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ORIGINAL ARTICLE

Lenalidomide plus dexamethasone treatment in Japanese patients with relapsed/refractory multiple myeloma

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Abstract We conducted a multicenter, open-label study to investigate the safety, efficacy, and pharmacokinetics of lenalidomide in Japanese patients with relapsed or refractory multiple myeloma The study was composed of the "monotherapy phase", a dose-escalation phase, to determine the tolerability to single agent lenalidomide and the "combination phase" to determine the safety and obtain preliminary data on the efficacy of lenalidomide plus dexamethasone. The primary end points were the tolerability to 25 mg lenalidomide and safety. Nine and six patients were enrolled in the monotherapy phase and the combination phase, respectively. Since 25 mg of monotherapy treatment did not satisfy the DLT criteria, this dose was employed in the combination phase. The major adverse event was myelosuppression. At the planned interim analysis (median study duration, 26.3 weeks), grade 3 or grade 4 neutropenia was observed with high frequency (66.7%). However, all adverse events observed were clinically manageable. In the combination cohort, the overall response rate (≥PR) was 100%. The pharmacokinetics of lenalidomide showed rapid absorption and elimination after both single and multiple doses. In conclusion, 25 mg of lenalidomide was given safely as a single agent or in combination with dexamethasone in Japanese patients. The good efficacy of the combination therapy was also demonstrated in this study.

Keywords Multiple myeloma · Lenalidomide · Dexamethasone · Pharmacokinetics

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1 Introduction

Lenalidomide is one of the immunomodulatory drugs (IMiD® brand drugs) developed by Celgene Corporation. Two phase III studies, MM-009 and MM-010, designed to compare lenalidomide plus high-dose dexamethasone combination therapy (LD therapy) with dexamethasone monotherapy (D therapy) in previously treated patients with multiple myeloma (MM) were conducted in US/Canada and Europe/Israel/Australia, respectively [1, 2]. In these studies, 25 mg of lenalidomide was administrated at days 1–21 of a 28-day cycle; 40 mg of dexamethasone was co-administrated with lenalidomide on days 1–4, 9–12, and 17–20 for the first 4 cycles, and on days 1–4 after the 4th cycle. Superiority of the LD regimen was demonstrated based on the following significant differences from D therapy [3]: overall response rate (≥PR) of 60.6% (vs. 21.9% for D therapy), time to progression (TTP) of 13.4 months (vs. 4.6 months), progression-free survival (PFS) of 11.1 months (vs. 4.6 months) and overall survival period of 38 months (vs. 31.6 months). With regard to the safety, the adverse events (AEs) of LD therapy were mainly related to bone marrow suppression, e.g., neutropenia, and all AEs were manageable by supportive care, dose reduction, or interruption of lenalidomide. These data led to approval of lenalidomide as a treatment in combination with dexamethasone for patients with MM who had been treated previously with at least one therapeutic regimen by the US Food and Drug Administration (FDA) in 2006, the European Medicines Evaluation Agency (EMEA) in 2007, and by regulatory agencies on many other countries. The combination therapy is recommended by the clinical practice guidelines of the National Comprehensive Cancer Network (NCCN) as a salvage therapy (category 1) for relapsed or refractory MM [4]. The combination therapy is also category 1 primary therapy for newly diagnosed MM.

Lenalidomide has not yet been approved in Japan. No data are available regarding the safety, efficacy, or pharmacokinetics of LD therapy in Japanese patients. This study was a multicenter, non-randomized, and open-label study to examine the safety, efficacy, and pharmacokinetics of lenalidomide as a single agent or in combination with dexamethasone in Japanese patients with relapsed/refractory MM. The study also examined the pharmacokinetics of higher dose dexamethasone (40 mg), which has not been previously reported.

2 Patients and methods

2.1 Patients

Patients who fulfilled the following inclusion criteria were enrolled in the study: Japanese MM patients aged 20 years or older who were previously treated with at least one prior therapy for myeloma and evaluated to have progressive disease (PD)/disease progression during or after the prior treatment; serum M protein ≥0.5 g/dL or urinary M protein (as measured in a 24-h urine sample) ≥0.2 g; Eastern Cooperative Oncology Group (ECOG) performance status of 0, 1, or 2. Patients were excluded from the study if they met any of the following exclusion criteria: patients with acute myocardial infarction within the past 6 months, or patients with a history of deep venous thrombosis (DVT) or pulmonary embolism within the past 3 years; pregnant or lactating females; absolute neutrophil count of less than 1,000/μL (1.0×10^9/L); platelet count of less than 75,000/μL (75×10^9/L); serum creatinine level of over 2.5 mg/dL.

2.2 Study design

The study design is based on the previously conducted MM-009/010 studies. Figure 1 illustrates the outline of the study design. The primary end points were the tolerability of 25 mg of single agent lenalidomide and the safety of lenalidomide given alone or in combination with dexamethasone in Japanese patients. The secondary end points

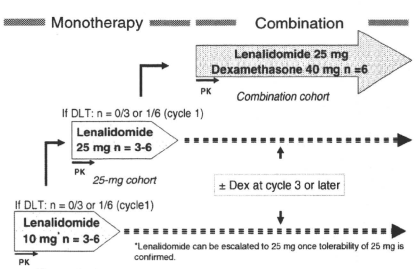

Fig. 1 Study design. This study was composed of the monotherapy phase and the combination phase. In the monotherapy phase, the standard "3 + 3" design of phase I study was employed to determine the safety of single agent lenalidomide. After confirming the tolerability of 25 mg dose, a combination cohort was initiated. In the combination cohort, 6 patients were enrolled. In the 10- or 25-mg monotherapy cohort, patients received 10 or 25 mg lenalidomide once daily, respectively, for 1–21 days a 28-day cycle. In the combination cohort, in addition to the lenalidomide administration, 40 mg of dexamethasone was co-administered with lenalidomide on days 1–4, 9–12, and 17–20 for the first 4 cycles and only on days 1–4 after the 4th cycle. Interim analysis was performed after all patients completed 24 weeks at least after the initiation of the study. Patients who participated in the 10- or 25-mg cohort were allowed to receive 40 mg of dexamethasone and 25 mg of lenalidomide from cycle 3 or later. Pharmacokinetic analysis was performed in cycle 1 in each cohort. Dexamethasone or lenalidomide was not administered on day 1 or 2, respectively, in cycle 1 in the combination cohort to evaluate plasma concentration of each drug when administered alone

were pharmacokinetics and efficacy (response rate, duration of response, and PFS). AEs were reported according to MedDRA Ver. 10. The grade of the AEs was evaluated according to the Common Terminology Criteria for Adverse Events, Ver. 3.0 (NCI-CTCAE). The relationship of AEs to drug was based on the investigators' assessment. Efficacy was evaluated according to the modified EBMT/IBMTR/ABMTR criteria [1, 2, 5].

The study consisted of two phases: the "monotherapy phase" during which patients received lenalidomide alone, followed by the "combination phase" during which lenalidomide was administered in combination with dexamethasone (Fig. 1). A treatment cycle with both phases consisted of 28 days. During each cycle, lenalidomide was administered orally once daily from days 1 to 21, followed by 7 days off therapy. In the monotherapy phase, patients were treated in the "3 + 3" design of phase I study at two different dose levels. DLT (dose limiting toxicity) evaluation was conducted at the end of cycle 1. In this study, DLT was defined as ≥grade 4 neutropenia or thrombocytopenia, or ≥grade 3 febrile neutropenia or non-hematological AEs. The initial dose level tested was 10 mg (10-mg cohort). If no DLTs occurred in 3 patients, a next cohort at 25 mg of lenalidomide was tested. If a DLT occurred in 1 of 3 patients, an additional 3 patients were enrolled; if incidence of DLT resulted in 1 of 6 patients, a 25-mg cohort was enrolled. As with the 10-mg cohort, if DLT did not occur in 3 patients or occurred in 1 of 6 patients in the 25-mg cohort, the 25 mg dose was used for future studies. After the safety of 25 mg of single agent lenalidomide was confirmed, a combination phase with 6 new patients was investigated for the safety and efficacy of LD treatment. In the combination phase, lenalidomide was administered at 25 mg from days 1 to 21 of each cycle. Dexamethasone at a strength of 40 mg (ten 4-mg tablets) was administered orally in combination with lenalidomide once daily from days 1 to 4, 9 to 12, and 17 to 20 for the first 4 cycles, and from days 1 to 4 after cycle 4. Treatment could be continued for up to 156 weeks (3 years) at the investigators' discretion. Patients who participated in the monotherapy phase were allowed to receive 40 mg of dexamethasone and a higher dose (25 mg) of lenalidomide from cycle 3 or later to evaluate the safety of long-term treatment, once the safety of 25 mg dose of lenalidomide was determined.

Interim analysis was performed after all patients completed at least 24 weeks of treatment. Administration of DVT prophylaxis was recommended for the patients who received combination treatment of lenalidomide and dexamethasone. Administration of G-CSF was permitted for treatment of neutropenia.

Dose reduction or dose interruption (temporary suspension of the treatment) of the study drugs due to the study drug-related adverse events was permitted. In accordance with the principles of Good Clinical Practice (GCP), the study protocol was approved by IRB of each institution, and written informed consent was obtained from each patient enrolled in the study.

2.3 Pharmacokinetics

Pharmacokinetic analysis was performed in cycle 1 in each cohort. In the 10- and 25-mg cohorts in the monotherapy phase, plasma concentration of lenalidomide following a single administration or multiple administrations was determined on day 1 or 12, respectively. In the combination phase, to determine the PK of lenalidomide and dexamethasone given alone and in combination, dexamethasone was held on day 1 to determine the plasma concentration of lenalidomide administered alone (day 1). On day 2, dexamethasone was administered but lenalidomide held, so as to determine the plasma concentration of dexamethasone administered alone. On day 12, plasma concentrations of both lenalidomide and dexamethasone were determined with the two drugs given concurrently. Drugs were administrated under fasting conditions.

Blood samples for lenalidomide were collected before and 0.5, 1, 1.5, 2, 4, 6, 9, 12, and 24 h after the drug administration. Blood samples for dexamethasone were collected before and 0.5, 1, 1.5, 2, 3, 4, 6, 9, 12, and 24 h after the drug administration. The concentrations of *R* and *S*-lenalidomide in plasma were determined by chiral liquid chromatography–tandem mass spectrometry (LC–MS/MS). The concentration of dexamethasone in plasma was determined by LC–MS/MS.

3 Results

3.1 Patient characteristics

A total of 15 patients were enrolled from July 2007 to August 2008. During the monotherapy phase, 3 patients were enrolled in the 10-mg cohort and 6 patients in the 25-mg cohort. After completion of the monotherapy phase, an additional 6 patients were enrolled in the combination cohort. The characteristics of the 15 patients are shown in Table 1. The median age of the patients was 64.0 years (range 43.0–81.0 years), and the median time from initial diagnosis to the screening was 2.0 years (range 0.8–7.6 years). Thirteen of the 15 patients had received more than or equal to 2 prior regimens for MM and 11 patients had previous autologous stem cell transplant (ASCT). Types of myeloma included 6 patients of IgG, 5 of IgA, 2 of IgD, 1 of Bence-Jones protein (BJP)-kappa, and 1 of BJP-lambda type. In patients whose cytogenetic data by fluorescence in

Table 1 Patient characteristics

Characteristic	10 mg ($n = 3$)	25 mg ($n = 6$)	Combo ($n = 6$)	Total ($n = 15$)
Age (years)				
Median	64.0	64.5	64.0	64.0
Range	54.0–68.0	43.0–76.0	47.0–81.0	43.0–81.0
Female:male (n)	1:2	2:4	2:4	5:10
Time since MM diagnosis (years)				
Median	4.4	2.1	1.9	2.0
Range	1.4–7.6	0.8–4.8	1.4–4.1	0.8–7.6
No. of previous therapies, n (%)				
1	0	2 (33.3)	0	2 (13.3)
≥2	3 (100)	4 (66.7)	6 (100)	13 (86.7)
Type of therapy, n (%)				
Thalidomide	1 (33.3)	1 (16.7)	1 (16.7)	3 (20.0)
Bortezomib	0	0	1 (16.7)	1 (6.7)
ASCT	2 (66.7)	4 (66.7)	5 (83.3)	11 (73.3)
Cytogenetic abnormality by FISH				
t(4;14)	1	2	1	4
t(11;14)	0	1	2	3
del(13q)	1	4	3	8

ASCT autologous stem cell transplant

situ hybridization (FISH) were available, t(4:14) (p16;q32) was detected in 4 of 9 patients and del(13q) (13S319) in 8 of 11 patients. Del(17p) was detected in none of the 7 patients.

The median study duration at the data cutoff was 48.3 (range 41.1–51.1), 24.0 (12.0–36.1), and 25.3 (22.7–28.0) weeks in the 10-mg, 25-mg, and the combination cohorts, respectively. It was 26.3 (12.0–51.1) weeks for all cohorts. Two patients had discontinued the study due to AE ($n = 1$) or PD ($n = 1$) in the 25-mg cohort, while none of the patients in the 10-mg or the combination cohort had discontinued the study at the time of data cutoff.

3.2 Safety in the monotherapy phase

Grade 3 or higher lenalidomide-related AEs reported during cycle 1 in the 10-mg cohort were anemia (grade 4, $n = 1$), leukopenia (grade 3, $n = 1$), lymphopenia (grade 3, $n = 1$), and neutropenia (grade 3, $n = 1$). Since none of the AEs corresponded to DLT, 6 patients were additionally enrolled to receive a higher dose of 25 mg.

≥Grade 3 AEs related to lenalidomide during cycle 1 in the 25-mg cohort were leukopenia (grade 3, $n = 1$), neutropenia (grade 3, $n = 1$), lymphopenia (grade 3, $n = 1$), and hypoxia (grade 3, $n = 1$). The grade 3 hypoxia corresponded to DLT. Since only one of the 6 patients in the 25-mg cohort developed DLT, the dose of lenalidomide to be used in Japanese patients with MM was set at 25 mg, as specified in the study protocol. The safety data of the 25 mg dose was reviewed and recommended for the combination cohort by the Independent Data Monitoring Committee. Patients who experienced DLT discontinued the study at cycle 1.

3.3 Safety

All patients who received at least one dose of lenalidomide ($n = 15$) were included in the safety evaluation. Grade 3 or 4 lenalidomide-related AEs were reported in 11 of 15 patients at the data cutoff (Table 2). The major AE was myelosuppression. Neutropenia ($n = 10$) was reported with the highest frequency. None of the patients in the combination cohort had any grade 4 AEs. Regarding neutropenia, median duration of grade 3 or 4 neutropenia was 15 days (range 3–29 days). Median frequency of grade 3 or 4 neutropenia per patient was 1.5 (range 1–5). To manage neutropenia, lenalidomide were interrupted in 2 patients. None of the patients experienced drug reduction due to neutropenia. As dexamethasone-related AEs, hyperglycemia (grade 3, $n = 2$), and osteomyelitis (grade 3, $n = 1$), which was considered equivalent to a recurrence of osteonecrosis of the jaw (ONJ), were reported. The patient who experienced ONJ had a history of bisphosphonate treatment. Peripheral neuropathy, DVT, pulmonary embolism, or thrombosis was not reported in any cohort. Seven of the 12 patients in whom lenalidomide and dexamethasone were administered received low-dose aspirin to prevent DVT.

None of the patients in the 25-mg or combination cohort experienced dose reduction of lenalidomide. Eight of the 15 patients, including the 3 in the 10-mg cohort, 3 in the

Table 2 Lenalidomide-related adverse events with NCI-CTCAE grade 3 or 4 ($n = 15$)

Events	Grade 3	Grade 4	Grade 3 + 4, *n* (%)
Patients with at least one ≤grade 3 adverse event	–	2 (13.3)	11 (73.3)
Anemia	3 (20.0)		2 (13.3)
Leukopenia	3 (20.0)[a]	–	3 (20.0)
Lymphopenia	9 (60.0)	1 (6.7)	3 (20.0)
Neutropenia	1 (6.7)	–	10 (66.7)
Hypoxia	1 (6.7)	–	1 (6.7)
Malaise[b]	1 (6.7)	–	1 (6.7)
Hepatic function abnormality[b]	1 (6.7)	–	1 (6.7)
Decreased blood phosphorus	1 (6.7)	–	1 (6.7)
Increased alanine aminotransferase[b]			1 (6.7)

A subject with multiple occurrence of an adverse event is counted once

[a] In 3 cases of lymphopenia, one was reported as a lenalidomide and/or dexamethasone-related AE

[b] Lenalidomide and/or dexamethasone-related AEs

25-mg cohort and 2 in the combination cohort experienced dose interruption of lenalidomide due to lenalidomide-related AEs including, anemia, neutropenia, malaise, pyrexia, and rash that developed in 2 patients each.

One patient in each cohort experienced lenalidomide-related serious AEs (SAEs) including malaise (grade 2) and pyrexia (grade 1) in the 10-mg cohort, hypoxia (grade 3) and interstitial pneumonia (grade 2) in the 25-mg cohort, and hepatic function abnormality (grade 3) in the combination cohort. None of the SAEs induced study discontinuation except interstitial pneumonia, which might have triggered the hypoxia, a DLT. All lenalidomide-related AEs observed in this study had been previously reported. Moreover, all the AEs were manageable by supportive care or dose interruption/reduction of study drugs.

3.4 Efficacy

All 6 patients in the combination cohort achieved PR at the data cutoff. The overall response rate (ORR), defined as partial or complete response, was 100%. Within 2 months of the interim analysis, CR was achieved in 2 of the 6 patients (33%) in the combination cohort. Median time to response, the time from study start to PR entry, was 4.1 weeks (range 4.0–4.3 weeks) in the combination cohort. Median response duration and progression-free survival (PFS) as determined by the Kaplan–Meier method were not estimable at the time of the interim analysis. The response was continued in 5 of the 6 patients in the combination cohort at the time of data cutoff. Patients in the combination cohort who had a history of prior bortezomib or thalidomide therapy achieved PR at the time of the interim analysis.

3.5 Pharmacokinetics

Mean plasma lenalidomide concentrations following single and multiple doses of 10 or 25 mg lenalidomide are shown in Fig. 2a. Mean plasma lenalidomide concentrations when administered alone (day 1) or in combination with dexamethasone (day 12) are shown in Fig. 2b. The plasma pharmacokinetic parameters of lenalidomide are listed in Table 3.

The profile of the plasma lenalidomide concentration over time was similar between days 1 and 12 following administration of the drug alone at 10 or 25 mg: The plasma concentration of lenalidomide reached a peak at approximately 1 h postdose and levels of lenalidomide declined rapidly in a monophasic manner at both dose levels and on both days. The C_{max} and AUC increased in a dose-dependent manner. Mean accumulation ratios between days 1 and 12 for C_{max} (AR [C_{max}]) or AUC_τ (AR [AUC_τ]) were nearly 1.00, suggesting no drug accumulation following multiple doses of lenalidomide. There were also no meaningful differences in the V_z/F, CL/F, or $t_{1/2}$ between the two dose levels as well as between days 1 and 12.

After oral administration of 25 mg lenalidomide in combination with dexamethasone (day 12), plasma lenalidomide C_{max} was observed at 1 h later than that observed after administration of lenalidomide alone (day 1). A higher variation in the t_{max} value was observed after co-administration of lenalidomide with dexamethasone (t_{max} = 0.53–4.02 h) compared to administration of lenalidomide alone (t_{max} = 1–1.97 h). A higher intersubject variability in C_{max} was also observed on day 12 (CV% = 46.1% on day 12 and 27.1% on day 1). There were no marked differences in $t_{1/2}$ when lenalidomide was administered with or without dexamethasone. The AUC_τ was slightly reduced from days 1 to 12. The accumulation ratio of day 12 to day 1 for the C_{max} and AUC_τ (AR [C_{max}] and AR [AUC_τ]) was 0.914 and 0.868, respectively. These observations indicate a modest change in lenalidomide oral absorption when co-administered with large quantities of dexamethasone tablets (4 mg × 10), which is not considered clinically relevant since other PK parameters were almost the same between days 1 and 12.

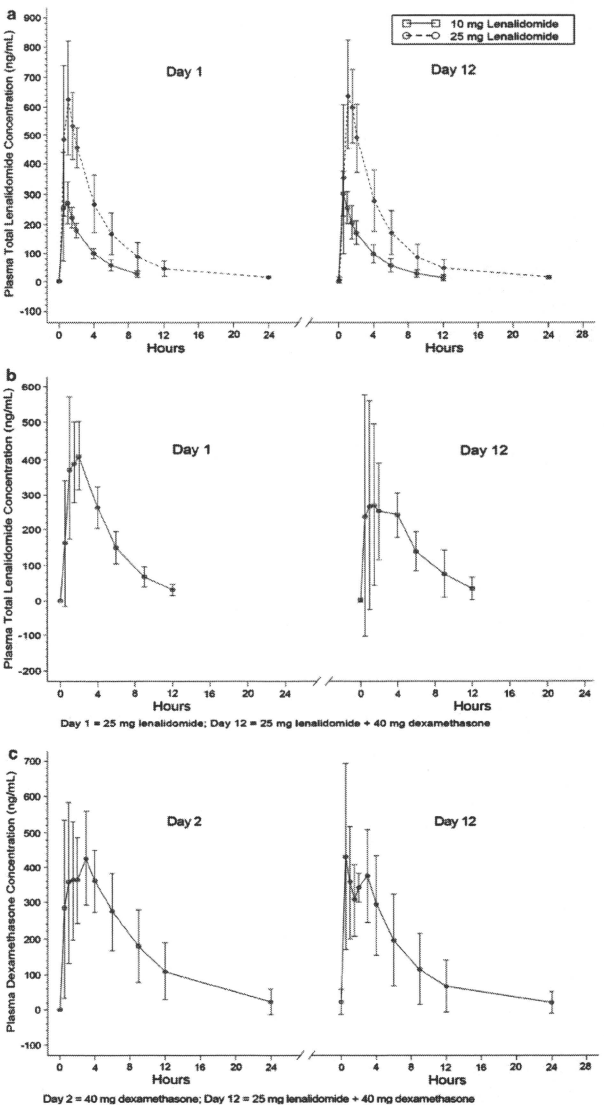

Fig. 2 **a** Mean (±SD) plasma lenalidomide concentrations versus time on days 1 and 12 (monotherapy cohorts). Plasma lenalidomide concentrations following single (day 1) and multiple doses (day 12) of 10 mg ($n = 3$) or 25 mg ($n = 6$) lenalidomide. Data points at 24 h in 10 mg lenalidomide are missing because of BLQ (below the limit of quantitation; 5 ng/mL). **b** Mean (±SD) plasma lenalidomide concentrations versus time on days 1 and 12 (combination cohort). Plasma lenalidomide concentrations when administered alone (day 1, $n = 6$) and in combination with dexamethasone (day 12, $n = 6$). Data points at 24 h are missing because of BLQ. **c** Mean (±SD) plasma dexamethasone concentrations versus time on days 2 and 12 (combination cohort). Plasma dexamethasone concentration when administered alone (day 2, $n = 6$) and in combination with lenalidomide (day 12, $n = 6$)

Table 3 Plasma lenalidomide pharmacokinetic parameters when administered alone or in combination with dexamethasone

Parameters	10-mg cohort		25-mg cohort		Combination cohort	
	Day 1 (single dose)	Day 12 (multiple dose)	Day 1 (single dose)	Day 12 (multiple dose)	Day 1 (single dose) Len	Day 12 (multiple dose) Len + Dex
Number of patients	3	3	3	6	6	6
t_{max} (h)[a]	0.93	0.50	1.01	0.97	1.70	2.76
	(0.50, 1.00)	(0.45, 1.00)	(0.43, 2.00)	(0.45, 1.47)	(1.00, 1.97)	(0.53, 4.02)
C_{max} (ng/mL)	315 (39.5)	311 (22.5)	622 (29.3)	714 (15.2)	474 (27.1)	433 (46.1)
AUC_t (ng h/mL)	962 (29.8)	973 (26.5)	2600 (39.0)	2687 (34.6)	2063 (10.9)	1817 (20.1)
AUC_τ (ng h/mL)	1034 (30.2)	1024 (27.6)	2686 (36.3)	2773 (32.1)	2177 (12.6)	1890 (17.4)
AUC_∞ (ng h/mL)	1037 (30.5)	NA	2710 (37.2)	NA	2183 (12.8)	NA
$t_{1/2}$ (h)	2.52 (24.3)	2.43 (16.5)	3.11 (25.8)	3.14 (30.6)	2.56 (14.0)	2.55 (23.0)
CL/F (mL/min)	161 (30.5)	163 (27.6)	154 (37.2)	150 (32.1)	191 (12.8)	221 (17.4)
V_z/F (L)	35.0 (17.4)	34.2 (10.8)	41.4 (18.8)	40.8 (17.8)	42.2 (11.9)	48.7 (17.2)
AR (C_{max})	NA	0.988 (27.6)	NA	1.15 (24.0)	NA	0.914 (25.1)
AR (AUC_τ)	NA	0.991 (13.8)	NA	1.03 (9.6)	NA	0.868 (7.59)

Geometric mean (CV%)

AR accumulation ratio, *Dex* dexamethasone, *Len* lenalidomide, *NA* not applicable

[a] Median (minimum–maximum)

Table 4 Plasma dexamethasone pharmacokinetic parameters when administered alone or in combination with lenalidomide

Parameters	Combination cohort	
	Day 2 (single dose) Dex	Day 12 (multiple dose) Dex + Len
Number of patients	6	6
t_{max} (h)[a]	2.49 (1.00, 4.00)	1.75 (0.47, 3.07)
C_{max} (ng/mL)	499 (33.0)	523 (33.9)
AUC_t (ng h/mL)	3528 (38.4)	2633 (44.5)
AUC_τ (ng h/mL)	3526 (38.3)	2687 (43.0)
AUC_∞ (ng h/mL)	3661 (43.8)	NA
$t_{1/2}$ (h)	4.24 (31.9)	3.85 (32.3)
CL/F (mL/min)	182 (43.8)	248 (43.0)
V_z/F (L)	66.9 (23.3)	82.7 (21.3)
AR (C_{max})	NA	1.05 (18.7)
AR (AUC_τ)	NA	0.762 (16.2)

Geometric mean (CV%)

AR accumulation ratio, *Dex* dexamethasone, *Len* lenalidomide, *NA* not applicable

[a] Median (minimum–maximum)

Mean plasma dexamethasone concentrations are demonstrated in Fig. 2c. Table 4 summarizes the plasma pharmacokinetic parameters for dexamethasone when administered alone (day 2) and in combination with lenalidomide (day 12). The mean C_{max} and median t_{max} values were comparable between days 2 and 12, indicating that the oral absorption rate of dexamethasone was not altered significantly after multiple doses. Mean AUC_τ was 24% lower (3526 ng h/mL on day 2 to 2687 ng h/mL on day 12), while mean CL/F was 36% higher on day 12 compared to day 2 (182 mL/min on day 2 to 248 mL/min on day 12). There were no considerable differences in $t_{1/2}$ when dexamethasone was administered with or without lenalidomide.

4 Discussion

In this study, the 25 mg dose of lenalidomide, which was the dose used in the pivotal phase III studies MM-009/010 of lenalidomide conducted outside of Japan, was found to be safe in Japanese patients with relapsed/refractory MM. Safety data in this study were also comparable with those reported in clinical studies conducted previously. The most common AE was neutropenia in this study. Ten of 15 patients (67%) experienced grade 3 or 4 neutropenia. Two patients experienced interruption of lenalidomide for 14 or 21 days, respectively, to manage neutropenia. In the patient in whom lenalidomide was interrupted for 21 days, neutropenia disappeared 10 days after treatment interruption. However, lenalidomide was not administered until the next cycle in accordance with the protocol. Although administration of G-CSF was permitted for neutropenia treatment, only one patient received G-CSF. Other treatment-related adverse events were also clinically manageable. Grade 2 interstitial pneumonia (IP) was reported in 1 patient in the 25-mg cohort on day 29. This patient had grade 3 hypoxia,

corresponding to DLT, and IP-like findings were seen on diagnostic imaging studies. Pulse steroid treatment was given for IP; after the steroid treatment, there was improvement clinically and based on imaging studies by day 40. Although the M protein was significantly reduced, this patient discontinued the study. Occurrences of IP-like syndrome associated with lenalidomide have previously been reported [6, 7]. Given the high incidence of thromboembolic events reported in several clinical trials of LD therapy, international groups of multiple myeloma experts including the International Myeloma Working Group (IMWG) have recommended prophylactic anticoagulation for patients treated with LD therapy according to the risk factors [8, 9]. In our study, dexamethasone and lenalidomide were co-administered to 12 patients and 7 of them received low-dose aspirin at the investigators' discretion. None of the patients developed DVT, pulmonary embolism, or thrombosis during the study. Patients with a prior history of DVT in the previous 3 years or ones with prior history of acute myocardial infraction in the previous 6 months were not enrolled in the study.

All 6 patients in the combination cohort achieved PR at the time of interim analysis. In addition, 2 of the 6 patients achieved CR after the interim analysis. The median time to response (TTR) in the combination cohort was 4.1 weeks. A recent compassionate use study of lenalidomide and dexamethasone reported a median TTR of about 4 weeks [10], which indicates rapid response of LD therapy in relapsed/refractory MM patients. In the monotherapy cohorts, all patients were on stable disease at the end of cycle 3. By cycle 3, the patients were not allowed to receive dexamethasone. At the interim analysis, one patient in the 10-mg cohort achieved minimal response. Two patients in the 25-mg cohort showed responses of PR. It should be noted that two patients in the 10-mg cohort and 4 patients in the 25-mg cohort started the combination therapy (lenalidomide + dexamethasone) by interim analysis. Duration of the combination therapy varied among the patients. Moreover, patients in the 10-mg cohort received 25 mg of lenalidomide after its tolerability was confirmed.

It has been reported that LD therapy was effective in patients with previous exposure to thalidomide or bortezomib [1, 2, 10, 11]. Patients who had a history of bortezomib or thalidomide therapy, respectively, achieved PR at the time of the interim analysis in the combination cohort. Responses were also seen in patients with high-risk cytogenetic features. In the combination cohort, a patient with t(4;14) achieved PR at the interim analysis and CR thereafter. This patient had del(13q) in addition to t(4;14). The other two patients with del(13q) achieved PR at the data cutoff in the combination cohort. Also, two patients with t(4;14) who were treated with lenalidomide in the 25-mg cohort showed responses of MR and PR, respectively, when combined with dexamethasone. In the MM-016 study, LD therapy induced durable responses among relapsed t(4;14) or del(13q) diseases [12].

When administered alone to Japanese MM patients under fasting conditions, the pharmacokinetics of lenalidomide show rapid absorption and elimination, with a t_{max} and $t_{1/2}$ of about 1 and 3 h, respectively after both single and multiple doses. The lack of a significant difference in any of the pharmacokinetic parameters between days 1 and 12 indicated no plasma accumulation after multiple doses of lenalidomide. The C_{max} and AUC increased in a dose-dependent manner. These pharmacokinetic characteristics are comparable to those reported for Japanese MDS patients and Caucasian healthy subjects [13, 14]. It is also reported that a majority of lenalidomide is eliminated unchanged through urinary excretion [15]. Co-administration with multiple doses of dexamethasone had no significant effect on the elimination of lenalidomide, as evidenced by the identical mean values of the $t_{1/2}$ for lenalidomide alone and lenalidomide plus dexamethasone. Judged by the wider range of t_{max} and the higher variability in C_{max}, the oral absorption of lenalidomide appeared mildly affected when co-administered with large quantities of dexamethasone tablets. These changes are considered negligible and not clinically relevant since there was no marked influence on the other PK parameters.

After a single oral 40 mg dose, dexamethasone showed a median t_{max} of 2.5 h and a mean $t_{1/2}$ of 4.2 h. These data are comparable to those reported at a lower oral dose (1 mg) in adult healthy subjects [16]. Dexamethasone is also known to be eliminated through urinary excretion [17]. Upon multiple dosing, dexamethasone displayed a 36% increase in CL/F and a 24% decrease in AUC_t with little change in t_{max} and C_{max}, indicating a slightly faster elimination from plasma after multiple doses. It has been known that higher doses of dexamethasone can induce CYP3A4 activity, the primary enzyme responsible for dexamethasone metabolism [18]. Thus, dexamethasone may accelerate its own metabolism via enzyme induction, thereby causing a lower exposure in plasma with multiple high doses. Lenalidomide is not likely to affect dexamethasone metabolism because it is not an inducer of the human cytochrome P450 enzymes.

Conflict of interest statement Conflicts of interest of all authors are as follows; H. Lau, K. Takeshita, and M. Takatoku are employees of Celgene Co., Ltd. S. Iida, T. Chou, S. Okamoto, H. Nagai, K. Hatake, H. Murakami, T. Takagi, K. Shimizu, and T. Hotta received acceptance research expenses for this trial from Celgene KK, Tokyo. S. Iida received research grants from Kyowa Hakko Kirin Co., Ltd. and Chugai Pharmaceutical Co., Ltd. H. Murakami received grants from Janssen Pharmaceutical K.K. and Novartis Pharma K.K. S. Iida declares honoraria from Janssen Pharmaceutical K.K.

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ORIGINAL ARTICLE

Bortezomib-resistant myeloma cell lines: a role for mutated *PSMB5* in preventing the accumulation of unfolded proteins and fatal ER stress

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Bortezomib is an effective agent for treating multiple myeloma (MM). To investigate the underlying mechanisms associated with acquired resistance to this agent, we established two bortezomib-resistant MM cell lines, KMS-11/BTZ and OPM-2/BTZ, the 50% inhibitory concentration values of which were respectively 24.7- and 16.6-fold higher than their parental cell lines. No activation of caspase and BH3-only proteins such as Noxa was noted in bortezomib-resistant cells after exposure to the drug. The accumulation of polyubiquitinated proteins was reduced in bortezomib-resistant cells compared with the parental cells, associated with avoidance of catastrophic ER stress as assessed by downregulation of CHOP expression. These resistant MM cells have a unique point mutation, G322A, in the gene encoding the proteasome β5 subunit (*PSMB5*), likely resulting in conformational changes to the bortezomib-binding pocket of this subunit. KMS-11 parental cells transfected to express mutated PSMB5 also showed reduced bortezomib-induced apoptosis compared with those expressing wild-type PSMB5 or the parental cells. Expression of mutated PSMB5 was associated with the prevention of the accumulation of unfolded proteins. Thus, a fraction of MM cells may acquire bortezomib resistance by suppressing apoptotic signals through the inhibition of unfolded protein accumulation and subsequent excessive ER stress by a mutation of the *PSMB5* gene.
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Keywords: bortezomib; drug resistance; MM; PSMB5; cell line

Introduction

Bortezomib, a proteasome inhibitor, is widely used in the treatment of multiple myeloma (MM), resulting in remarkable response rates in both relapsed/refractory MM and newly diagnosed MM.[1,2] However, bortezomib treatment often achieves only very short-duration responses and drug resistance rapidly develops.[3,4] Therefore, understanding the mechanisms underlying this drug resistance is necessary to develop novel approaches to overcome this problem. Bortezomib was originally developed as a proteasome inhibitor, which blocked the degradation of ubiquitinated IκBα, a negative regulator of the canonical nuclear factor (NF)-κB pathway, and prevented its translocation into the nucleus.[5] However, several investigators have proposed additional mechanisms for its antitumor effects, especially focusing on the expression of BH3-only proteins, including Noxa, Bid, puma and Bik,[6–9] and on misfolded protein accumulation followed by endoplasmic reticulum(ER) stress-associated apoptosis.[10,11] When proteasome function is inhibited, damaged proteins including unfolded or oxidatively modified proteins accumulate in the intracellular environment, which causes ER overload, well recognized as an ER stress.[12,13] This in turn induces cellular protective responses, so-called 'unfolded protein responses' (UPR) that promote refolding or elimination of unfolded proteins, but can ultimately trigger apoptosis by activating CHOP, caspase-4 and caspase-12 if the accumulation of damaged protein is excessive.[14] Administration of low doses of proteasome inhibitors can disrupt this mechanism, protecting cells from the effects of damaged protein accumulation, particularly effectively in cells such as MM and pancreatic tumors, which actively secrete proteins.[15] For this reason, modifications to the mechanism for disposal of misfolded proteins and avoidance of catastrophic ER stress caused by their accumulation may be one of the means by which MM cells acquire bortezomib resistance.

Several investigators have reported on the mechanisms of bortezomib tolerance in different tumor cell lines induced by continuous exposure to stepwise-increasing doses of bortezomib. Lu *et al.*[16] and Oerlemans *et al.*[17] have proposed either mutation of the gene for the proteasome β5 subunit (*PSMB5*) (a single G to A nucleotide shift at the position 322) or overexpression of this protein as possible mechanisms associated with bortezomib resistance in the T-lymphoblastic/leukemia cell line JURKATB, and the monocytic/macrophage cell line, THP1/BTZ, respectively. In another study, Rückrich *et al.*[18] proposed that the suppression of protein biosynthesis contributes to the adaptation to impaired proteasome activity in myeloid leukemia HL60a cells, which have acquired resistance to bortezomib. These investigators also established a bortezomib-resistant MM cell line, designated AMO-1a, but did not report any details with regard to resistance mechanisms. To the best of our knowledge, there have been no published studies on the mechanisms responsible for bortezomib resistance in MM cells. Here, we established two bortezomib-resistant MM cell lines, which tolerated the drug even at doses 10-fold higher than the 50% inhibitory concentration (IC_{50}) values for parental cells. Using these new resistant lines, we investigated the alteration of *PSMB5*, misfolded protein accumulation, ER stress and apoptosis signals including BH3-only proteins at clinically achievable drug concentrations. Our study demonstrates that preventing the accumulation of misfolded proteins and avoidance of catastrophic ER stress has a crucial role in bortezomib resistance by suppressing apoptosis-inducing signals in MM cells. We also document that the mechanism for this effect involves a unique point mutation of *PSMB5* in bortezomib-resistant MM cells, which contributes to reducing the accumulation of misfolded proteins and alleviates catastrophic ER stress in MM cells.

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Materials and methods

Establishment of bortezomib-resistant MM cell lines

KMS-11 was kindly provided by Professor T Otsuki, Kawasaki Medical University (Okayama, Japan). OPM-2 was purchased from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). Two bortezomib-resistant MM cell lines, KMS-11/BTZ and OPM-2/BTZ, were established from their parental lines, KMS-11 and OPM-2, under continuous exposure to bortezomib in RPMI-1640 medium supplemented with 10% fetal bovine serum over a half year. During this time, the concentration of bortezomib was increased stepwise weekly after confirmation of the maintained viability of the cells at the previous dose. After their establishment, the bortezomib-resistant cell lines were incubated in bortezomib-free medium for 2 weeks to confirm the stability of resistance trait, and then subjected to all assays used in our study.

Antibodies, reagents and western blot analysis

Bortezomib was purchased from Toronto Research Chemicals (North York, Ontario, Canada). Antisera against caspase-12 and CHOP were purchased from Cell Signaling Technology, Inc (Danvers, MA, USA). Antisera against Bcl-Xl/s, Mcl-1, Noxa, ubiquitin and actin were purchased from Santa Cruz Biotechnology Inc (Santa Cruz, CA, USA). Antisera against Bid, Bcl-2, β-galactosidase, V5-tag and caspase-4 were purchased from Medical & Biological Laboratories (Nagoya, Japan). Antisera against β5, β1 and β2 subunits of the 26S proteasome were purchased from BIOMOL International, L.P. (Plymouth Meeting, PA, USA).

Western blot analysis was performed as previously described.[8] In brief, protein samples were electrophoresed by SDS polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. After blocking with 5% nonfat milk, membranes were incubated with primary antibody followed by a horseradish peroxidase-conjugated secondary antibody. The protein band was detected using chemiluminescent substrate.

Cell proliferation and apoptosis assays

The cell proliferation assay was previously described.[8] Calculation of the IC_{50} value used XLfit 4.2 curve-fitting software for Excel. Apoptotic cells after exposure to bortezomib were evaluated using Annexin V-FITC Apoptosis Detection Kit I (BD Pharmingen, Franklin Lakes, NJ, USA).

Chymotrypsin-like activity assay

A total of 5×10^5 cells were incubated with or without 10 nM bortezomib for the indicated times. After washing twice with cold phosphate-buffered saline, cells were resuspended in 50 mM Tris (pH 7.4) buffer containing 5 mM $MgCl_2$ and 0.2 μg/ml digitonin, which permeabilizes the cell membrane without disrupting it. Cells were transferred into black 96-well flat-bottom plates at a final concentration of 4×10^4 cells in 160 μl of buffer in each well. Thereafter, 40 μl of fluorogenic substrate, Suc-LLVY-amc (BIOMOL International, L.P.), was added to each well. After incubation for 3 h at 37 °C, fluorescence was measured at 380 nm excitation wavelength and 460 nm emission wavelength.

DNA sequencing

Total RNA extraction from MM cell lines, followed by reverse transcription into cDNA, was performed as previously reported.[8] Exon II of the *PSMB5* gene was amplified by means of PCR using the following primer set: forward, 5′-TTCCGCCATGGAGTCA TA-3′; and reverse, 5′-GTTGGCAAGCAGTTTGGA-3′. After purification, the PCR product was directly sequenced by the dye terminator method with the aid of an ABI377 (Applied Biosystems, Foster City, CA, USA).

Transfection by lentiviral infection

The cDNA encoding wild-type or mutated *PSMB5* was obtained from KMS-11 or KMS-11/BTZ cells, respectively, by PCR amplification using 5′-attB-added PCR primers (Gateway Technology, Invitrogen, Carlsbad, CA, USA) followed by sequencing. The lentivirus-based expression vector was constructed from the combination of each cDNA-containing entry vector, cytomegalovirus (CMV) promoter-containing vector, and plenti6.4/R4R2/V5-DEST multisite gateway vector by attB-attP and attL-attR reaction. The 293FT packaging cell line was transfected with plenti/CMV/wPSMB5/V5 or plenti/CMV/mPSMB5/V5 plasmids for 24 h and each viral supernatant was collected. After equalization of viral titer, KMS-11 cells were infected by each viral supernatant for 24 h and then selected by incubation with 5 μg/ml blasticidin. After selection, KMS-11 cells stably expressing wild-type *PSMB5* (KMS-11/wPSMB5-V5) or mutated *PSMB5* (KMS-11/mPSMB5-V5) were assayed for apoptosis after exposure to bortezomib, and were also used for immunoblot analysis. As a control for lentiviral expression, plenti/CMV/lacZ/V5 plasmid was similarly constructed and transfected into KMS-11 cells (KMS-11/lacZ-V5).

Results

Two MM cell lines, KMS-11/BTZ and OPM-2/BTZ, demonstrate acquired resistance to bortezomib treatment

Two bortezomib-resistant MM cell lines, KMS-11/BTZ and OPM-2/BTZ, showed a 24.7-fold (IC_{50} 148.3 nM) and 16.6-fold (IC_{50} 51.6 nM) higher resistance to bortezomib, respectively, compared with their parental cells, KMS-11 (IC_{50} 6 nM) and OPM-2 (IC_{50} 3.1 nM) after a 72-h exposure (Figure 1a). In addition, these cells showed cross-resistance against another proteasome inhibitor, MG132, but not against doxorubicin (Figure1a and Table 1). In experiments measuring apoptosis induced by dose-escalated bortezomib treatment (Figure 1b), KMS-11/BTZ showed remarkable tolerance to between 3.3 and 100 nM of the drug, and OPM-2/BTZ between 3.3 and 33.3 nM, whereas the parental cells underwent apoptosis even at lower concentrations, 10 nM in KMS-11 and 3.3 nM in OPM-2. We next investigated the biological differences between resistant and parental cells at 10 nM bortezomib in further analyses.

Bortezomib-resistant cells do not activate apoptosis-executing signals induced by bortezomib treatment

To compare the apoptosis-regulating signals following bortezomib treatment, we investigated alterations in the expression of different caspases, Bcl-2 family members, BH3-only proteins, NF-κB activation, and ER stress signaling in the bortezomib-resistant cells and their parental cells in the presence of 10 nM bortezomib for 48 h. As shown in Supplementary Figure S1 (also refer Supplementary Materials and methods), immunoblot analysis indicated that KMS-11/BTZ and OPM-2/BTZ cells failed to activate caspases, that is, cleavage of caspase-3, -8 and -9, which did occur in the parental cells after bortezomib exposure. In Figure 2a, two bcl-2 family proteins, Bcl-2 and Bcl-xL, are

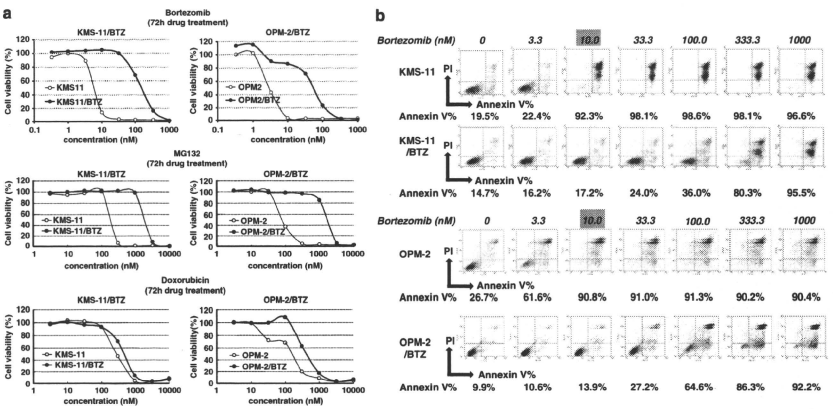

Figure 1 Bortezomib-resistant myeloma cell lines, KMS-11/BTZ and OPM-2/BTZ. (**a**) Cell viability of bortezomib-resistant cell lines, KMS-11/BTZ and OPM-2/BTZ, and their parental cell lines after exposure to different concentrations of three drugs, bortezomib, MG132 and doxorubicin for 72 h. (**b**) Bortezomib-induced apoptosis at 72 h after exposure to different concentrations of the drug assessed by flow cytometric analysis of Annexin V and PI double staining.

Table 1 The IC_{50} of KMS11/BTZ and OPM-2/BTZ and their parental cell lines in each drug

	IC_{50} (nM)		*IC_{50} ratio*	*IC_{50} (nM)*		*IC_{50} ratio*
	KMS-11	*KMS-11/BTZ*		*OPM-2*	*OPM-2/BTZ*	
Bortezomib	6	148.3	24.7	3.1	51.6	16.6
MG132	260	2000	7.7	110	1900	17.27
Doxorubicin	300	450	1.5	190	370	1.94

Abbreviation: IC_{50}, 50% inhibitory concentration.
The IC_{50} of KMS-11/BTZ and OPM-2/BTZ and their parental cell lines when exposed to each of the drugs for 72 h. The ratio of IC_{50} value of resistant to parental cell line is shown on the right.

shown to be overexpressed in OPM-2/BTZ cells compared with their parental cells, and this was maintained during exposure to bortezomib. However, this was not the case for KMS-11/BTZ cells. We also noted that another Bcl-2 family protein, Mcl-1, was not altered in the bortezomib-resistant cells after exposure to bortezomib, whereas parental cells showed accumulation of long, short and cleaved forms of Mcl-1 after treatment. A BH3-only protein, Noxa, which was rapidly upregulated by bortezomib treatment in parental cells, was significantly suppressed in bortezomib-resistant cells. Similarly, the basal level of Bid, a caspase-8-dependent BH3-only protein, which declined in parental cells after bortezomib exposure, was not changed in bortezomib-resistant cells. Most recently, Wang *et al.*[19] proposed that Noxa was transcriptionally activated by the complex consisting of ATF3 and ATF4, which were upregulated by an inhibitor of ER-associated protein degradation or by bortezomib treatment in tumor cells. As shown in Supplementary Figure S2 (also refer Supplementary Materials and methods), there were no obvious differences in the induced levels of ATF3 and ATF4 between KMS-11/BTZ and the parental KMS-11 line. Moreover, both OPM-2/BTZ and OPM-2 showed low levels of ATF3 expression before and after bortezomib treatment.

Gel shift assays demonstrated that even in the presence of bortezomib, resistant cells maintained NF-κB activation as represented by three (KMS-11/BTZ) or four (OPM-2/BTZ) different DNA–protein complexes, and one of them became abundant at 48 h after bortezomib exposure (depicted by an asterisk in Supplementary Figure S3 and refer Supplementary Materials and methods). On the other hand, in the parental cells, NF-κB activity was suppressed after bortezomib treatment, whereas only one of the complexes (indicated by three asterisks) showed a transient increase at 6 h after bortezomib exposure, followed by a decrease at 12 h and later. Most recently, Hideshima *et al.*[20] have proposed that bortezomib activates the canonical NF-κB pathway through activation of IKKβ resulting in IκBα phosphorylation and degradation, a process mediated by the proteasome. Following this report, the over-expressed band (depicted by triple asterisks in Supplementary Figure S3a) after bortezomib exposure may correspond to the one resulting from activation of the canonical NF-κB pathway. In fact, we have also demonstrated that bortezomib treatment resulted in phosphorylation of p65 and IκBα followed by degradation of IκBα in bortezomib-sensitive MM cells even when they are committed to cell death (Supplementary Figure S3b). On the other hand, two bortezomib-resistant MM cells

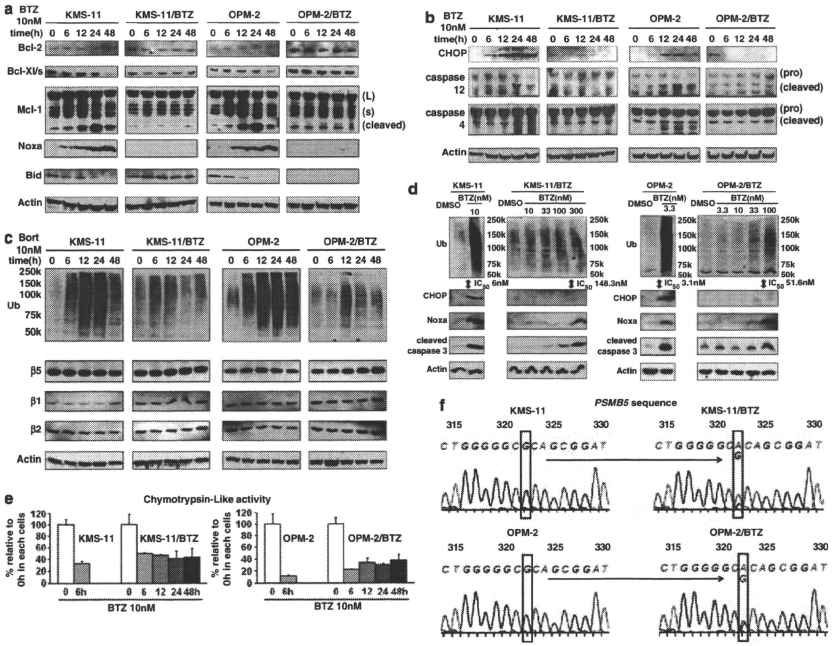

Figure 2 Kinetic changes of apoptosis-related protein expression, chymotrypsin-like activity, and sequencing of the proteasome β5 subunit in parental KMS-11, OPM-2, and the bortezomib-resistant KMS-11/BTZ and OPM-2/BTZ lines. (**a**) Altered expression of antiapoptotic Bcl-2 family proteins such as Bcl-2, Bcl-XL and Mcl-1 and proapoptotic proteins, that is, BH3-only proteins, such as Noxa and bid. (**b**) Kinetic changes of CHOP expression and cleavage of caspase-12 and -4 evaluated by western blot analysis in KMS-11/BTZ, OPM-2/BTZ and their parental cell lines. (**c**) Accumulation of polyubiquitinated proteins and altered expression of 20S proteasome subunits, including β1, β2 and β5 before and after exposure to 10 nM bortezomib for the indicated times, evaluated by western blot analysis. (**d**) Dose-dependent alterations of accumulated polyubiquitinated proteins, expression levels of CHOP and Noxa, and activation of cleaved caspase-3 in bortezomib-resistant cells and in their parental cells. (**e**) Chymotrypsin-like activity measured using specific fluorogenic peptides after exposure to 10 nM bortezomib for the indicated times. The values represent the means of three independent experiments. (**f**) At nucleotide position 322, wild-type *PSMB5* in KMS-11 and OPM-2 indicates only G, whereas a double peak (G/A) is present at the same site in KMS-11/BTZ and OPM-2/BTZ cells.

showed neither phosphorylation of IκBα and p65 nor degradation of IκBα, indicating that the canonical NF-κB pathway in these two resistant lines was not altered by bortezomib exposure. Our study demonstrated that even in the presence of bortezomib, resistant cells maintained constitutively active NF-κB.

We next examined the expression of ER stress-related markers including cytosolic and ER chaperones, which might prevent the aggregation of misfolded proteins and promote their refolding.[14] We also assessed the activation of CHOP and caspase. As shown in Supplementary Figure S4 (also refer Supplementary Materials and methods), compared with the parental cells, bortezomib-resistant cells had neither upregulated cytosolic chaperones nor ER chaperones such as Bip and Grp94, thioredoxin family (PDI) or lectin family proteins (calreticulin) with the exception of calnexin. On the other hand, CHOP expression was upregulated immediately after bortezomib treatment in the two parental MM cells. The already-low levels of CHOP completely disappeared in bortezomib-resistant cells (Figure 2b). In addition, the resistant cells showed no activation of caspase-4 and -12, which were induced in susceptible cells.

The ubiquitin–proteasome pathway is altered in bortezomib-resistant cells

To determine whether unfolded proteins accumulated after exposure to bortezomib, intracellular misfolded proteins recognized as polyubiquitinated were assessed by western blot analysis in bortezomib-resistant MM and parental cells before and after bortezomib treatment. As shown in Figure 2c, both KMS-11 and OPM-2 cells gradually accumulated polyubiquitinated proteins after bortezomib treatment. However, only transient accumulation of polyubiquitinated proteins was observed both in KMS-11/BTZ and OPM-2/BTZ cells, which

had returned to basal level by 24 and 48 h after exposure, respectively. We next investigated the status of protein biosynthesis in resistant and parental cells. Newly synthesized proteins were labeled and measured before and after bortezomib treatment (Supplementary Materials and methods and Supplementary Figure S5). After bortezomib treatment, the two parental lines continued to synthesize protein 6 h after treatment but this was reduced by 24 h because of progressive apoptosis. In contrast, the two resistant lines both maintained continuous protein synthesis throughout the treatment. These results suggest that bortezomib-resistant MM cells maintain the same level of protein synthesis, unlike the bortezomib-adapted myeloid leukemia HL60a cell that was previously reported.[18] We also determined the expression levels of each of the 20S proteasome subunits, β1, β2 and β5. The total amount of all three subunits was comparable between resistant and parental cells before bortezomib exposure (Figure 2c). Only the amount of β2 subunit was slightly increased after bortezomib treatment in the resistant cells, whereas it was gradually decreased in the parental cells.

We next investigated the dose-dependent alteration of accumulated polyubiquitinated proteins and expression levels of CHOP and Noxa, and activation of caspase-3, in bortezomib-resistant and parental MM cells treated with the drug. At a higher concentration of bortezomib than the IC_{50} value, the two resistant lines showed activation of caspase-3 and Noxa expression, indicating progression to apoptosis. However, they showed moderate accumulation of polyubiquitinated proteins, which was not followed by activation of CHOP. This finding may indicate that intracellular stresses different from proteasome inhibition have occurred, which trigger apoptosis independently of ER stress before excessive unfolded protein accumulation can take place at high concentrations of bortezomib.

To compare bortezomib-induced proteasome inhibition, chymotrypsin-like activity was measured. This was found to decrease in KMS-11 cells to 30–37% of the control level after a 6-h exposure, whereas KMS-11/BTZ cells retained 47–51% of the activity even after a 48-h exposure. In OPM-2 cells, chymotrypsin-like activity on 6-h exposure was reduced to 10–13%, whereas OPM-2/BTZ cells retained 21–23% of the level in unexposed cells and maintained that until 48 h after exposure. These results indicate that the degree of proteasome inhibition is slightly weaker in bortezomib-resistant cells than their parental cells after bortezomib exposure. This subtle difference may contribute to the avoidance of fatal unfolded protein accumulation.

Expression levels of ubiquitin specific proteases, a lysosomal protease,[18] before and after bortezomib exposure were also examined. As shown in Supplementary Figure S5 (also refer Supplementary Materials and methods), neither bortezomib-resistant line overexpressed USPs before or after exposure to bortezomib compared with their parental cells. This indicates that USPs have little effect in compensating for impaired proteasome activity in bortezomib-resistant cells.

Alteration of the proteasome β5 subunit in bortezomib-resistant MM cells

Most recently, mutation of the *PSMB5* gene (G322A) has been proposed as a possible mechanism responsible for bortezomib resistance in T-cell lymphoblastic/leukemia and myeloid leukemia cells adapted to bortezomib treatment.[16,17] To investigate the presence or absence of genetic alterations in the *PSMB5* gene in our MM cells, exon 2 encoding the conserved bortezomib-binding pocket regions in the β5 subunit was sequenced. As shown in Figures 2d and a, substitution at nucleotide position 322 (G/A), which corresponds to the amino-acid change (Ala49Thr) same as previously reported,[16,17] was identified in both the bortezomib-resistant MM cell lines, but not in the parental cells. This reflects the appearance of a G322A-mutated allele in addition to the remaining wild-type allele in bortezomib-resistant MM cells.

The G322A-mutated PSMB5 *reduces bortezomib-induced apoptosis through the prevention of ubiquitinated protein accumulation and fatal ER stress in MM cell*

To investigate the role of the *PSMB5* mutation (G322A) in bortezomib resistance of the MM cell lines, we transfected a G322A-mutated *PSMB5* expression construct into KMS-11 cells using a lentiviral vector. As controls, wild-type *PSMB5* or *lacZ* constructs were similarly transfected into KMS-11 cells. In Figure 3a, these transfected cells can be seen to have similar expression levels of V5-tag, which indicates that the transfection efficiency was essentially the same for the mutated *PSMB5* and the other genes. After 72-h bortezomib treatment, KMS-11 mPSMB5-V5 cells showed significant reduction of apoptosis compared with KMS-11- wPSMB5-V5-, KMS-11 lacZ-V5-transfected and nontransfected KMS-11 cells (Figure 3b). Similar result was shown in growth inhibition assay when treated with various concentrations of bortezomib for 72 h. The IC_{50} values were 8.88, 26.38, 8.83 and 113.63 nM in KMS-11 wPSMB5-V5, KMS-11 mPSMB5-V5, parental KMS-11 and KMS-11/BTZ cells, respectively (Figure 3c). There was much less accumulation of polyubiquitinated proteins in m*PSMB5*-transfected cells compared with wild-type control cells (Figure 3d). Similarly, expression levels of CHOP and Noxa were lower in mutated PSMB5-transfected KMS-11 cells after bortezomib exposure (Figure 3d). These results indicate that G322A-mutated *PSMB5* contributed to a reduction in bortezomib-induced apoptosis by preventing ubiquitinated protein accumulation and fatal ER stress in these MM cells.

Discussion

We have established two novel bortezomib-resistant MM cell lines, KMS-11/BTZ and OPM-2/BTZ, both of which tolerated the drug even at high concentrations and were also resistant to a different proteasome inhibitor, MG132. These two bortezomib-resistant MM cell lines did not overexpress the β5 proteasome subunit compared with the parental lines, unlike what has been reported in bortezomib-resistant cells other than MM cells.[16,17] However, a unique point mutation, G322A, was identified in the *PSMB5* gene as has also been found in previous studies of bortezomib-resistant cells of other hematopoietic lineages.[16,17] In addition, we demonstrated that the G322A point mutation in *PSMB5* actually contributes to resistance against bortezomib-induced apoptosis, which is mediated by prevention of polyubiquitinated protein accumulation and fatal ER stress signaling followed by the downregulation of BH3-only protein, that is, Noxa expression. However, the degree of bortezomib resistance in mutated PSMB5-transfected KMS-11 cells was not as great as that of KMS-11/BTZ cells, indicating that as-yet unidentified mechanisms other than *PSMB5* mutation may partly contribute to the bortezomib resistance in KMS-11/BTZ cells.

A point mutation of the *PSMB5* gene (G322A) identified in both bortezomib-resistant MM cell lines results in replacement of the codon 49 Thr for Ala at the amino-acid level. This would

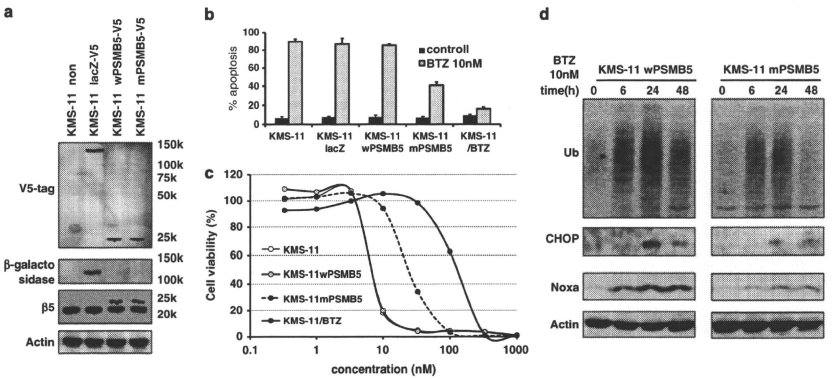

Figure 3 Comparison of bortezomib-induced apoptosis, accumulation of polyubiquitinated proteins, and the expression of CHOP between mutated PSMB5-expressing KMS-11 and wild-type PSMB5-expressing KMS-11 cells. (**a**) The expression of V5-tagged lacZ, V5-tagged wild-type (w) PSMB5 and V5-tagged mutated(m) PSMB5 in transfected KMS-11 cells. (**b**) Comparison of bortezomib-induced apoptosis at 72 h in mutated PSMB5-, wild-type PSMB5-, lacZ-, non-transfected KMS-11 cells and bortezomib-resistant cell (KMS-11/BTZ). Apoptotic cells were evaluated as Annexin V-positive cells. (**c**) Cell viability of mutated PSMB5-, wild-type PSMB5-, non-transfected KMS-11 cells and bortezomib-resistant KMS-11 cells (KMS-11/BTZ) after exposure to various concentrations of bortezomib for 72 h. (**d**) The time-dependent alteration of accumulated polyubiquitinated proteins, CHOP and Noxa expression in mutated PSMB5- and wild-type PSMB5-transfected KMS-11 cells in the presence of 10 nM bortezomib.

give rise to a conformational change of the bortezomib-binding pocket in the β5 subunit, resulting in the partial disruption of contact between bortezomib and the chymotrypsin-like active site.[16,21] Our study first proved that in MM cells, the G322A point mutation in PSMB5 contributes to resistance against bortezomib-induced apoptosis. Moreover, our study does not support the notion that overexpression of wild-type PSMB5 alone led to bortezomib resistance, unlike previous reports using a bortezomib-adapted monocytic/macrophage cell line.[19] Among various hematological malignant cell types, MM cells have the greatest sensitivity to bortezomib even at low doses, probably due to preexisting ER overload. Thus, moderately upregulated PSMB5 may not be able to impart the resistance against bortezomib. In fact, as the mutated PSMB5 is not overexpressed in either of the bortezomib-resistant MM cell lines, the presence of this mutated form may have a major role in acquired resistance against bortezomib. Although bortezomib is a highly selective inhibitor of the chymotrypsin-like activity of PSMB5, another proteasome catalytic site, PSMB1, is also inhibited to some extent by bortezomib. Another subunit, PSMB6, is known to act as a scaffold to stabilize the interaction between bortezomib and the active site of PSMB5.[21] However, we could not find any mutations in *PSMB1* and *PSMB6* in our bortezomib-resistant MM cells (data not shown).

We have not found any *PSMB5* gene mutations in a small number ($n=4$) of clinically available specimens derived from MM patients who showed resistance against bortezomib so far. However, further investigations using a larger number of patients who have acquired bortezomib resistance during or after treatment are required.

In conclusion, we have established two different stable bortezomib-resistant MM cell lines, both of which were found to have acquired exactly the same point mutation (G322A) in the bortezomib-binding pocket of *PSMB5*. These cells, unlike their parental cells, did not accumulate misfolded proteins and thus avoided the catastrophic ER stress, which triggers CHOP expression and caspase-4 and -12 activations. Apoptosis triggered by Noxa induction was also suppressed. These cell lines will provide tools for the better understanding of the underlying mechanisms of bortezomib resistance, and may lead to the development of novel treatment strategies for overcoming bortezomib resistance in patients with MM.

Conflict of interest

TN, HM and YS are employees of Kyowa Hakko Kirin Co., Ltd., Japan. SI received research funding from Kyowa Hakko Kirin. SI declares honoraria from Janssen Pharmaceutical K.K., Dainippon Sumitomo Pharmaceutical Co., Ltd, Chugai Pharmaceutical Co., Ltd and Novartis Pharma K.K.

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Risk of Japanese carriers of hyperphosphorylated paratarg-7, the first autosomal-dominantly inherited risk factor for hematological neoplasms, to develop monoclonal gammopathy of undetermined significance and multiple myeloma

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Hyperphosphorylated paratarg-7 (pP-7) is a frequent target of paraproteins in German patients with monoclonal gammopathy of undetermined significance (MGUS)/multiple myeloma (MM). The frequency of MGUS/MM is lower in Japan than in Europe. As pP-7, the first molecularly defined autosomal-dominant risk factor for any hematological neoplasm, is inherited in a dominant fashion, we determined the incidence of the pP-7 carrier state in a Japanese population, and compared the frequency of pP-7-specific paraproteins and the pP-7 carrier state in Japanese and German patients with MGUS/MM. Peripheral blood from 111 Japanese patients with MGUS/MM and 278 healthy blood donors was analyzed for the pP-7 carrier state by isoelectric focusing and for pP-7-specific antibodies by ELISA. The Japanese group was compared with 252 German MGUS/MM patients and 200 healthy controls. Five of 111 (4.5%) Japanese and 35/252 (13.9%) German IgA/IgG MGUS/MM patients had a pP-7-specific paraprotein ($P = 0.009$). The prevalence of healthy pP-7 carriers in the Japanese study group was 1/278 (0.36%), whereas it was 4/200 in the German group ($P = 0.166$). The relative risk for pP-7 carriers developing MGUS/MM had an odds ratio of 13.1 in the Japanese and 7.9 in the German group. In conclusion, the fraction of pP-7 carriers with a pP-7-specific paraprotein is lower among Japanese than in German patients with MGUS/MM, but pP-7 carriers in both ethnic groups have a high risk of developing MGUS/MM. (*Cancer Sci* 2011; 102: 565–568)

Multiple myeloma (MM) is a B-lymphocyte-derived malignancy characterized by a monoclonal proliferation of plasma cells that produce a clonal immunoglobulin. Monoclonal gammopathy of undetermined significance (MGUS) is an asymptomatic precursor condition commonly preceding MM.[1] Recent studies show that family members of MGUS and MM patients have a two to threefold higher risk of developing MGUS/MM.[2,3] Environmental influences, chance occurrence, and inherited factors might all contribute to familial clusters. Also, the incidence of MM and the prevalence of MGUS are reportedly two to threefold higher among Black people than White people,[4–6] and lower in Asians.[6,7] Until now, the pathogenesis of MGUS/MM has remained obscure. A causal relationship between MGUS/MM and chronic antigenic stimulation has been suggested by the results of several studies,[8–12] hence the identification of the antigenic stimuli of B-cell neoplasms might be of considerable importance. Antigenic targets of paraproteins were discovered accidentally due to clinical symptoms caused by the paraprotein (e.g. chronic cold agglutinin disease or cryoglobulinemia[13] or bleeding disorders[14]), because of interference of the paraprotein with laboratory tests ordered for the clinical work-up of the patient (e.g. HIV-1 p24 antigen in an HIV-infected patient with myeloma),[15] and/or by screening paraproteins against pre-defined antigens (e.g. anti-streptolysin, anti-DNA, or anti-IgG[13]). The first systematic studies covering a broad spectrum of potential antigens used serological identification of antigens by expression cloning (SEREX), which allows for the systematic screening of putative antibody–antigen interactions, even if neither the antigen nor the antibody are known.[16] cDNA libraries derived from human testis, lung, and breast cancer, bovine and porcine muscle, and wheat germ were expressed in *Escherichia coli* and investigated by SEREX for reactivity with paraproteins from the sera of 114 patients with MGUS or MM. More than 6×10^8 paraprotein–antigen interactions were probed, resulting in the identification of only four antigens, each recognized by the paraprotein of only one patient.[17,18] In a complementary approach using a human fetal brain-derived macroarray and IgA or IgG paraprotein-containing sera, the paraproteins of 29 (15.1%) consecutive MGUS and MM patients reacted with paratarg-7 (P-7).[19] Paratarg-7 is identical to STOML2 (stomatin [EPB72]-like), also known as HSPC108 or stomatin-like protein and SLP-2,[20] that has also been reported to be expressed in all human tissues and overexpressed[21,22] in several cancers.[23] Other investigators have reported that P-7 modulates T cell activation,[24] and in a recent publication it was claimed that SLP-2 is required for stress-induced mitochondrial hyperfusion.[25] In an extension of our earlier study, the high frequency of P-7-specific paraproteins in the sera of MGUS/MM patients (35/252; 14%) was confirmed in a subsequent study.[26] Moreover, it was shown that all patients with P-7-specific paraproteins were carriers of a hyperphosphorylated version of the protein (pP-7) and that this hyperphosphorylation is inherited in a dominant fashion.[26,27] As only 2% of healthy Germans are carriers of pP-7, the pP-7 carrier state is associated with an increased risk (odds ratio, 7.9) to develop MGUS/MM. Thus, pP-7 is the first molecularly defined inherited risk factor known for any hematological neoplasm. Because of the autosomal-dominant inheritance of pP-7, it is of interest to determine the prevalence of the pP-7

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carrier state and the frequency of pP-7-specific paraproteins in other ethnic groups. As the incidence of MGUS and MM is lower in Asians than in Europeans,[6,7] it was the aim of this study to compare a German population with a Japanese population with respect to the prevalence of the pP-7 carrier state and the incidence of P-7-specific paraproteins in MGUS and MM.

Materials and Methods

Patients and controls. This study was approved by the local German ethical review board (Ethikkommission der Ärztekammer des Saarlandes, Saarbrücken, Germany) and the internal review board of Nagoya City University and Nagoya City Midori Municipal Hospital (Nagoya, Japan). Serum samples were taken from 111 Japanese patients with MGUS/MM treated at Nagoya City University Hospital or Nagoya City Midori Municipal Hospital and 252 German patients treated at Saarland University Medical School (Homburg/Saar, Germany). Serum protein electrophoresis identified a monoclonal spike in these samples, which was shown to contain a monoclonal IgA, IgD, or IgG paraprotein by immunofixation. The German control group consisted of 200 healthy employees of Saarland University Medical School and the Japanese control group consisted of 278 Japanese healthy blood donors. "Healthy" was defined as being healthy in the pre-donation medical check-up and having no monoclonal immunoglobulin by serum electrophoresis and immunofixation. In addition, the healthy donors who were identified by isoelectric focusing (IEF) as pP-7 carriers were contacted and they confirmed that they were without any major health problems and explicitly without malignant disease, in particular.

The same criteria were applied in our previous study to the German population of 252 MGUS/MM patients. The German control group was the same as in the previous study. There was no difference with respect to age, gender (all patients), progression to MM, or stage of the disease between patients with a P-7-specific paraprotein. Patients were listed under MGUS if the first blood sample was obtained while they had still MGUS, even if they later progressed into MM. Whenever possible, human materials were obtained during routine diagnostic or therapeutic procedures and stored at −80°C until use.

Western blot sample preparation. For Western blot analyses, blood samples were centrifuged and washed with PBS. The pellet was resuspended in 8 M urea, 0.1 M phosphate, 10 mM Tris–HCl (pH 8.0), and 0.1% NP-40 and incubated at 20°C for 15 min.

Isoelectric focusing for determination of pP-7 carrier state. Blood samples were centrifuged and washed with PBS followed by lysis in lysis buffer containing 8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris–HCl, and 0.1% NP40 (15 min, 20°C) and stored at −20°C until use. Equal volumes of sample and loading buffer were mixed. Samples were analysed by IEF on a gel with a fixed pH gradient (pH 3.0–10.0) according to the manufacturer's instructions (Novex pH 3.0–10.0; Invitrogen, Karlsruhe, Germany) followed by an immunoblot screening.

Immunoblot staining. After lysates from whole peripheral blood were separated by IEF or SDS-PAGE under reducing conditions, the proteins were transferred to an Immobilon-P PVDF membrane (Immobilon; Millipore, Eschborn, Germany) by semi-dry blotting. The membrane was blocked overnight at 4°C in TBST/milk buffer (10% [v/v] milk in 10 mM Tris–HCl [pH 7.5], 150 mM NaCl, and 0.1% [v/v] Tween 20), washed and incubated for 1 h at room temperature with serum in TBST (paraprotein-containing serum from patients at a dilution of $1:10^8$ and from controls at a dilution of $1:10^3$). After three washings in TBST, the membranes were incubated for 1 h at room temperature with mouse anti-human IgG–POX antibody (BioRad, Dreieich, Germany) diluted 1:3000 in TBST. The membranes were then washed in TBST, followed by detection using Pharmacia's ECL (enhanced chemiluminescence) system (General Electric Healthcare, Fairfield, CT, USA).

Paratarg-7 ELISA for detection of paraproteins with specificity to P-7. To determine the antigen specificity of the paraproteins, the P-7 ELISA using full-length recombinant paratarg-7 was carried out as described previously.[19]

Results

Thirty-five of 252 (13.9%) paraproteins from the German MGUS/MM patients were shown by ELISA to react with paratarg-7. In contrast, only 5/111 Japanese patients (4.5%) had a paraprotein specific for paratarg-7 (Table 1) and were carriers of pP-7 (Fig. 1). This difference is significant with a *P*-value of 0.009 (chi-square test). The anti-paratarg-7 reactivity of these sera had titres ranging from $1:10^8$ to $1:10^{10}$. None of the sera from healthy controls reacted at a dilution of $\leq 1:10^2$. Lower serum dilutions were not tested because they cause too much background in the P-7 ELISA. As was the case with the German IgG paraproteins with specificity for P-7,[19] all Japanese P-7 reactive IgG paraproteins (4/78) belonged to the IgG_3 subtype, with 4/38 IgG_3 (10.5%) paraproteins displaying this specificity. The prevalence of healthy pP-7 carriers in the healthy Japanese population, as determined by IEF (Fig. 1) followed by immunoblot staining, was lower (1/278 or 0.36%) than in the German (4/200 or 2.0%) population. This is a strong trend but, due to the low prevalence of a pP-7 carrier state in healthy controls, did not reach significance ($P = 0.166$; Fisher's exact test). Nevertheless, the relative risk for pP-7 carriers to develop MGUS/MM is significant in both ethnic groups with an odds ratio of 13.1 (95% confidence interval, 1.5–113.1; $P = 0.020$) in

Table 1. Detection of paratarg-7-specific paraproteins in 252 German and 111 Japanese consecutive patients with monoclonal gammopathy of undetermined significance (MGUS) and multiple myeloma (MM)

	MGUS (%)	MM (%)	Total (%)
Japanese patients			
IgA	0/3 (0)	1/22 (4.5)	1/25 (4.0)
IgD	0/0 (0)	0/8 (0)	0/8 (0)
IgG	0/8 (0)	4/70 (5.3)†	4/78 (5.1)†
Total	0/11 (0)	5/100 (5.0)	5/111 (4.5)
German patients			
IgA	2/24 (8.3)	4/21 (19.0)	6/45 (13.3)
IgD	0/0 (0)	0/0 (0)	0/0 (0)
IgG	15/117 (12.8)†	14/90 (15.5)†	29/207 (14.0)†
Total	17/141 (12.1)	18/111 (16.2)	35/252 (13.9)

†All pP-7 reactive IgG paraproteins were of the IgG_3 subclass.

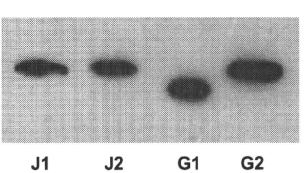

Fig. 1. Western blot analysis after isoelectric focusing of paratarg-7 (P-7) derived from Japanese and German patients with P-7-reactive paraproteins. Bands of whole peripheral blood lysates from two Japanese patients with multiple myeloma (J1, J2) with anti-P-7 paraproteins, one German patient with a P-7-specific paraprotein (G2) and one German patient with a non-P-7-specific paraprotein (G1) migrated differently.

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the Japanese and 7.9 (95% confidence interval, 2.8–22.6; $P = 0.0001$) in the German population.

Discussion

This is the first study to show that pP-7, which was identified as the first dominantly inherited risk factor for any hematological neoplasm in White people, is also found in Japanese patients with MGUS/MM and is a strong and highly significant risk factor for developing MGUS/MM in both Japanese and German pP-7 carriers. A causal relationship between MGUS/MM and chronic antigenic stimulation has been suggested by the results of several studies; however, results have generally been inconsistent.[4,5,10,11,28–37] The specificity of the P-7 binding paraproteins was extensively discussed in our previous published study[19] and has recently been confirmed by cloning the B-cell receptors of two patients with a P-7-specific paraprotein.[38]

The MGUS/MM patients in this study carrying pP-7, and those with paraproteins that did not bind to P-7, showed no significant difference with respect to age, sex, or course of disease (data not shown). Even though the frequency of P-7 as a paraprotein target is only 4.5% in Japanese patients, it is still much higher than expected by chance and suggests a direct or indirect role of pP-7 in the pathogenesis of these diseases in both the Japanese and German populations. All IgG paraproteins with specificity for P-7 belonged to the IgG_3 subclass, both in the Japanese patients (4/38 or 10.5%) and in the German patients (24/57 or 42.1%).[19] The reason for this is unknown, but indicates that additional factors might be necessary for the recognition of pP-7 as an auto-antigen by the autologous immune system. Recent results from our laboratory show that the difference between "wild-type" and pP-7 is due to a phosphorylation at a single site (serine 17 of the molecule; unpublished data). Because of the dominant inheritance of P-7 hyperphosphorylation, a polymorphism in one of the plethora of kinases is more likely to be responsible for the phosphorylation differences than a deficiency or decreased activity of a phosphatase, which should be compensated by the second allele.

Several reports suggest that gene mutations or genetic polymorphisms might be associated with the risk of MM.[39–41] However, results have been inconsistent and significant findings have not been replicated convincingly.[42] Hyperphosphorylated P-7 is the first molecularly characterized structure that provides a plausible explanation for the familial clustering of cases of MGUS/MM, at least in cases with a P-7-specific paraprotein. Indeed, we observed two pedigrees with familiar MGUS/MM, and all affected members in these two families were carriers of pP-7.[43] It is now possible to investigate whether previously reported cases of familial MGUS/MM[2,3,5,35] can also be explained by the carrier state of pP-7.

The frequency of the carrier state of pP-7 among patients with MGUS/MM and in healthy controls reveals a 13.1-fold increased risk to develop MGUS/MM for Japanese carriers and a 7.9-fold increased risk for German carriers. These are, to the best of our knowledge, the highest odds ratios for an MGUS/MM risk factor reported to date in either ethnic group.[2,4,5,44] The number of families with MGUS/MM patients carrying pP-7 is still too small to estimate the risk of a family member carrying pP-7; it is at least 13.1 and 7.9 in the Japanese and German population, respectively, but is probably much higher, because other, yet unidentified, genetic factors shared between family members might further increase the risk for MGUS/MM among family members.

The odds ratios for carriers of pP-7 to develop MGUS/MM was significant for the German study group (4/200 *vs* 35/252; $P = 0.0001$; chi-square test) and Japanese study group (1/278 *vs* 5/111; $P = 0.008$; Fisher's exact test). Testing of family members for the pP-7 carrier state by simple IEF enables the identification of family members who are at increased risk of developing MGUS/MM.

In contrast to the carrier state of pP-7, which is under exclusive genetic control, the nature of the immune response against pP-7 is complex and might involve both genetic and environmental factors. The fact that genetic factors are relevant is suggested by the previous findings that all IgG paraproteins with specificity for P-7, analyzed to date, are of the IgG_3 subclass and 42.1% of all IgG_3 paraproteins react with pP-7.[19] The frequency of pP-7 as an antigenic target and/or stimulus for paraprotein-producing clones and the availability of many families with MGUS/MM patients with the pP-7 carrier state now allow for the analysis of tumor-host interactions in the presence and absence of the antigen in the respective patients and family members, and to study more specifically the role of environmental factors and immunoregulatory deficiencies, such as the recently reported dysfunction of regulatory T cells[45] in patients with MGUS and MM.

The fact that pP-7 functions as the antigenic target of the paraproteins of all MGUS/MM patients with pP-7, suggests that the hyperphosphorylated protein plays a role in the development of sporadic and familial MGUS/MM. The hyperphosphorylation of P-7 appears to be the most obvious likely reason for its autoimmunogenicity. Whether pP-7 induces the development of MGUS/MM by chronic antigenic stimulation or whether it is only a marker or an epiphenomenon of another dominantly inherited susceptibility to develop MGUS/MM can now be investigated in the respective patients and their (not yet) affected relatives.

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Disclosure Statement

KDP and MP have applied for a relevant patent. None of the other authors have a conflict of interest.

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