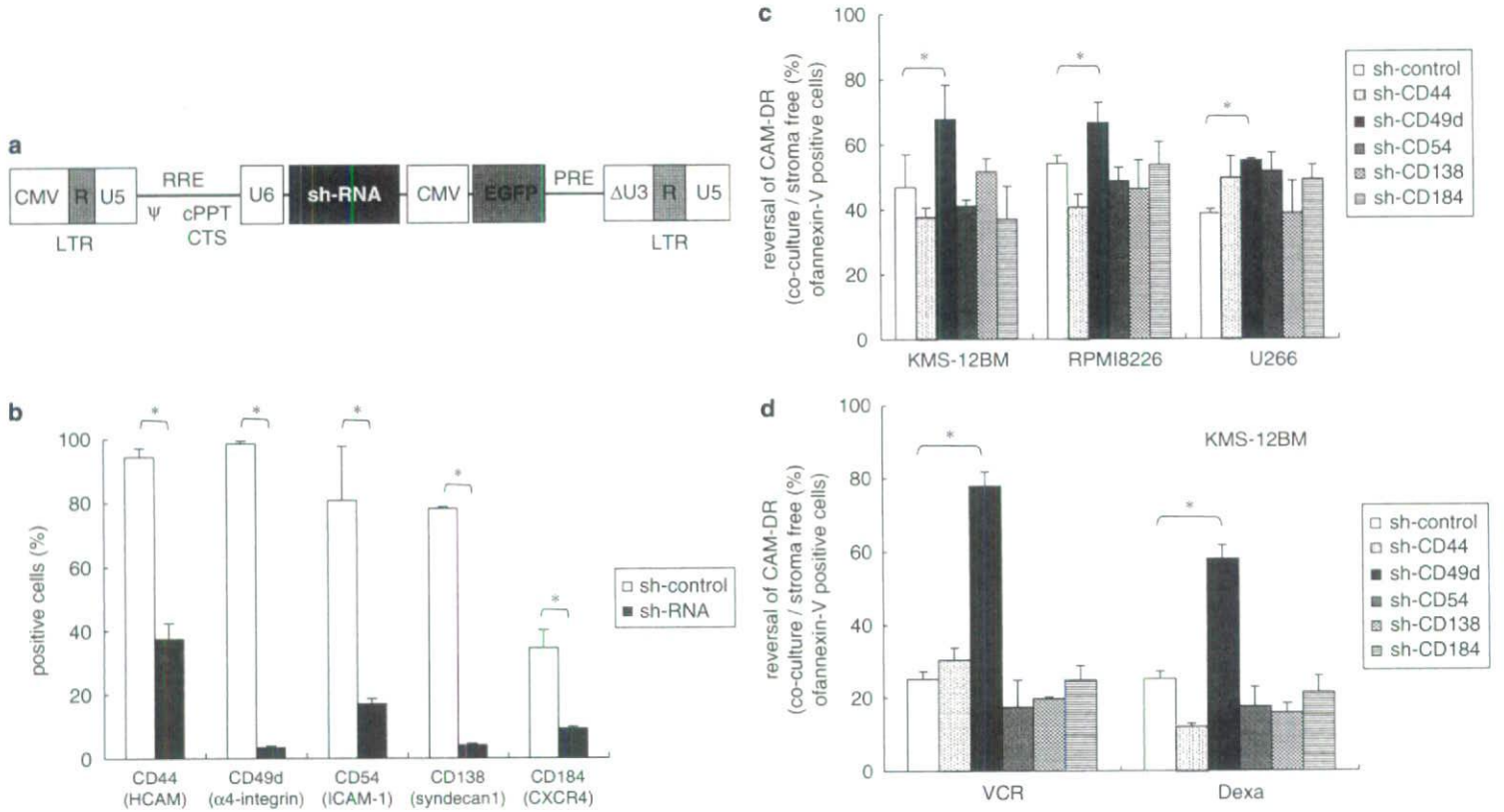


Figure 3 Effects of short hairpin RNA (shRNA)-mediated knockdown of adhesion molecules on adhesion-mediated drug resistance (CAM-DR) in multiple myeloma (MM) cells. (**a**) Schematic representation of a pLL3.7 lentiviral shRNA expression vector: U5 and U6 indicate U5 and U6 promoters, respectively; EGFP, enhanced green fluorescent protein; ψ, a packaging signal; RRE, rev-responsive element; cPPT, central polypurine tract; CTS, central termination sequence; CMV, cytomegalovirus promoter; PRE, wood-chuck hepatitis virus post-transcriptional regulatory element and LTR, long terminal repeat. See Materials and methods for details of construction. (**b**) KMS-12BM cells were transfected with either pLL3.7-sh-CD44, sh-CD49d, sh-CD54, sh-CD138, sh-CD184 or sh control vector. GFP-positive cells were collected by FACSaria flow cytometer, and stained with phycoerythrin (PE)-conjugated anti-CD44, anti-CD49d, anti-CD54, anti-CD138 and anti-CD184 antibodies, or PE-conjugated mouse and rat IgG isotype-matched controls. The means ± s.d. (bars) of three independent experiments are shown. The *P*-values were calculated by Student's *t*-test. $^*P<0.05$ against the sh control. (**c**) MM cell lines stably transfected with shRNA vectors were cultured with 2 nM bortezomib in the presence (co-culture) or absence (stroma free) of stromal cells. After 48 h, MM cells were harvested by pipetting, stained with annexin-V/PE, and subjected to flow cytometric analysis. The *Y* axis shows the reversal of CAM-DR as a ratio (%) of annexin-V positivity under co-culture vs stroma-free conditions. When annexin-V reactivity under co-culture condition is equal to that of stroma-free condition, the reversal of CAM-DR is 100%. The means ± s.d. (bars) of three independent experiments are shown. The *P*-values were calculated by Student's *t*-test. $^*P<0.05$ against the sh control. (**d**) KMS-12BM sublines stably transfected with shRNA vectors were cultured with 1 nM vincristine (VCR) or 50 nM dexamethasone (Dexa) in the presence (co-culture) or absence (stroma free) of stromal cells. After 48 h, the reversal of CAM-DR was examined as described above. $^*P<0.05$ against the sh control.

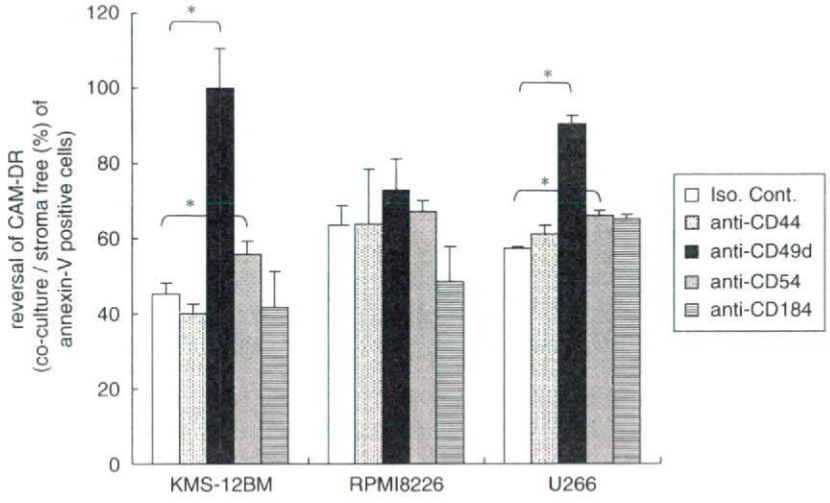


Figure 4 Effects of blocking antibodies against adhesion molecules on adhesion-mediated drug resistance (CAM-DR) in multiple myeloma (MM) cells. MM cell lines were treated with either antibodies against CD44, CD49d, CD54 and CD184 or isotype-matched controls (iso. cont.) at 10 μg/ml at 37 °C for 1 h. After treatment, cells were cultured with 2 nM bortezomib in the presence (co-culture) or absence (stroma free) of stromal cells for 48 h. The reversal of CAM-DR was determined as described in the legend of Figure 3. The means ± s.d. (bars) of three independent experiments are shown. The *P*-values were calculated by Student's *t*-test. $^*P<0.05$ against isotype-matched controls.

p50 inhibitory peptide, suggesting that bortezomib-mediated downregulation of CD49d is not a direct consequence of the inhibition of NF-κB activity (Supplementary Figure S6).

The reversal of CAM-DR to conventional anti-myeloma drugs by pretreatment with bortezomib

Given that bortezomib decreases the expression of CD49d, which plays a crucial role in CAM-DR, pretreatment of MM cells with bortezomib could overcome CAM-DR to conventional anti-myeloma drugs. Finally, we tested this hypothesis using the co-culture system for CAM-DR. MM cells were pretreated with bortezomib for 24 h, followed by exposure to either vincristine or dexamethasone for additional 24 h in the presence or absence of stroma cells. Pretreatment with bortezomib significantly reversed CAM-DR to both vincristine and dexamethasone in all three cell lines tested (Figure 6a), which coincided with the detachment of myeloma cells (Figure 6b). In particular, CAM-DR to VCR was almost completely inhibited in KMS-12BM and RPMI8226 cells: reversal ratios were 87.4 ± 3.2 and 104.6 ± 19.4%, respectively. In U266 cells, the effects of bortezomib were moderate but significant. This may be attributable to the relatively weak effects of bortezomib on CD49d expression in U266 cells (see Figure 5d). It should be emphasized that the combination of bortezomib with either vincristine or dexamethasone at the doses used in this experiment did not show additive effects under stroma-free condition (data not shown).

Discussion

In this study, we have clearly demonstrated that VLA-4, a heterodimer of CD49d/CD29, plays a critical role in CAM-DR of MM using a unique strategy involving myeloma cell lines in which individual adhesion molecules were stably knocked down by the aid of shRNA. In support of our finding, several studies described the roles of VLA-4 in the pathophysiology of MM. For example, CD29-mediated adhesion of MM cells to fibronectin upregulated the expression of the CDK inhibitor p27 and induced NF-κB activation, both of which confer CAM-DR to MM (Chauhan *et al.*, 1996; Hazlehurst *et al.*, 2000; Landowski *et al.*, 2003). Two independent groups reported that administration of anti-CD49d antibody suppressed the growth of MM cells in murine xenograft models (Mori *et al.*, 2004; Olson *et al.*, 2005). In line with these experimental findings, Schmidmaier *et al.* (2006) found that MM patients with primary multidrug resistance showed significantly higher concentrations of serum VLA-4 and ICAM-1 than responders. The involvement of VLA-4 in CAM-DR was also demonstrated in AML by the seminal study of Matsunaga *et al.* (2003), in which the combination of cytosine arabinoside and anti-CD49d antibody achieved a 100% survival rate in mice transplanted with AML cells. These findings strongly suggest that VLA-4-mediated signaling is important for the development of DR in MM and AML cells both *in vitro* and *in vivo*.

In addition, we obtained evidence indicating that bortezomib can overcome CAM-DR by selectively downregulating CD49d expression in myeloma cells. This ability was specific for bortezomib and was not observed in other commonly used anti-myeloma drugs such as vincristine, doxorubicin and dexamethasone. Moreover, we have found that bortezomib represses the expression of CD49d at mRNA levels. Regarding the mechanisms of this phenomenon, the direct involvement of NF-κB is unlikely because the p50 inhibitory peptide

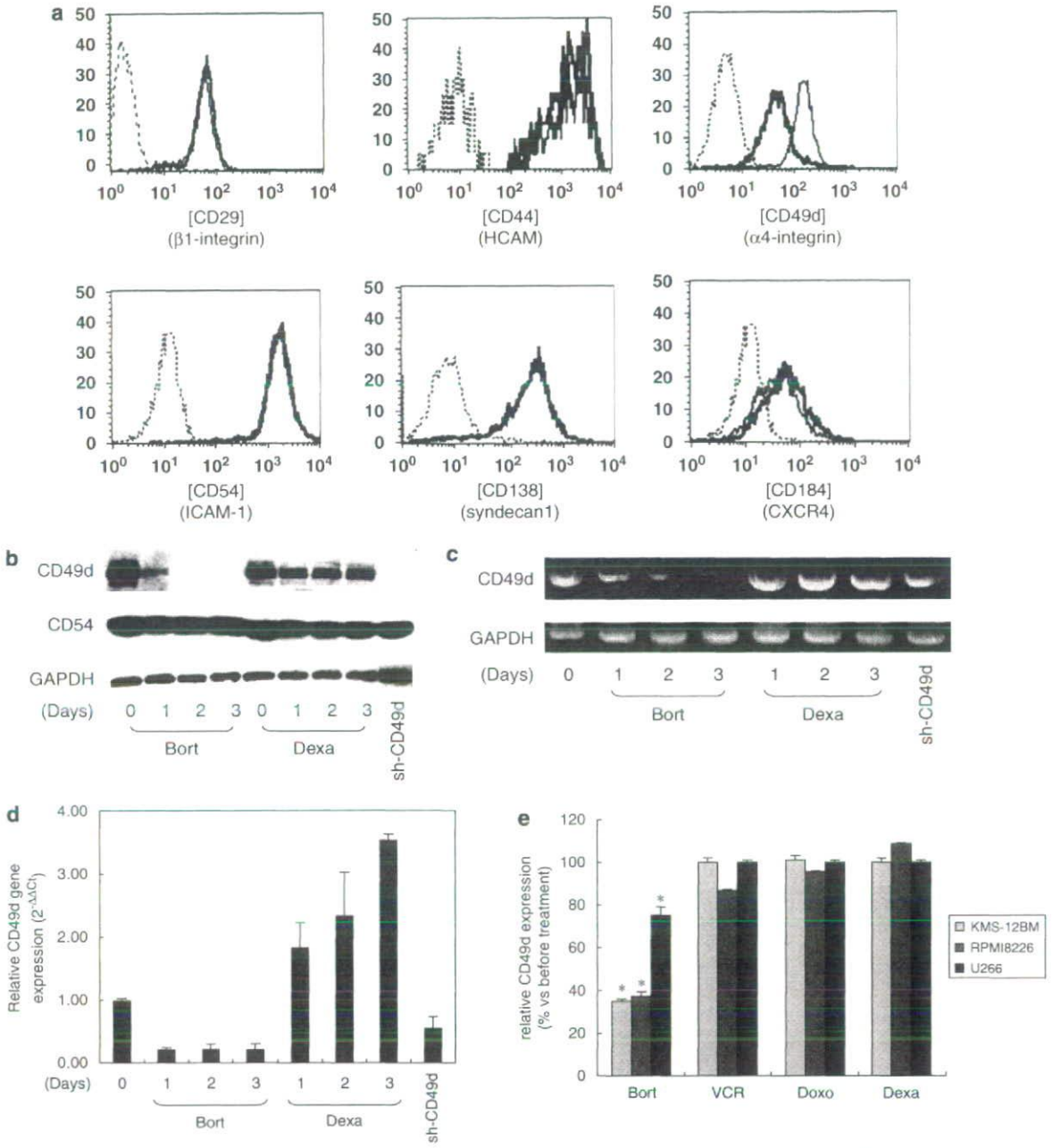


Figure 5 Effects of anti-myeloma drugs on the expression of adhesion molecules in multiple myeloma (MM) cells. (**a**) Surface expression of CD29, CD44, CD49d, CD54, CD138 and CD184 was detected by flow cytometry on KMS-12BM cells before and after treatment with 5 nM bortezomib for 48 h. Thin lines, bold lines and dotted lines show plots before treatment, after treatment and of isotype-matched controls, respectively. Representative histograms of three independent experiments are shown. (**b**) KMS12-BM cells were treated with either 5 nM bortezomib (Bort) or 50 nM dexamethasone (Dexa) for up to 3 days. We used a KMS12-BM subline transfected with pLL3.7-sh-CD49d (sh-CD49d) as a control. Cells were harvested at the indicated time points, and subjected to immunoblot analysis for the expression of CD49d, CD54 and GAPDH (loading control). (**c**) Total cellular RNA was isolated simultaneously at the experiments described in (**b**), and subjected to semiquantitative reverse transcription (RT)-PCR for the expression of CD49d and GAPDH (loading control). (**d**) Total cellular RNA was isolated simultaneously at the experiments described in (**b**), and subjected to real-time quantitative reverse transcription (RT)-PCR. The expression of CD49d was normalized to that of GAPDH and quantified by the $2^{-\Delta\Delta Ct}$ method. (**e**) The expression of CD49d was detected before and after bortezomib treatment in KMS-12BM, RPMI8226 and U266 MM cells by flow cytometry. The concentrations of bortezomib were 5, 2 and 2 nM for KMS-12BM, RPMI8226 and U266 cells, respectively. Data shown are the means ± s.d. (bars) of relative CD49d expression (ratio (%) of CD49d positivity after vs before treatment) of three independent experiments. The *P*-values were calculated by Student's *t*-test. $^*P<0.05$ against the values obtained before treatment.

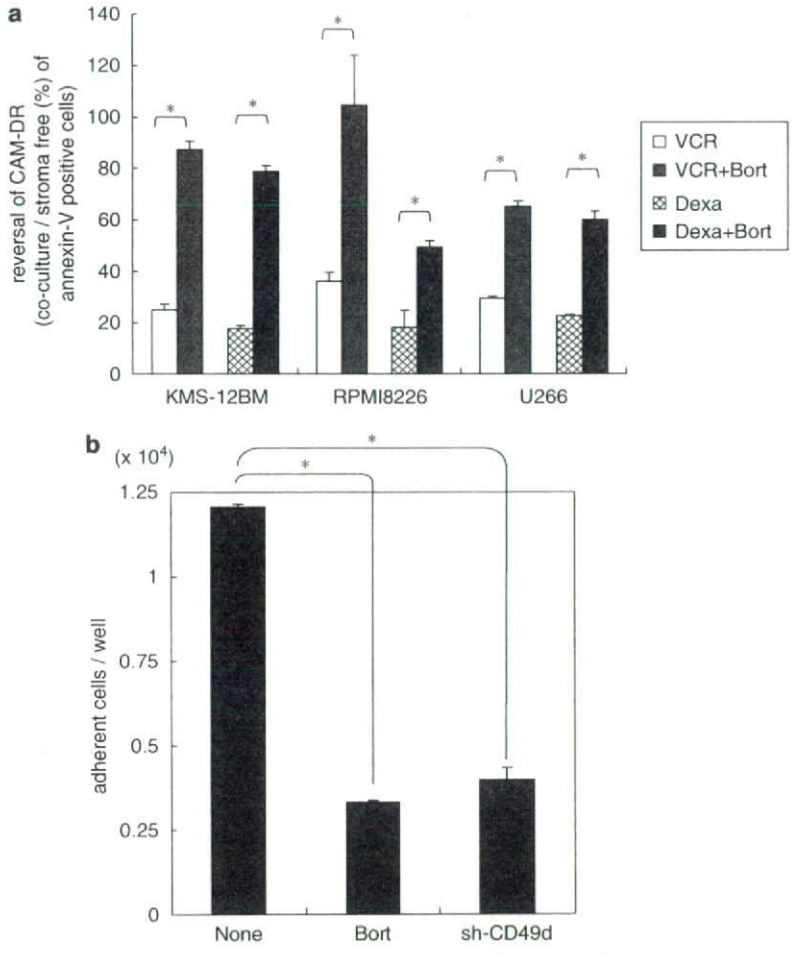


Figure 6 Bortezomib overcomes adhesion-mediated drug resistance (CAM-DR) to vincristine and dexamethasone in multiple myeloma (MM) cells. (**a**) Three myeloma cell lines were treated with 2 nM bortezomib for 24 h. After washing, we cultured cells with either vincristine (Bort + VCR) or dexamethasone (Bort + Dexa) in the absence or presence of stromal cells for additional 24 h. As controls, we carried out the same experiments without pretreatment of bortezomib (VCR and Dexa). The reversal of CAM-DR was determined as described in the legend of Figure 3. (**b**) Green fluorescent protein (GFP)-transduced KMS12-BM cells were cultured in the absence (None) or presence (Bort) of 5 nM bortezomib for 3 days. We used a KMS12-BM subline transfected with pLL3.7-sh-CD49d (sh-CD49d) as a control. Cells were then co-cultured with UBE6T-7 cells at 37 °C for 15 min. Non-adherent cells were washed off with phosphate-buffered saline (PBS), and resultant adherent cells were harvested with trypsin-EDTA. We determined GFP positivity (%) in non-adherent cells using flow cytometer. The means ± s.d. (bars) of three independent experiments are shown. The *P*-values were calculated by Student's *t*-test (*$P<0.05$).

did not downregulate the expression of CD49d in KMS-12BM cells, in which NF-κB is constitutively activated through the classical pathway (Keats *et al.*, 2007). The CD49d promoter contains canonical binding sites for Sp1, Ets and WT1 (Rosen *et al.*, 1994; Zutter *et al.*, 1997; Kirschner *et al.*, 2006). We are currently investigating whether these factors are implicated in bortezomib-mediated downregulation of CD49d mRNA expression.

The reversal of CAM-DR by bortezomib may underlie its broad range of synergy with other anticancer drugs as recently described (Horton *et al.*, 2006; Noborio-Hatano *et al.*, 2007). Unfortunately, severe pulmonary complications have been reported in Japanese patients treated with bortezomib (Miyakoshi *et al.*, 2006). Dose reduction by drug combination is one method to minimize side effects of bortezomib. Furthermore, it has been shown that the contact of MM cells with BM stromal cells through VLA-4/vascular cell adhesion molecule-1 (VCAM-1) interactions enhanced the production of osteoclastogenesis factors and that disruption of this cell-to-cell contact suppressed the development of osteoclastic osteolysis associated with MM (Michigami *et al.*, 2000; Pearse *et al.*, 2001). As bortezomib can suppress CD49d expression, this drug may also be effective for the amelioration of bone lesions in MM.

Although our findings provide rationale for safe and effective treatment strategies for refractory myeloma, further investigation is required to define suitable combinations involving not only conventional anti-myeloma drugs but also new drugs such as lenalidomide.

Previous studies revealed that both bortezomib and dexamethasone induced apoptosis primarily through caspase-9, whereas lenalidomide did so through caspase-8 (Mitsiades *et al.*, 2002). Indeed, we found that caspase-8 was not activated in MM cells by the drugs used in this study (data not shown). On the basis of these findings, the combination of lenalidomide with either bortezomib or dexamethasone is expected to trigger dual apoptotic signaling pathways and reverse DR, which results in the achievement of clinical responses in patients resistant to single agents. In support of this view, we have reported that bortezomib is effective for myeloma patients refractory to thalidomide therapy (Takatoku *et al.*, 2004). The combination of bortezomib with other anti-myeloma drugs, including thalidomide and its derivatives, is effective for overcoming CAM-DR and may improve the prognosis of MM patients.

Materials and methods

Cells and cell culture

We used four *bone fide* human MM cell lines, KHM-1B, KMS-12BM, RPMI8226 and U266, in this study (Drexler *et al.*, 2003). These cell lines were purchased from the Health Science Research Resources Bank (Osaka, Japan), and maintained in RPMI1640 medium (Sigma Co., St Louis, MO, USA) supplemented with 10% heat-inactivated fetal calf serum (Sigma Co.,) and antibiotics. Human BM-derived stromal cell line UBE6T-7 (Mori *et al.*, 2005), which was transduced with a human telomerase catalytic protein subunit, was kindly provided by Dr Akihiro Umezawa (National Research Institute for Child Health and Development, Tokyo, Japan), and used as stromal cells in co-culture experiments. Primary MM cells were isolated from the BM of patients at the time of diagnostic procedure. CD38-positive/CD45-low or negative ($CD38^+/CD45^{lo/neg}$) cells were purified from the BM of healthy volunteers, and used as normal plasma cells. Informed consent was obtained in accordance with the Declaration of Helsinki, and the protocol was approved by the Institutional Review Board.

Drugs and adhesion-blocking antibodies

Bortezomib (Velcade) was provided by Millennium Pharmaceuticals (Cambridge, MA, USA). We used vincristine (Shionogi Co. Ltd., Tokyo, Japan), doxorubicin (Meiji Co. Ltd., Tokyo, Japan) and dexamethasone (Sigma Co.) as conventional anti-myeloma drugs. All drugs were dissolved in RPMI1640 medium at appropriate concentrations and stored at −80 °C until use. We used the following adhesion-blocking antibodies in co-culture experiments: anti-CD44 (clone Hermes-1; Endogen, Rockford, IL, USA), anti-CD49d (2B4; R&D Systems, Minneapolis, MN, USA), anti-CD54 (Ab-2; Labvision Corp., Fremont, CA, USA), anti-CD184 (44717.111; R&D Systems), and mouse or rat IgG isotype controls (eBioscience, San Diego, CA, USA). Blocking experiments were performed as described earlier. In brief, 1×10^6 of MM cells were incubated with 10 μg/ml antibodies or isotype-matched controls in 100 μl of RPMI1640 medium for 1 h, then diluted to 2×10^5 cells/ml in complete medium, and added into culture wells preseeded with stromal cells (Matsunaga *et al.*, 2003).

Flow cytometric analysis of adhesion molecules

Cells were stained with specific monoclonal antibodies and analysed using BD-LSR or FACSaria flow cytometer (Becton Dickinson, Oxford, UK) as described earlier (Kikuchi *et al.*, 2005). The antibodies used were phycoerythrin (PE)-conjugated antibodies against CD11a (clone HI 111; BioLegend, San Diego, CA, USA), CD29 (4B4; Beckman Coulter, Fullerton, CA, USA), CD18 (6.7), CD22 (HIB22), CD29 (HUTS-21), CD49e (IIA1), CD56 (B159), CD138 (MI15) and CD184 (12G5) (all from Becton Dickinson), CD40 (5C3), CD44 (G44-26), CD49d (9F10) and CD54 (HA58) (all from eBioscience), allophycocyanine-conjugated antibodies against CD38 (HB7; Becton Dickinson) and PE-Cy7-conjugated antibodies against CD45 (2D1; Becton Dickinson).

Cell proliferation assay

Cell proliferation was monitored using Cell Counting Kit-8 (Dojin Chemical, Tokyo, Japan). In brief, cells were seeded in 96-well flat-bottomed microplates at a density of 5×10^4 per well and incubated with or without anti-myeloma drugs for 48 h at 37 °C. After incubation, 10 μl of WST-8 solution was added to each well at a final concentration of 1 μg/ml. Cells grown in complete medium alone were used as controls. After incubation at 37 °C for 4 h, absorbance was measured at a wavelength of 450 nm using a microplate reader.

Co-culture of MM cells with stromal cells and the assessment of cell death

For co-culture experiments, we established GFP-expressing MM cell lines by transfecting GFP expression plasmids to distinguish MM cells from stromal cells. UBE6T-7 stromal cells were seeded in 48-well plates to near confluence. After stromal cells were washed once with fresh medium, GFP-transduced MM cells were added to the plates and cultured in the absence or presence of anti-myeloma drugs. After 48 h, the cells were harvested by pipetting, washed with phosphate-buffered saline, and stained with PE-conjugated annexin-V (annexin-V/PE) (Biovision, Mountain View, CA, USA). Cell death/apoptosis was judged by annexin-V reactivity in GFP-positive populations using flow cytometer (Yanamandra *et al.*, 2006).

Construction and production of lentiviral shRNA/siRNA expression vectors

We used a lentiviral shRNA/siRNA expression vector pLL3.7. for knockdown of adhesion molecules. siRNA target sequences were designed to be homologous to wild-type cDNA sequences. Oligonucleotides were chemically synthesized, annealed, terminally phosphorylated and inserted into pLL3.7 vector. Oligonucleotides containing siRNA target sequences were shown in Supplementary Table S1. Ineffective sequences were used as sh controls. These vectors were co-transfected into 293FT cells with packaging plasmids (purchased from Invitrogen, Carlsbad, CA, USA). Infectious lentiviruses in culture supernatants were harvested, concentrated and infected as described earlier (Kikuchi *et al.*, 2007). Lentiviruses were then added into cell suspensions in the presence of 8 μg/ml polybrene, and transduced for 24 h. Transduction efficiencies were monitored by GFP expression using a flow cytometer.

Immunoblotting

Immunoblotting was carried out according to the standard method using the following antibodies: anti-CD49d (Novus Biologicals, Littleton, CO, USA), anti-CD54 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-caspase-8, anti-caspase-9 (Becton Dickinson) and anti-GAPDH (Santa Cruz Biotechnology) (Odgerel *et al.*, 2008).

PCR

We performed semiquantitative RT–PCR and real-time quantitative RT–PCR to estimate the expression of CD49d at mRNA levels. For the former, PCR amplification was carried out with 1 μl of cDNA solution (corresponding to 50 ng total RNA) in a 50-μl reaction mixture containing 5 U of *Taq* polymerase, 10 mM Tris-HCl (pH 8.5), 50 mM KCl, 1.5 mM $MgCl_2$ and 100 mM dNTPs in the presence of specific primer pairs (200 nM each) as follows; CD49d, forward: 5′-GGATGT GAACAGAAAGGCAGA-3′, reverse: 5′-GCCAGTGTTGA TAACATGGAAA-3′; GAPDH (internal control), forward: 5′-CCACCCATGGCAAATTCCATGGCA-3′, reverse: 5′-TCTAGACGGCAGGTCAGGTCCACC-3′. PCR products were resolved on 2% agarose gels, and visualized by staining with ethidium bromide. The results of 40 amplification cycles are shown. For the latter, we used the same primer pairs in the SYBR Green PCR system (Applied Biosystems, Foster City, CA, USA). Data quantification was carried out by the $2^{-\Delta\Delta C_t}$ method (Pfaffl, 2001).

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ORIGINAL ARTICLE

A phase II trial of weekly chemotherapy with paclitaxel plus gemcitabine as a first-line treatment in advanced non-small-cell lung cancer

Kiyoshi Mori · Hiroyuki Kobayashi · Yukari Kamiyama · Yasuhiko Kano · Tetsuro Kodama

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Abstract
Purpose The efficacy and toxicity of combined paclitaxel (PTX) and gemcitabine (GEM) was evaluated as a protocol for first-line chemotherapy in 40 patients with advanced non-small-cell lung cancer (NSCLC).
Methods Paclitaxel, 100 mg/m^2, was administered intravenously (IV) as a 1-h infusion, followed by GEM, 1,000 mg/m^2, IV over 30 min on days 1 and 8 of a 21-day cycle. The median age of patients was 66 years with a range of 33–75 years. Nearly all patients (39/40) had an ECOG performance status of 0 or 1. Thirteen patients (32%) had initial stage IIIB disease and 27 patients (68%) had stage IV disease. Histological subtypes were adenocarcinoma (73%) and squamous cell carcinoma (25%).
Results Twenty-two patients (55%) achieved a partial response and none achieved a complete response, giving an overall response rate of 55% (95% confidence interval: 38.2–71.8%). Disease stability was achieved in 14 patients (35%), and 4 patients (10%) had progressive disease. The median survival time was 11.9 months (95% CI: 10.3–14 months), with a 1-year survival rate of 47.5%. Grade 3 or 4 hematological toxicities observed included neutropenia in 37.5%, anemia in 2.5%, and thrombocytopenia in 5.0% of these patients. Non-hematologic toxicities were mild, with the exception of grade 3 and 4 pneumonitis. There were no deaths due to toxicity.
Conclusion Weekly chemotherapy with PTX plus GEM is effective and is acceptable for the first line treatment of advanced NSCLC.

Keywords Non-small-cell lung cancer · First-line chemotherapy · Weekly chemotherapy · Gemcitabine · Paclitaxel

K. Mori (✉) · H. Kobayashi · Y. Kamiyama · Y. Kano · T. Kodama
Division of Thoracic Oncology,
Department of Medical Oncology, Tochigi Cancer Center,
4-9-13, Yohnan, Utsunomiya, Tochigi 320-0834, Japan
e-mail: kmori@tcc.pref.tochigi.jp

H. Kobayashi
e-mail: kobahiro@jichi.ac.jp

Y. Kamiyama
e-mail: ykamiyam@tcc.pref.tochigi.jp

Y. Kano
e-mail: ykano@tcc.pref.tochigi.jp

T. Kodama
e-mail: tkodama@tcc.pref.tochigi.jp

Introduction

Lung cancer ranks among the most commonly occurring malignancies and currently is the leading cause of cancer-related deaths worldwide [21]. In Japan lung cancer is responsible for approximately 55,000 cancer-related deaths per year [5]. Even though the clinical usefulness of first-line chemotherapy has been established for the cases of advanced non-small-cell lung cancer (NSCLC), the prognosis is still extremely poor.

A number of new agents have become available recently for the treatment of unresectable and metastatic NSCLC in Japan, including the taxanes, gemcitabine (GEM), and vinorelbine. In randomized phase III trials, these agents in combination with a platinum compound have been associated with improved survival of patients having advanced NSCLC [8, 17, 23, 24]. However, a platinum compound is associated with a greater toxicity than other drugs used to treat NSCLC. In addition to nausea and vomiting, it causes neuropathy, profound fatigue, and renal toxicity. Some

patients are unable to tolerate the drug toxicity and terminate treatment early. Based on these observations, non-platinum regimens have been proposed as an alternative to the platinum-based combinations for treatment of advanced NSCLC [13].

Paclitaxel (PTX) and GEM are new anti-cancer agents having significant single-agent activity against advanced NSCLC. A recent clinical phase II study of 122 patients with previously untreated, unresectable stage III or IV NSCLC receiving a 3-h infusion of PTX at a dose of 210 mg/m^2 showed a good response rate of 35% [25]. Although PTX is usually given once every 3 weeks, Chan et al. [10] demonstrated that weekly administration of PTX at a dose of 80–90 mg/m^2 provides similar tolerability and a possible increase in efficacy.

Gemcitabine, a novel deoxycytidine analog, had a response rate of 20% with a single weekly administration in previously untreated advanced NSCLC [4]. As a first-line treatment, single-agent GEM has been shown to have anti-tumor activity equal to that of cisplatin/etoposide, resulting in less toxicity and a slightly better quality of life [27].

These agents have different mechanisms of action, and their toxicities are partially non-overlapping. Although the usual administration of PTX is once every 3 weeks, a weekly administration can increase efficacy with good tolerability [1, 2]. We demonstrated that weekly administration with PTX and GEM is a tolerable and active regimen for patients with advanced NSCLC previously treated with platinum-containing chemotherapy regimens [20]. Based on these findings, we designed a phase II trial to examine the efficacy and tolerance of the non-platinum-based combination of PTX and GEM administered weekly for patients with untreated advanced NSCLC.

Patients and methods

Patient selection

All patients with histologically or cytologically confirmed advanced NSCLC were eligible for this phase II trial. The subjects of this study were patients with clinical stage IV NSCLC or stage III with unresectable disease or for whom radiotherapy with curative intent is not possible. Patients with unresectable disease or radiotherapy with curative intent is not possible include those with pleural effusion and dissemination, those with intrapulmonary metastasis within the ipsilateral lobe, those with an irradiation field exceeding one-half of one lung, those with metastasis to the contralateral hilar lymph nodes, and those with reduced lung function. Other eligibility criteria included: age older than 20 years and younger than 76 years; Eastern Cooperative Oncology Group (ECOG) performance status (PS) of 0–2; measurable lesions; life expectancy $\geq$12 weeks; adequate bone marrow reserve with a WBC count $\geq$4,000 per mm^3; platelet count $\geq 10 \times 10^4$ per mm^3; and hemoglobin level $\geq$9.0 g/dL; liver function with a AST and ALT $\leq 2.5 \times$ upper normal limit, unless as a result of liver metastases; and adequate renal function with a serum creatinine level $\leq$1.5 mg/dL. No prior radiotherapy treatment was allowed if the irradiated area was not the site of measurable lesion and the therapy was completed at least 2 weeks before enrollment into the study.

Patients were excluded for the following indications: $\geq$76 years of age (vinorelbine as single agent treatment), severe cardiovascular or cerebrovascular disease, uncontrolled diabetes or hypertension, active infection, pulmonary fibrosis, massive pleural effusion or ascites, active peptic ulcer, and severe neurological disorders. Patients were also excluded in case of previous malignancy and any evidence or history of hypersensitivity or other contraindications for the drugs used in this trial. Written informed consent was obtained from all patients.

Treatment

Paclitaxel, 100 mg/m^2, was administered IV during a 1-h infusion, followed by GEM, 1,000 mg/m^2, IV over 30 min on days 1 and 8 of 21-day cycle. Premedication for PTX consisted of dexamethasone 20 mg, diphenhydramine 50 mg, and ranitidine 50 mg IV for 30 min before PTX infusion. After the premedication for PTX was completed, a serotonin receptor antagonist was given as a 30-min infusion for prophylactic antiemetic therapy. Treatment was repeated every 3 weeks until maximum response plus two cycles or unacceptable toxicity. In stable disease, patients received a maximum of six cycles. At the investigator's discretion, patients were treated with up to eight cycles of the drug combination.

Dose modifications were planned according to hematologic and severe non-hematologic toxic effects. Once the doses were reduced, they were not increased. Patients who experienced grade 4 neutropenia, grade 4 thrombocytopenia, reversible grade 2 neurotoxicity, or liver dysfunction received reduced doses of both PTX, 75 mg/m^2, and GEM, 800 mg/m^2, for the next cycle. The next course of chemotherapy was started after 3 weeks when the leukocyte count was 3,000 per mm^3 or greater, the neutrophil count was 1,500 per mm^3 or greater, the platelet count was 75,000 per mm^3 or greater, serum creatinine was less than 1.5 mg/dL, GOT and GPT were less than twice the upper limit of the normal range, and the neurotoxicity was grade 1 or less. If hematologic recovery was not achieved by day 35 of treatment, the patient was withdrawn from the study.

Evaluation of responses and toxicity

Responses and toxicity were evaluated on the basis of tumor images obtained by computerized tomography (CT), laboratory results, subjective/objective symptoms, signs before, during, and after administration of the study drugs and during the period from completion of treatment to the final analysis. Measurable disease parameters were determined every 4 weeks by various means such as CT. Evaluation was performed in compliance with the response evaluation criteria in solid tumors (RECIST) guidelines for anti-tumor activity. Adverse events were assessed using the Common Terminology Criteria for Adverse Events version 3.0 (CTCAEv3.0). Patients were withdrawn from the study if evidence of tumor progression was observed. The institutional ethical review committee gave approval to the study.

Statistical analysis

The primary end point of the study was the response rate. Simon's two-stage design was used to determine sample size and decision criteria. It was assumed that a response rate of 40% in eligible patients would indicate potential usefulness, while a rate of 20% would be the lower limit of interest; $\alpha = 0.05$ and $\beta = 0.10$. Using these design parameters, the first stage of the study was to enroll 24 patients, and the regimen was rejected if fewer than five patients had an objective response. If six or more patients responded, the accrual was continued until 45 patients were enrolled (45 patients were required because of anticipated percentage of dropout cases). Combination therapy was considered effective if ≥ 14 of the 45 patients showed a response in the final analysis. Secondary end points were toxicity and overall survival. Response and survival rates were both calculated on an intent-to-treat basis. Overall survival and time to progression were measured from the start of this treatment until time of death or the date of the last follow-up clinical assessment. Survival curves were constructed using the Kaplan–Meier method (Fig. 1).

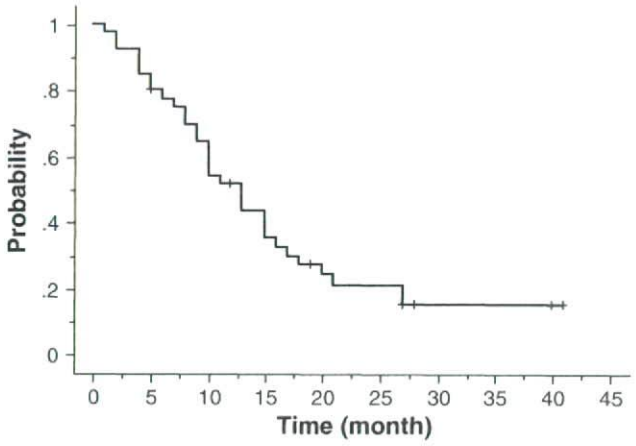

Fig. 1 Kaplan–Meier estimated overall survival curves. Median survival time, 11.9 months; 1-year survival rate, 47.5%

Results

Patient characteristics

A total of 40 patients were enrolled in the study between September 2001 and July 2004. The majority of patients were treated as outpatients. The clinical characteristics of the patients are listed in Table 1. The median age was 66 years with a range of 33–75 years. Nearly two-thirds of the patients were men. Twenty-four patients had an PS of 0, and 15 had PS of 1. Histological subtypes were 73% (29/40) adenocarcinoma and 25% (10/40) squamous cell carcinoma.

Table 1 Patient characteristics

Eligible patients	40
Gender	
Male	26
Female	14
Age (years)	
Median	66
Range	33–75
Performance status	
0	24
1	15
2	1
Histology	
Adenocarcinoma	29
Squamous cell	10
Large cell	1
Stage	
III	13
IV	27
Number of metastatic sites	
Median	2
Range	0–3
Location of metastases	
Bone	12
Lung nodules	10
Liver	9
Lymph nodes	8
Adrenals	6
Brain	3
Subcutaneous	1