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IJC
International Journal of Cancer

Over one-third of African-American MGUS and multiple myeloma patients are carriers of hyperphosphorylated paratarg-7, an autosomal dominantly inherited risk factor for MGUS/MM

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As hyperphosphorylated paratarg-7 (pP-7) carrier state was shown to be the first molecularly defined autosomal dominantly inherited risk factor for monoclonal gammopathy of unknown significance (MGUS) and multiple myeloma (MM) in a European population, the prevalence of pP-7 carrier state among African-Americans who have a significantly higher incidence of MGUS/MM is of interest. We therefore determined pP-7 carrier state and paraproteins with specificity for P-7 in African-American, European and Japanese patients with MGUS/MM and healthy controls. By isoelectric focusing and ELISA, a paratarg-7-specific paraprotein and the associated pP-7 carrier state was observed in 30/81 (37.0%) African-American, 42/252 (16.7%) European and 7/176 (4.0%) Japanese MGUS/MM patients ($p < 0.001$). A pP-7 carrier state was found in 11/100 (11.0%) African-American, 8/550 (1.5%) European and 1/278 (0.4%) Japanese healthy controls ($p < 0.001$), resulting in an odds ratio for MGUS/MM of 4.8 ($p < 0.001$) among African-American, 13.6 among European ($p < 0.001$) and 11.5 ($p = 0.023$) among Japanese carriers of pP-7. We conclude that pP-7 carriers are most prevalent among African-Americans, but a pP-7 carrier state is the strongest molecularly defined single risk factor for MGUS/MM known to date in all three ethnic groups. The high prevalence of pP-7 carriers among African-American patients emphasizes a predominant role of this genetic factor in the pathogenesis of these diseases. The large number of pP7 African-American patients and controls should facilitate the identification of the SNP or mutation underlying the pP-7 carrier state.

Epidemiology

A causal relationship between monoclonal gammopathy of unknown significance (MGUS) and multiple myeloma (MM) and chronic antigenic stimulation has been suggested by the results of several studies[1]; hence, the identification of the antigenic stimuli of B-cell neoplasms is of interest. In a modification of SEREX[2] using a commercially available human fetal brain-derived protein macroarray and IgA or IgG paraprotein-containing sera, paratarg-7 was shown to be a frequent antigenic target of paraproteins from European patients with MGUS, MM and Waldenstrom's macroglobulinemia.[3–5] All patients with paratarg-7-specific paraproteins were carriers of a hyperphosphorylated version of the protein (pP-7) and this hyperphosphorylation is inherited in a dominant fashion.[3–5] The hyperphosphorylation of paratarg-7 in these patients is due to an inactivation of protein phosphatase 2A resulting in the failure to dephosphorylate the physiologically occurring phosphorylation of p-7 at serine 17.[6] Paratarg-7 is identical to STOML2 (stomatin [EPB72]-like), also known as HSPC108 or stomatin-like-protein and SLP-2,[7] which is expressed in all human tissues.[8] The physiological function of SLP-2 is unknown. As few healthy Europeans are carriers of pP-7, pP-7 carrier state is associated with an

Key words: MGUS, multiple myeloma, risk factors, genetics
*These authors contributed equally to this work
Grant sponsors: Wilhelm Sander-Stiftung (a charity organization), Deutsche Forschungsgemeinschaft (German Research Society, DFG Association), Deutsche Krebshilfe (a charity organization)
DOI: 10.1002/ijc.28731
History: Received 13 Nov 2013; Accepted 19 Dec 2013; Online 20 Jan 2014
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What's new?

African-Americans are more than twice as likely as the general population to develop monoclonal gammopathy of unknown significance (MGUS) and multiple myeloma (MM). A hyperphosphorylated form of the protein paratarg-7, called pP-7, is a dominantly inherited risk factor for MGUS/MM. In this study, the authors found that more than one third of African-American MGUS/MM patients expresspP-7. This suggests that pP-7 might play a role in the pathogenesis of MGUS/MM, and should facilitate the identification of the SNP or mutation responsible for the pP-7 carrier state.

increased risk for MGUS/MM. Thus, pP-7 is the first molecularly defined inherited risk factor for any hematological neoplasm known to date. The incidence of MGUS and MM is lower in Asians and higher in African-Americans than in Europeans[9,10]; therefore, we compared these three ethnic groups with respect to the prevalence of a pP-7 carrier state and the incidence of P-7-specific paraproteins in MGUS/MM and healthy controls.

Material and Methods

Patients and controls

This study was approved by the local ethical review boards of the participating institutions. Consecutive patients with MGUS/MM with an IgA, IgD or IgG paraprotein were included. Healthy European, Japanese and African-American blood donors served as controls. Healthy was defined as having no monoclonal immunoglobulin by serum electrophoresis and immunofixation, and being healthy as diagnosed by the Medical Officer in the pre-donation check-up. Samples of African-American MGUS/MM patients and patients with other malignant diseases were collected at Emory University Hospital in Atlanta, GA, as were healthy African-American controls. Autoimmune-disease blood samples were obtained from patients diagnosed and treated at the Autoimmune Disease Clinics of the Department of Internal Medicine I, Saarland University Medical School. Written informed consent was obtained from all patients and controls. Peripheral blood was centrifuged, and plasma and cells were stored at −20°C.

Isoelectric focusing for the determination of the pP-7 carrier state

The isoelectric focusing to determine the pP-7 carrier state was performed as described before.[4,6,11]

Paratarg-7 ELISA for the detection of paraproteins with specificity to paratarg-7

The paratarg-7 ELISA using full-length recombinant paratarg-7 was performed as described previously.[3]

Statistical methods

Odds ratios with 95% confidence intervals (CIs) and p-values are presented to describe the risk for MGUS/MM (in relation to healthy controls) in pP-7 carriers separately for each of the three ethnic groups. To compare the odds ratios between ethnic groups Breslow–Day tests were performed. Chi-square and if necessary Fisher's exact tests were used to test differences between ethnic groups regarding the prevalence of pP-7, separately for MM/MGUS patients and healthy controls. In case of significant global test over all three ethnic groups pairwise tests were performed. For comparison of prevalence of pP-7 carriers among healthy controls and MGUS/MM patients within each of the ethnic groups we used chi-square and if necessary Fisher's exact tests. For differences regarding patient characteristics, we calculated chi-square and if necessary Fisher's exact tests for qualitative data and Wilcoxon rank sum tests for quantitative data (Table 2). The significance level was $p = 0.05$. Statistical analyses were done with IBM SPSS Statistics 20.

Results

A total of 252 European, 176 Japanese and 81 Afro-American MGUS and MM patients were included in this study. About 30/81 (37.0%) African-American, 42/252 (16.7%) European and 7/176 (4.0%) Japanese MGUS/MM

Table 1. Detection of paratarg-7 specific paraproteins in consecutive African-American, European and Japanese patients with MGUS/MM

	MGUS (%)	MM (%)	Total (%)
African-Americans			
IgA	2/6 (33.3)	2/4 (50)	4/10 (40.0)
IgD	0/0	0/1	0/1
IgG[1]	8/22 (36.4)	18/42 (42.9)	26/64 (40.6)
Light chain	0/0	0/6	0/6
Total	10/28 (35.7)	20/53 (37.7)	30/81 (37.0)
Europeans			
IgA	1/7 (14.3)	4/24 (16.7)	5/31 (16.1)
IgD	0/0	0/0	0/0
IgG[2]	5/45 (11.1)	32/176 (18.2)	37/221 (16.7)
Total	6/52 (11.5)	36/200 (18.0)	42/252 (16.7)
Japanese			
IgA	0/4	1/32 (3.1)	1/36 (2.8)
IgD	0/0	0/11	0/11
IgG[2]	0/13	6/116 (5.2)	6/129 (4.7)
Total	0/17	7/159 (4.4)	7/176 (4.0)

[1]All pP-7 reactive IgG paraproteins were of the IgG_3 subclass except 1 IgG_1.
[2]All pP-7 reactive IgG paraproteins were of the IgG_3 subclass.

Table 2. Characteristics of African-American MGUS and MM patients who are carriers of hyperphosphorylated paratarg-7 and those who are not

	pP7$^+$ $n = 30$ (%)	pP7 $n = 51$ (%)	p-value[1]
Age (years)			
Median	64	64	0.325
Range	43–89	32–84	
Diagnosis			
MGUS	10 (33.3)	17 (33.3)	1.000
Multiple Myeloma	19 (63.3)	32 (62.7)	0.958
Stage I	3 (15.8)	3 (9.7)	0.778[2]
Stage II	7 (36.8)	11 (35.5)	
Stage III	9 (47.4)	17 (54.8)	
Unknown		1 (3.1)	
Plasmacytoma	1 (3.3)	2 (3.9)	1.000
Paraprotein			
IgG	26 (86.7)	38 (74.5)	0.194
IgG_1	1 (3.3)	23 (45.1)	<0.001
IgG_2	0 (0.0)	0 (0.0)	–
IgG_3	25 (83.3)	15 (29.4)	<0.001
IgG_4	0 (0.0)	0 (0.0)	–
IgA	4 (13.3)	6 (11.8)	1.000
IgD	0 (0.0)	1 (2.0)	1.000
Light chain	0 (0.0)	6 (11.8)	0.080
Lytic lesions			
Yes	14 (48.3)	27 (52.9)	0.688[3]
No	15 (51.7)	24 (47.1)	
Unknown		1 (3.3)	
Hemoglobin (g/dl)			
Median	11.1	11.3	0.491
Range	7.2–14.9	6.7–15.8	
Creatinine (mg/dl)			
Median	1.1	1.4	0.082
Range	0.7–5.0	0.6–20.0	
Calcium (mg/dl)			
Median	10.0	9.4	0.221
Range	2.9–13.0	7.0–14.0	
ß2 microglobulin (mg/l)			
Median	3.0	3.6	0.637
Range	2.0–31.6	1.5–19.9	
Cytogenetics			
Normal	10 (33.3)	25 (49.0)	0.169
Trisomy 3	1 (3.3)	0 (0.0)	0.370
Trisomy 8	0 (0.0)	1 (2.0)	1.000
Complex	12 (40.0)	12 (23.5)	0.117
Unknown	7 (23.3)	13 (25.5)	

[1]For qualitative data chi-square test and if necessary Fisher's exact test and for quantitative data Wilcoxon rank sum test were used.
[2]p-value for 'Stages I–III'.
[3]p-value without 'unknown'.

Table 3. Prevalence of pP-7 carrier state in African-American patients with hematological neoplasms other than MGUS/MM

Acute lymphocytic leukemia	0/2
Acute myeloid leukemia	0/4
Chronic lymphocytic leukemia	2/13
Chronic myeloid leukemia	2/6
Diffuse large B-cell lymphoma	2/24
Follicular lymphoma	0/7
Hodgkin lymphoma	0/8
Lymphomas, others	2/8
Total	8/72

Table 4. Prevalence of pP-7 carrier state in European patients with autoimmune diseases

Autoimmune disorder	
- Rheumatoid arthritis	1/100
- Systemic lupus erythematosus	0/30
- Polymyalgia rheumatic	0/15
- Granulomatosis with polyangiitis	0/10
- Multiple sclerosis	0/10
- Crohn's disease	0/10
- Churg–Strauss syndrome	0/5
- SAPHO syndrome	0/5
- Sjögren's syndrome	0/5
Total autoimmune disorders	1/190 (0.5%)
European healthy controls	8/550 (1.5%)

patients had a paraprotein reacting with paratarg-7. All these patients were pP-7 carriers (Table 1). Notably, the 30 African-American patients with a pP-7 specific paraprotein was the first among the total of 69 patients with an IgG paraprotein who did not belong to the IgG_3, but rather to the IgG_1 subtype. The characteristics of African-American MGUS/MM patients with p-7 specific paraproteins and with paraproteins that did not bind to paratarg-7 showed no significant differences (Table 2). The differences between ethnic groups regarding the prevalence of pP-7 among MM/MGUS patients were significant ($p < 0.001$) for the global test, as were the differences between African-American (37.0%) and European (16.7%; $p < 0.001$), African-American and Japanese (4.0%; $p < 0.001$) and European and Japanese patients ($p < 0.001$).

The prevalence of healthy pP-7 carriers was 11/100 (11.0%) among healthy African-Americans, 8/550 (1.5%) in Europeans and 1/278 (0.4%) in Japanese ($p < 0.001$ in the global test). The prevalence of pP-7 carriers in the European and Japanese controls was not different ($p = 0.286$), but the prevalence of healthy pP-7 carriers in the African-Americans were significantly higher than in the European and Japanese population ($p < 0.001$ and $p < 0.001$), respectively.

The prevalence of pP-7 carriers was lower in healthy controls than in MGUS/MM patients in all three ethnic groups (African-Americans: 11.0% vs. 37.0%, $p < 0.001$; Europeans: 1.5% vs. 16.7%, $p < 0.001$; Japanese 0.4% vs. 4.0%, $p = 0.007$) resulting in an elevated risk for MGUS/MM among healthy pP-7 carriers (odds ratio: African-Americans: 4.8 [95% CI: 2.2–10.3], $p < 0.001$; Europeans: 13.6 [95% CI: 6.3–29.3], $p < 0.001$; Japanese 11.5 [95% CI: 1.4–94.1], $p = 0.023$). Thus, pP-7 carrier state is the strongest molecularly defined single risk factor for MGUS/MM known to date in all three ethnic groups. The p-value for differences in the odds ratios for MGUS/MM among healthy pP-7 carriers was 0.058 between African-Americans and Europeans, 0.430 between African-Americans and Japanese and 0.884 between Europeans and Japanese.

Discussion

This study is the first that investigated pP-7 carrier state in African-American patients. The high prevalence of pP-7 carriers among African-American MGUS/MM patients is astonishing and intriguing. The frequency of paratarg-7 as a paraprotein target in more than one-third of all African-American MGUS/MM patients suggests a role of pP-7 in the pathogenesis of these diseases in all three ethnic groups. The fact that carriers of pP-7 are only at a higher risk for MGUS/MM and Waldenstrom's macroglobulinemia (which was not included in this study due to the very low incidence of Waldenstrom's macroglobulinemia in African-Americans), but not for other malignancies in neither the European[5] nor the African-American population (Table 3) suggests that the contribution of pP-7 to the pathogenesis is more likely to be mediated by chronic antigenic stimulation and not due to a higher intrinsic potential of cells carrying the hyperphosphorylated paratarg-7 for malignant transformation. This is also supported by the fact that while all 79 patients included in this study with a paraprotein reacting with paratarg-7 were carriers of the hyperphosphorylated version of paratarg-7, none of the 430 MGUS/MM patients with paraproteins not reacting with paratarg-7 were carriers of pP-7.

Notably, the autoimmunity in pP-7 carries appears to be specific for P-7, because we found no increased frequency of pP-7 carriers among 190 patients with autoimmune disease (Table 4).

We do not know the age of the healthy blood donors, but presumably they were considerably younger than the patients. The younger age of the donors does not affect the prevalence data, because pP-7 carriership is inherited and present from birth to death. However, some of the healthy donors might develop MGUS/MM later in their life, but this would only increase the odds ratios for MGUS/MM among healthy pP-7 carriers. We also checked the healthy carriers for anti-P-7 antibodies, because we would expect a polyclonal anti-P-7 reactivity followed by the appearance of an immortalized clone. However, so far we have not (yet) detected such a polyclonal anti-paratarg-7 reactivity in the sera of healthy pP-7 carriers.

Although the differences in the risk for MGUS/MM failed to become significant between the three ethnic groups due to the limited number of patients, there was a strong trend ($p = 0.058$) for a difference between African-Americans and Europeans, which was weaker for the smaller number of African-American and Japanese probands. The reasons for the different risk ratios to develop MGUS/MM in patients of different ethnic background remain to be determined, and might be due to either environmental or additional genetic factors. In this respect, studies of African populations from Africa or Japanese from Hawaii would be very interesting, but even though we tried hard, we got no access to such populations.

We expect the risk of a pP-7 carrier in the family of a pP-7 patient to be even considerably higher, but the number of families with multiple cases of pP-7 MGUS/MM in our database is (still) too small to prove this with a solid statistics. Nevertheless, because two robust tests are available (IEF for the identification of a pP-7 carrier state and ELISA for the detection of paraproteins with specificity for paratarg-7), the identification of family members from a pP-7 MGUS/MM patient at risk, i.e., carriers of pP-7 is an easy task.

With the frequency of pP-7 carriers among healthy African-Americans and patients with MM/MGUS, sufficient numbers of pP-7 MGUS/MM patients and healthy wtP-7 and pP-7 family members should now be available to scrutinize tumour–host interactions in the presence and absence of the antigenic pP-7 in these individuals. Most importantly, the large number of African-American pP-7 carriers both among MGUS/MM patients and healthy controls should now facilitate the dissection of the break-down of tolerance against the autoantigenic pP-7 and the identification of the SNP or mutation responsible fore the inactivation of PP2A, which causes and maintains the hyperphosphorylation of paratarg-7 in individuals with a pP-7 carrier state.[6]

Acknowledgements

The authors thank all patients and their families for participating in the study.

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ORIGINAL

International Journal of Myeloma 5(1): 1-7, 2015

Induction of endoplasmic reticulum stress by bortezomib sensitizes myeloma cells to DR5-mediated cell death

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TNF-related apoptosis-including ligand/Apo2 (TRAIL)-mediated immunotherapy is an attractive anti-tumor modality with high tumor specificity. In order to improve its therapeutic efficacy, we further need to implement a novel maneuver for sensitization of malignant cells to TRAIL. Bortezomib (BTZ), a novel anti-myeloma (MM) agent, potently induces endoplasmic reticulum (ER) stress to cause apoptosis. Here, we explored the roles of BTZ in the cytotoxicity of anti-TRAIL receptor agonistic antibodies against MM cells with special reference to ER stress. BTZ enhanced the expression of death receptor 5 (DR5) but not DR4 in MM cells at surface protein as well as mRNA levels. However, the DR5 expression was not affected by BTZ without ER stress induction in MM cells with a point mutation in a BTZ-binding proteasome β_5 subunit. Tunicamycin, an ER stress inducer, was able to enhance the DR5 expression even in the BTZ-resistant MM cells, suggesting the role of ER stress in up-regulation of DR5 expression. Interestingly, BTZ facilitated extrinsic caspase-mediated apoptosis by anti-DR5 agonistic antibody in MM cells along with reducing c-FLICE-like interleukin protein, a caspase 8 inhibitor. These results suggest that BTZ enhances DR5 expression and its downstream apoptotic signaling through ER stress to sensitize MM cells to TRAIL-mediated immunotherapy.

Key words: multiple myeloma, bortezomib, TRAIL, DR5, ER stress

Received: August 7, 2014, accepted: September 21, 2014
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Introduction

Multiple myeloma (MM) still remains incurable because of its resistance to chemotherapeutic drugs and an escape from tumor immune surveillance, although new agents as well as high dose chemotherapy followed by stem cell transplantation have been introduced into a clinical practice with better quality of therapeutic response and outcome. Therefore, alternative approaches are needed to overcome drug resistance to improve the therapeutic outcome in patients with MM.

Immunotherapies have been getting generally accepted as attractive treatment options for yet incurable malignancies by conventional chemotherapeutic agents. One such approach is a TNF-related apoptosis-including ligand/Apo2 (TRAIL)-mediated immunotherapy[1-3]. TRAIL binds to two different proapoptotic receptors, death receptor 4 (DR4) and DR5. Unlike Fas ligand and TNF-α, TRAIL is able to induce cell death in malignant cells with marginally affecting normal tissues; TRAIL-mediated immunotherapy is, therefore, regarded as an attractive tumor-specific strategy against various cancers, including MM[4-6].

However, weak expression of the TRAIL receptors as well as the suppression of their downstream pro-apoptotic signaling often cause malignant cell resistance to TRAIL; and sensitization of malignant cells to TRAIL has become a major issue in the TRAIL-mediated immunotherapy. To restore the sensitivity to TRAIL, we need to develop novel therapeutic maneuvers to up-regulate surface TRAIL receptors along with stimulation of DR-mediated pro-apoptotic signaling.

The proteasome inhibitor bortezomib (BTZ) is widely used in treatment of MM with improved response rates in patients with both relapsed/refractory and newly diagnosed MM[7]. BTZ induces misfolded protein accumulation in MM cells followed by endoplasmic reticulum (ER) stress-associated apoptosis[8,9]. However, the effects of ER stress induced by BTZ on TRAIL-mediated MM cell death are largely unknown. In the present study, we therefore aimed to clarify the role of BTZ on TRAIL receptor editing and TRAIL-mediated cell death in MM cells with special reference to ER stress. We demonstrated here that BTZ enhanced the surface expression of DR5 but not DR4 in MM cells and its downstream apoptotic signaling through the induction of ER stress to sensitize MM cells to an anti-DR5 agonistic antibody.

Materials and Methods

Reagents

Bortezomib was purchased from Millenium Pharmaceuticals, Inc. (Cambrigde, MA, USA). Rabbit monoclonal antibodies against caspase 9, caspase 3, cleaved caspase 3, poly (ADP-ribose) polymerase (PARP), and mouse monoclonal antibodies against activating transcription factor 4 (ATF4) and C/EBP-homologous protein (CHOP) were purchased from Cell Signaling Technology Japan (Tokyo, Japan). Mouse monoclonal antibodies against caspase 8, c-FLICE-like interleukin protein (c-FLIP) and β-actin were obtained from Medical and Biotechnological Laboratories (Nagoya, Japan), Santa Cruz Biotechnology (Santa Cruz, CA), Abcam (Cambridge, UK) and Sigma (Saint Louis, MO), respectively. FITC-conjugated mouse monoclonal antibodies against human DR4 and DR5 were from Biolegend (San Diego, CA). Horseradish peroxidase-conjugated goat anti-mouse antibody was from Invitrogen Life Technologies (Carlsbad, CA). The human monoclonal anti-DR5 agonistic antibody R2-E11 was a kind gift from from Kyowa Hakko Kirin Co. Ltd. (Tokyo, Japan).

Cells and cultures

The use of human samples was approved by the Institutional Review Board at University of Tokushima (Tokushima, Japan), and informed consent was obtained according to the Declaration of Helsinki. Peripheral blood mononuclear cells (PBMCs) were isolated from fresh peripheral blood from healthy donors[10]. Primary MM cells were purified from bone marrow mononuclear cells (BMMCs) using CD138 microbeads and a magnetic cell sorting system (Miltenyi Biotec, Auburn, CA). Human MM cell lines, RPMI 8226, KMS-11 and U266, were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The human MM cell line OPC was established in our laboratory[11]. The human MM cell line INA-6 was kindly provided by Dr. Renate Burger (University of Kiel, Kiel, Germany). The human MM cell lines OPM-2 was purchased from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). BTZ-resistant MM cell lines with a point mutation in the β_5 subunit of a 26S proteasome, KMS-11/BTZ and OPM-2/BTZ, were kindly provided from Kyowa Hakko Kirin Co. Ltd. (Tokyo, Japan)[12]. Cells were cultured in RPMI1640 (Sigma) supplemented with 5% FCS (Life Technologies, Grand island, NY), penicillin (100 units/mL), and streptomycin (100 μg/mL) at 37°C in a humidified atmosphere with 5% CO_2.

Flow cytometry

Cell preparation and staining for flow cytometry were performed as described previously[13]. Briefly, cells were incubated in 100 μl PBS with 2% human γ-globulin with saturating concentrations of different FITC-conjugated monoclonal antibodies on ice for 40 minutes. They were then washed and analyzed by flow cytometry using EPICS-Profile (Coulter Elecronics, Hialeah, FL).

Cell viability and apoptosis assay

MM cells were incubated with various concentrations of BTZ with or without TRAIL agonistic antibody at 37°C for 48 hours. Viable cell numbers were measured by a cell proliferation assay using 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-8; Kishida Chemical, Osaka, Japan). Apoptosis in MM cells was evaluated by staining the cells with an annexinV-FITC and propidium iodide labeling kit (MEBCYTO Apoptosis Kit; MBL, Nagano, Japan) according to the manufacturer's instruction.

Western blot analysis

Cells were collected and lysed in a lysis buffer (Cell Signaling, Beverly, MA) supplemented with 1 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail solution (Sigma). The cell lysates were subjected to SDS-PAGE on a 10% polyacrylamide gel, and then transferred to polyvinylidene difluoride membranes (Millpore, Billerica, MA). The membranes were blocked with 5% non-fat dry milk in TBS with 0.01% Tween 20 for 1 hour at room temperature and incubated for 16 hours at 4°C with the primary antibodies. After washing, a secondary horseradish peroxidase-conjugated antibody was added

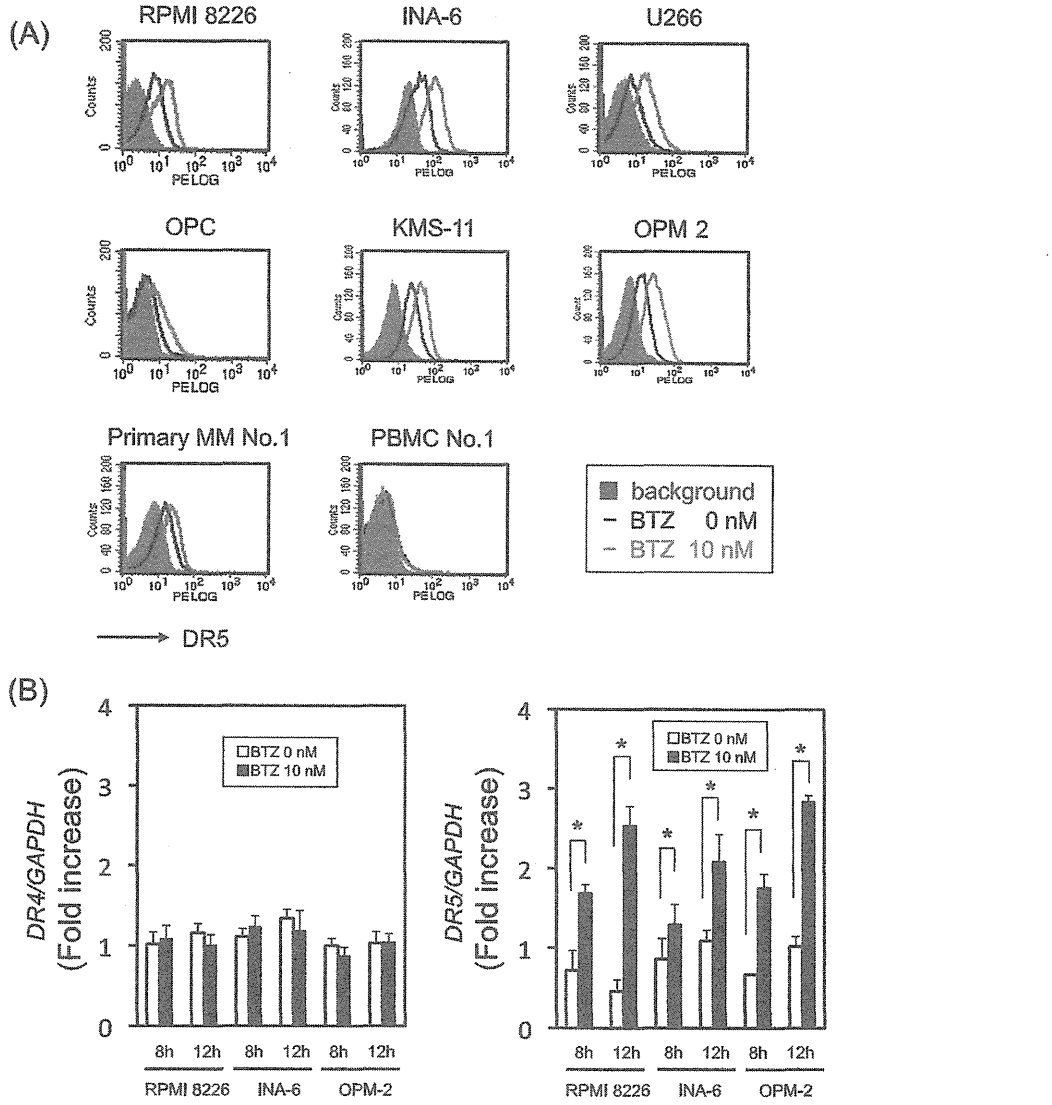

Figure 1. Up-regulation of DR5 expression in MM cells by BTZ. (A) MM cell lines, primary MM cells, and peripheral blood mononuclear cells (PBMCs) were incubated with BTZ at 10 nM for 24 hours, and the surface expression of DR5 was analyzed by flow cytometry. (B) RPMI 8226, INA-6 and OPM-2 cells were incubated with BTZ at 10 nM for different periods as indicated. *DR4* and *DR5* mRNA expression was determined by real time RT-PCR. *GAPDH* was used as an internal control. *$P < 0.05$.

and the membranes were developed using the enhanced chemiluminescence plus Western blotting detection system (American Biosciences, Piscataway, NJ).

Quantitative real-time PCR

Cells were harvested and total RNA was extracted from cells using TRIZOL reagent (Invitrogen). Equal amounts of total RNA were subjected to reverse transcription using Superscript II (Invitrogen). Real-time PCR was performed using Platinium SYBR Green qPCR SuperMix UDG with Rox (Invitrogen) with the following amplification program: one cycle of 50°C for 2 minutes and 95°C for 2 minutes and 40 cycles of 95°C for 15 seconds and 60°C for 30 seconds. The reaction was followed by a melting curve protocol according to the specifications of the ABI 7300 (Applied Biosystems, Foster City, CA, USA). Primers used were as follows: *DR4* sense 5′-AAGTTTGTCGTC GTCGGGGTCCT-3′ and antisense 5′-GGTGGACACACTCTCCCA AAGGGC-3′, *DR5* sense 5′-TCTCCTGAGATGTGCCGGAAGTGCC-3′ and antisense 5′-GCTGGGACTTCCCCACTGTGCTTT-3′, *GAPDH* sense 5′-AATCCCATCACCATCTTCCA-3′ and antisense 5′-TGGAC TCCACGACGTACTCA-3′. Products were run on 2% agarose gels containing ethidium bromide.

Statistical analysis

Comparisons between experimental data were performed by one-way analysis of variance (ANOVA) or one-sided, paired t-test. *P* below .05 was considered statistically significant.

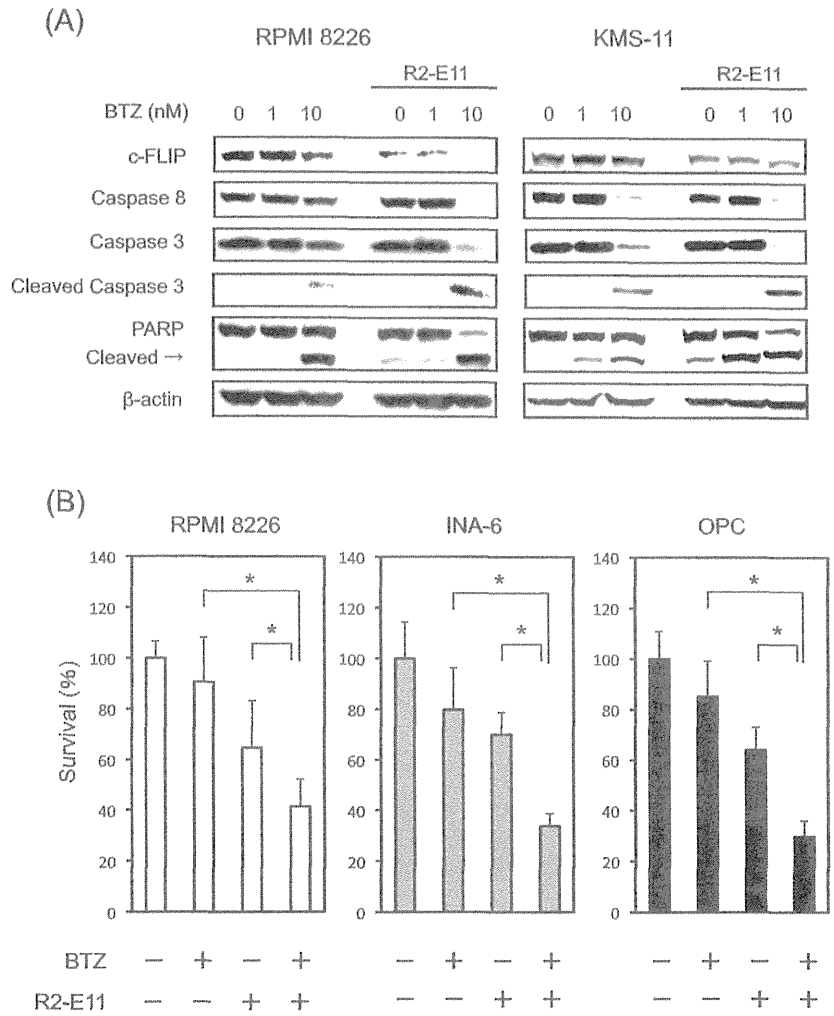

Figure 2. Induction of caspase activation and cell death in MM cells. (A) RPMI 8226 and KMS-11 cells were treated with BTZ at the indicated concentrations or the anti-DR5 agonistic antibody R2-E11 at 100 ng/mL alone or both in combination for 24 hours. The protein levels of c-FLIP, caspase 8, caspase 3, and PARP were analyzed by Western blotting. β-actin was used as a protein loading control. (B) RPMI 8226, INA6 and OPC cells were treated with BTZ or the anti-DR5 agonistic antibody R2-E11 at 100 ng/mL alone or both in combination for 48 hours. Cell viability was analyzed by WST-8 assay. *$P < 0.05$.

Results and Discussion

BTZ up-regulates DR5 expression in MM cells

We first examined whether BTZ affects the surface expression of TRAIL receptors, DR4 and DR5, on MM cells. BTZ at 10 nM up-regulated the surface level of DR5 on primary MM cells as well as all MM cell lines tested (Fig. 1A). However, BTZ did not up-regulate the surface level of DR4 on MM cells (data not shown). Real time RT-PCR demonstrated BTZ increased the *DR5* mRNA expression by BTZ in RPMI 8226, INA-6 and OPM-2 MM cells (Fig. 1B), suggesting the up-regulation of DR5 at transcriptional levels.

BTZ enhances anti-DR5 agonistic antibody-mediated activation of the extrinsic apoptotic pathway and death in MM cells

Because BTZ up-regulates DR5 expression in MM cells, we next looked at the effects of BTZ on anti-DR5 agonistic antibody-mediated activation of the extrinsic apoptotic pathway and death in MM cells. BTZ at 10 nM induced the activation of caspase 8 and caspase 3, and the cleavage of caspase 3 and PARP along with decreasing c-FLIP protein levels in RPMI 8226 and KMS-11 cells (Fig. 2A). Treatment with the anti-DR5 agonistic antibody R2-E11 at 100 ng/mL in combination with BTZ at 10 nM markedly reduced c-FLIP protein levels and enhanced the activation of caspase 8 and the cleavage of caspase 3 and PARP, although the anti-DR5

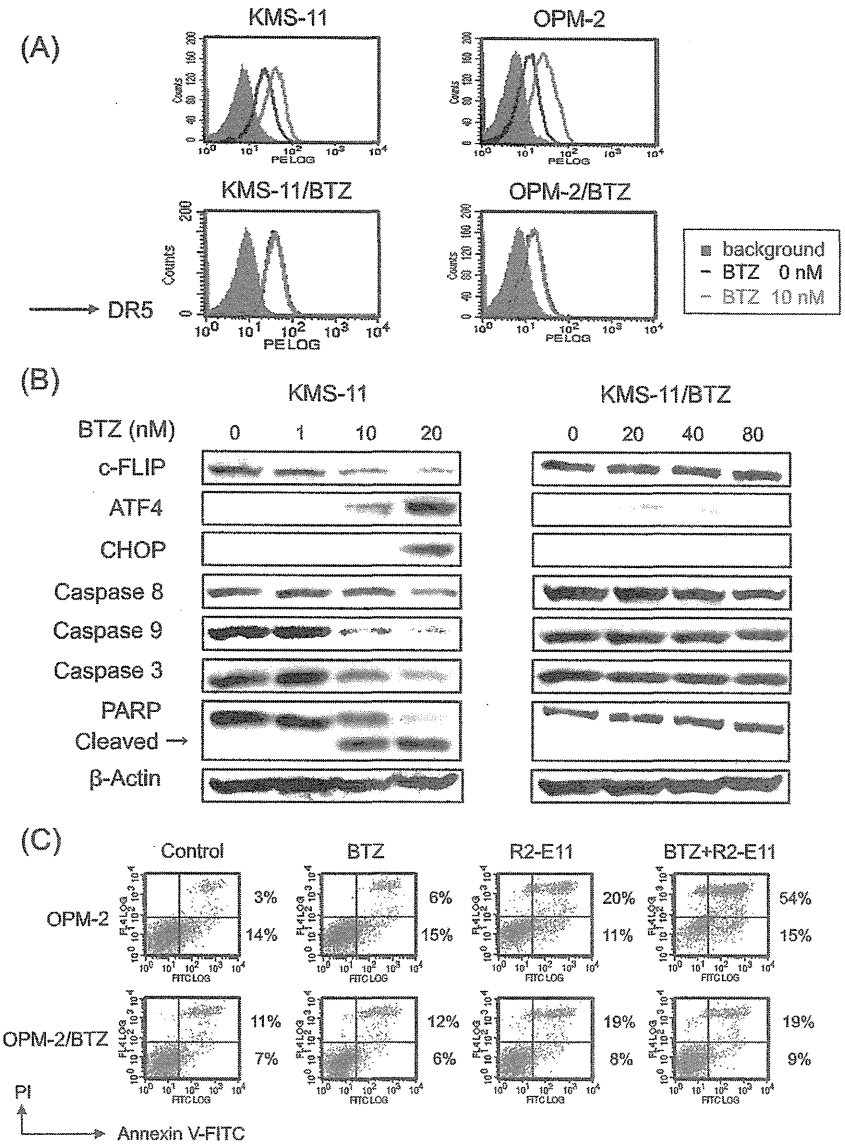

Figure 3. Up-regulation of DR5 as well as ER stress by BTZ was absent in BTZ-resistant MM cells. (A) KMS-11 and OPM-2 cells, and their derived BTZ-resistant cell lines with a point mutation in a β_5 subunit (KMS-11/BTZ and OPM-2/BTZ cells, respectively) were treated with BTZ at 10 nM for 24 hours. The surface expression of DR5 was analyzed by flow cytometry. (B) KMS-11 and KMS-11/BTZ cells were treated with BTZ at the indicated concentrations for 24 hours. The protein levels of c-FLIP, ATF4, CHOP, caspase 8, caspase 9, caspase 3, and PARP were analyzed by Western blotting. β-actin was used as a protein loading control. (C) OPM-2 and OPM-2/BTZ cells were treated with BTZ or the anti-DR5 agonistic antibody R2-E11 at 500 ng/mL alone or both in combination for 48 hours, and stained with annexin V-FITC and propidium iodide (PI). Cells were then analyzed by flow cytometry to determine the percentage distribution of cells displaying annexin V staining (early apoptosis) or both annexin V and PI staining (late apoptosis).

agonistic antibody R2-E11 alone showed only marginal effects on these caspase and PARP cleavage. Consistently, BTZ and R2-E11 in combination cooperatively enhanced cell death in RPMI 8226, INA6 and OPC cells, whereas BTZ or R2-E11 alone at this experimental condition only partially induced MM cell death (Fig. 2B). These results suggest that BTZ potentiates DR5-mediated activation of the extrinsic apoptotic pathway and cell death in MM cells.

BTZ does not up-regulate DR5 expression in MM cells with a β_5 subunit mutation

To further clarify the role of ER stress induced by BTZ on DR5 expression and DR5-mediated cytotoxicity, we examined the effects of BTZ, using KMS-11 and OPM-2 cells with a point mutation in the β_5 subunit of a 26S proteasome, KMS-11/BTZ and OPM-2/BTZ, respectively, which are resistant to BTZ-induced cell death. Although BTZ up-regulated DR5 expression on parental KMS-11 and OPM-2 cells, the DR5 up-regulation by BTZ was completely absent in the BTZ-

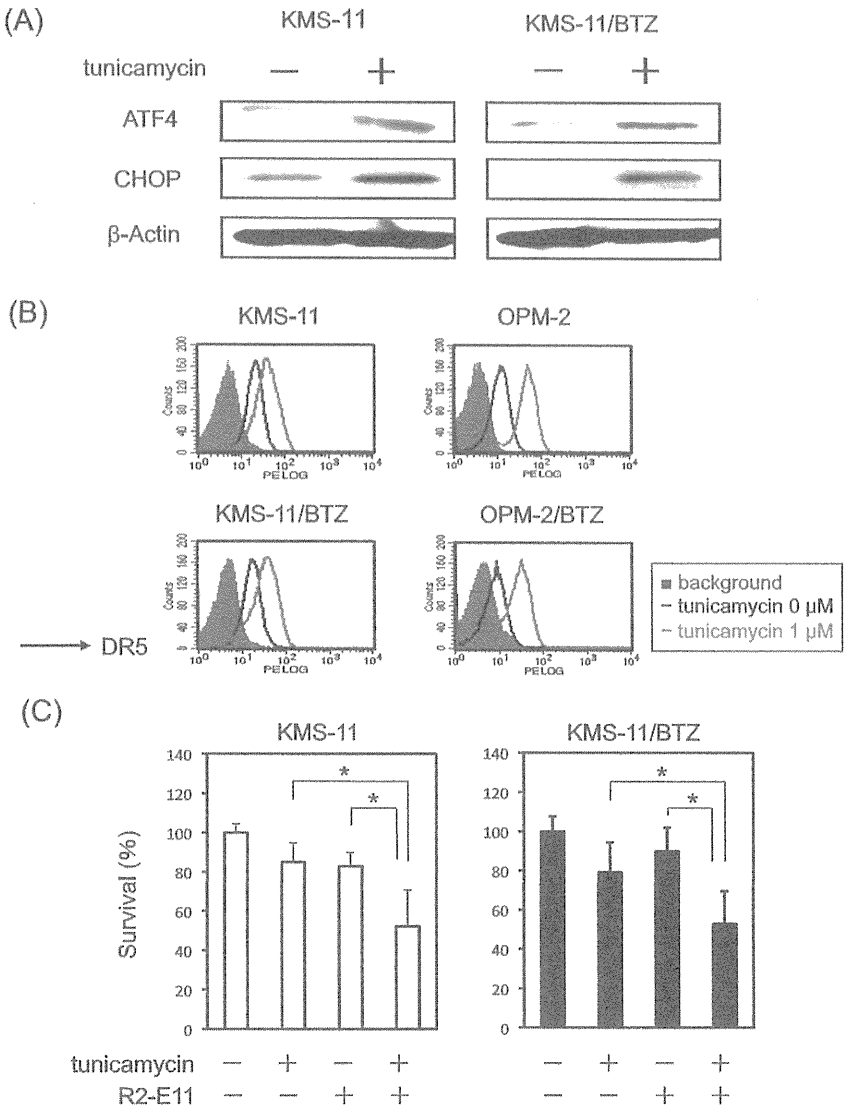

Figure 4. Induction of ER stress and DR5 in BTZ-resistant MM cells by tunicamycin. (A) KMS-11 and KMS-11/BTZ cells were incubated with tunicamycin at 1 μM for 24 hours. Cell lysates were then extracted, and the protein levels of ATF4 and CHOP were analyzed by Western blotting. (B) KMS-11 and OPM-2 cells, and their derived BTZ-resistant cell lines (KMS-11/BTZ and OPM-2/BTZ cells, respectively) were treated with tunicamycin at 1 μM for 24 hours. The surface expression of DR5 was analyzed by flow cytometry. (C) KMS-11 and KMS-11/BTZ cells were treated with tunicamycin at 1 μM or the anti-DR5 agonistic antibody R2-E11 at 100 ng/mL alone or both in combination for 48 hours. Cell viability was analyzed by WST-8 assay.*$P < 0.05$.

resistant KMS-11/BTZ and OPM-2/BTZ cells (Fig. 3A). Treatment with BTZ at 10 nM or more increased ATF4 and CHOP protein levels along with the activation of caspase 8, caspase 9 and caspase 3, and the cleavage of PARP in KMS-11 cells (Fig. 3B). However, these effects of BTZ were not observed in the BTZ-resistant KMS-11/BTZ cells. Consistently, BTZ did not induce apoptosis in the OPM-2/BTZ cells (Fig. 3C). The cytotoxic effects of the anti-DR5 agonistic antibody R2-E11 were equally observed in the parental and mutated OPM-2 cells. However, the enhancement of cell death by R2-E11 in combination with BTZ was only observed in the parental OPM-2 cells but not in the mutated ones. Therefore, the induction of ER stress and thereby DR5 up-regulation appears to be responsible for the enhancement of anti-MM effects of the combinatory treatment with an anti-DR5 agonistic antibody and BTZ.

DR5 expression is up-regulated in the BTZ-resistant MM cells under ER stress by tunicamycin

In order to further clarify the relationship between ER stress and the up-regulation of DR5, we looked at the effects of ER stress induced by the ER stress inducer tunucamycin on DR5 expression and death in the parental and β_5 subunit-mutated MM cells. Tunicamycin at 1 μM was able to increase ATF4 and

CHOP protein levels in BTZ-resistant β_5 subunit-mutated KMS-11/BTZ cells as well as their parental cells (Fig. 4A), and up-regulate the DR5 expression on the surface of both the parental and mutated KMS-11 and OPM-2 cells (Fig. 4B). Consistent with the up-regulation of the surface DR5 expression, tunicamycin at 1 μM was able to induce significant cytotoxic effects equally on both KMS-11 cells and bortezomib-resistant KMS-11/BTZ cells in combination with R2-E11 at 100 ng/mL, although tunicamycin or R2-E11 alone only minimally affected the viability of these cells in this experimental condition (Fig. 4C). These results further corroborated the critical role of ER stress in the up-regulation of DR5 in MM cells and anti-DR5 agonistic antibody-mediated MM cell death.

Because *DR5* has been demonstrated to be one of the target genes of CHOP [14,15] and ATF3 [16] induced downstream of ATF4, the up-regulation of DR5 in MM cells by BTZ is suggested at least in part due to the induction of ATF4 through ER stress. Collectively, BTZ enhances DR5 expression and its downstream apoptotic signaling through ER stress to sensitize MM cells to TRAIL-mediated immunotherapy. Furthermore, BTZ also induces death receptor-independent apoptosis as a result of excessive ER stress, which may cooperatively enhance MM cell death in combination with anti-DR5 agonistic antibody.

We have previously reported that MM cells post-translationally down-modulate the cell surface expression of DR4 but not DR5 through ectodomain shedding by endogenous TNF-α converting enzyme (TACE), and that TACE inhibition is able to restore cell surface DR4 levels and the susceptibility of MM cells to TRAIL or an agonistic antibody against DR4 [17]. TACE-mediated shedding appears to be an important mechanism for the reduction of surface DR4 levels on MM cells, which may blunt TRAIL-mediated apoptosis by surrounding immune cells expressing TRAIL to protect MM cells. Thus, DR4 and DR5 editing and expression on the surface of MM cells appear to be differentially regulated. BTZ and TACE inhibitors seem good options to revitalize TRAIL-mediated immunotherapy whose therapeutic efficacy has been limited as a single treatment modality. The combination of TRAIL-mediated immunotherapy with BTZ and/or TACE inhibitors is warranted for further study in patients with MM.

Acknowledgments

This work was supported in part by JSPS KAKENHI grant numbers 23591390, 25860789 and 26461422. The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. The authors thank Kyowa Hakko Kirin Co. Ltd. (Tokyo, Japan) for providing the human monoclonal anti-DR5 agonistic antibody R2-E11 and BTZ-resistant MM cell lines, KMS-11/BTZ and OPM-2/BTZ.

Conflicts of Interest Disclosures

The authors declare no competing financial interests related to this work.

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Cancer Science
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Changing trends in prognostic factors for patients with multiple myeloma after autologous stem cell transplantation during the immunomodulator drug/proteasome inhibitor era

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Key words
Autologous stem cell transplantation, immunomodulator drugs, International Staging System, multiple myeloma, proteasome inhibitors

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Funding Information
This study was supported by the International Myeloma Foundation Japan.

Received August 25, 2014; Revised November 24, 2014; Accepted December 4, 2014

Cancer Sci (2015)

doi: 10.1111/cas.12594

We evaluated the clinical significance of prognostic factors including the International Staging System (ISS) and modified European Group for Blood and Marrow Transplantation response criteria in 1650 Japanese patients with multiple myeloma (MM) who underwent upfront single autologous stem cell transplantation (ASCT). We categorized patients into two treatment cohorts: pre-novel agent era (1995–2006) and novel agent era (2008–2011). The combined percentage of pre-ASCT complete response and very good partial response cases (463 of 988, 47%) significantly increased during the novel agent era compared with the pre-novel agent era (164 of 527, 31%; $P < 0.0001$). The 2-year overall survival (OS) rate of 87% during the novel agent era was a significant improvement relative to that of 82% during the pre-novel agent era ($P = 0.019$). Although significant differences in OS were found among ISS stages during the pre-novel agent era, no significant difference was observed between ISS I and II ($P = 0.107$) during the novel agent era. The factors independently associated with a superior OS were female gender ($P = 0.002$), a good performance status ($P = 0.024$), lower ISS ($P < 0.001$), pre-ASCT response at least partial response ($P < 0.001$) and ASCT during the novel agent era ($P = 0.017$). These results indicate that the response rate and OS were significantly improved, and the ISS could not clearly stratify the prognoses of Japanese patients with MM who underwent upfront single ASCT during the novel agent era.

The prognosis of patients with multiple myeloma (MM) has improved since the introduction of novel treatment agents such as bortezomib, thalidomide and lenalidomide. Bortezomib is classified as a proteasome inhibitor and thalidomide/lenalidomide as immunomodulator drugs. During the pre-novel agent era, an international collaborative project developed the International Staging System (ISS) based on serum albumin and β2-microglobulin levels.[1] This system has been widely used in both young and elderly patients with MM treated with either conventional chemotherapy or autologous stem cell transplantation (ASCT) after high-dose melphalan conditioning during the novel agent era. However, the validity of the ISS for prognostic predictions has not been verified in Asian patients with MM. We analyzed the prognostic factors of a large cohort of newly diagnosed Japanese patients with MM who underwent upfront single ASCT after high-dose melphalan (200 mg/m^2; Mel 200) treatment during both the pre-novel and novel agent eras.

Materials and Methods

Data source and patients. For this retrospective observational study, data were collected and analyzed using the Transplant Registry Unified Management Program (TRUMP) of the Japan Society for Hematopoietic Cell Transplantation (JSHCT). Patient consent is not required for JSHCT TRUMP registration because the registry data comprise anonymized clinical information. This study was approved by the data management committees of JSHCT and the institutional review boards of the Kanazawa University Graduate School of Medical Science, Japan.

Because bortezomib, thalidomide and lenalidomide were released for treatment of relapse/refractory MM in Japan in December 2006, February 2009 and July 2010, respectively,

we categorized the patients into two treatment cohorts: pre-novel (1995–2006) and novel agent eras (2008–2011). The study participants included 1650 Japanese patients (936 men and 714 women) with a median age of 58 years (range: 18–73 years) who underwent upfront single ASCT after Mel 200 treatment for newly diagnosed symptomatic MM; all patients underwent an ASCT in Japan between October 1995 and December 2011. Because bortezomib was released for the treatment of relapse/refractory MM on 1 December 2006 and approved for the treatment of previously untreated MM on 16 September 2011, most patients who underwent an ASCT in 2007–2011 were first treated with conventional chemotherapies, such as VAD (infusional vincristine, doxorubicin and pulsed dexamethasone) or high-dose dexamethasone, but when patients did not achieve a sufficient response, they were then treated with novel agents before ASCT. Patients who underwent an ASCT in 2007 were excluded, because bortezomib was released in December 2006 and a relatively large number of these patients were assumed to have received induction chemotherapy without the use of novel agents. The overall survival (OS) curve of patients who underwent an ASCT in 2007 was located between that in 1995–2006 and that in 2008–2011 (data not shown). All patients were diagnosed with MM based on institutional assessment. When ASCT was performed between January 2004 and December 2011, patient responses to therapy were assessed based on the criteria of the European Group for Blood and Marrow Transplantation,[2] which was modified to include very good partial response (VGPR) and stable disease (SD), and categorized as either a complete response (CR), VGPR, partial response (PR), SD or

Table 1. Patient characteristics

	ASCT during pre-novel agent era (until 31 December 2006) (n = 654)		ASCT during novel agent era (after 1 January 2008) (n = 996)	P-value
	October 1995–December 2003 (n = 117)	January 2004–December 2006 (n = 537)	January 2008–December 2011	
Median age, years (range) at ASCT	54 (23–68)	57 (22–70)	59 (18–73)	<0.001
Age ≤65 at ASCT, n (%)	113 (96.6)	517 (96.3)	937 (94.1)	0.0497
Male, n (%)	62 (53.0)	297 (55.3)	577 (58.0)	0.223
Performance status at ASCT, n (%)				
0 or 1	100 (85.5)	475 (88.5)	906 (91.0)	0.789
>1	7 (6.0)	51 (9.5)	87 (8.7)	
Unknown	10 (8.5)	11 (2.0)	3 (0.3)	
ISS stage at diagnosis, n (%)				
I	21 (17.9)	148 (27.6)	342 (34.3)	0.992
II	21 (17.9)	167 (31.1)	376 (37.8)	
III	19 (16.2)	90 (16.8)	216 (21.7)	
Unknown	56 (47.9)	132 (24.6)	62 (6.2)	
Myeloma type, n (%)				
Light-chain only	23 (19.7)	81 (15.1)	182 (18.3)	0.194
IgA	21 (17.9)	109 (20.3)	198 (19.9)	
IgG	65 (55.6)	316 (58.8)	553 (55.5)	
IgD	5 (4.3)	16 (3.0)	28 (2.8)	
IgM	0	1 (0.2)	2 (0.2)	
Non-secreting	0	9 (1.7)	31 (3.1)	
Unknown	3 (2.6)	5 (0.9)	2 (0.2)	
Planned post-ASCT therapy, n (%)				
Thalidomide	0	1 (0.2)	28 (2.8)	<0.001
Bortezomib	0	0	24 (2.4)	<0.001
Lenalidomide	0	0	31 (3.1)	<0.001
Pre-ASCT response, n (%)		CR 45 (8.4)	CR 123 (12.3)	
nCR	18 (15.4)	VGPR 119 (22.2)	VGPR 340 (34.1)	
PR	64 (54.7)	PR 285 (53.1)	PR 434 (43.6)	
SD	11 (9.4)	SD 62 (11.5)	SD 76 (7.6)	
PD	4 (3.4)	PD 16 (3.0)	PD 15 (1.5)	
NA	20 (17.1)	NA 10 (1.9)	NA 8 (0.8)	
CR + VGPR	NA	164 (31.1)	463 (46.9)	<0.001
Non-CR + Non-VGPR	NA	363 (68.9)	525 (53.1)	
Post-ASCT response, n (%)	NA	CR 45 (15.9)	182 (26.4)	<0.001
		Non-CR 238 (84.1)	508 (73.6)	
		NA 254	306	

P-value, comparison between pre-novel and novel agent eras. Because the response of patients (n = 4) who underwent ASCT between October 1995 and December 1996 was based on institutional assessment, we excluded them from the pre-ASCT response assessment. ASCT, autologous stem cell transplantation; CR, complete response; ISS, International Staging System; NA, not assessed; nCR, near complete response; PD, progressive disease; PR, partial response; SD, stable disease; VGPR, very good partial response or better.

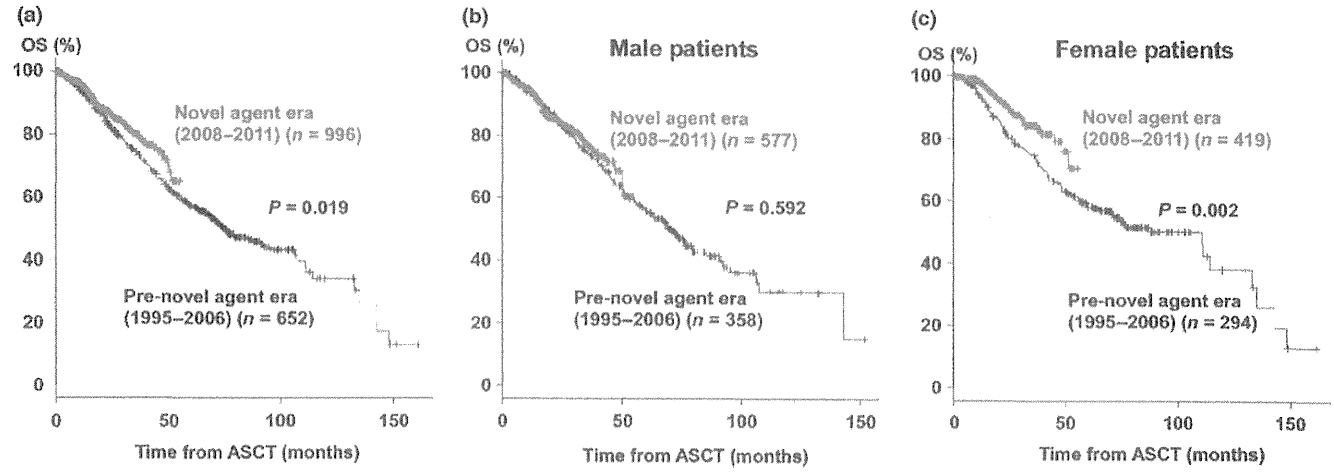

Fig. 1. Overall survival (OS) from the time of autologous stem cell transplantation (ASCT) of patients who underwent ASCT during the pre-novel and novel agent eras (a); males (b) and females (c).

Table 2. Comparison of factors associated with survival

	2-years survival (%) (95% CI)	*P*-value
Age ≤65 at ASCT	84.5 (82.3–86.4)	0.603
Age >65 at ASCT	83.2 (70.5–90.8)	
Male	83.9 (81.0–86.3)	0.014
Female	85.2 (81.9–87.9)	
Performance status at ASCT		
0 or 1	85.7 (83.6–87.7)	<0.001
>1	74.0 (65.0–81.1)	
ISS stage at diagnosis		
I	90.1 (86.6–92.7)	<0.001
II	83.2 (79.3–86.5)	
III	79.4 (73.9–83.9)	
Pre-ASCT response		
CR	85.3 (77.3–90.6)	<0.001
VGPR	88.1 (84.2–91.1)	
PR	85.3 (82.1–88.0)	
SD	78.6 (69.6–85.1)	
PD	51.6 (31.4–68.6)	
Post-ASCT response		
CR	90.6 (84.8–94.3)	0.001
Non-CR	85.4 (82.3–88.1)	
ASCT during pre-novel agent era	82.0 (78.7–84.8)	0.019
ASCT during novel agent era	86.8 (84.1–89.2)	

Pre-ASCT and post-ASCT responses were analyzed using the data of pre-novel (January 2004–December 2006) and novel agent eras (January 2008–December 2011). The overall survival was calculated from the time of ASCT. ASCT, autologous stem cell transplantation; CR, complete response; ISS, International Staging System; PD, progressive disease; PR, partial response; SD, stable disease; VGPR, very good partial response or better.

progressive disease (PD). Because we could not exclude the possibility that immunofixation electrophoresis tests were not performed in some VGPR cases, VGPR or better is indicated in this study. VGPR was defined by a ≥90% reduction in M-component levels in the serum by electrophoresis (EP) in addition to PR criteria, and SD was defined as minor response (MR) plus no change (NC). In contrast, when ASCT was performed between January 1997 and December 2003, the responses to therapy were assessed as follows: near CR required the absence of detectable M-component levels in the serum and urine by EP and plasmacytomas, which was maintained for a minimum of 4 weeks without the emergence of new lesions, together with ≤5% plasma cells in the bone marrow on the recovery of peripheral white blood cell counts, platelet counts, and Hb to $\geq 2.5 \times 10^9$/L, $\geq 100 \times 10^9$/L and ≥10 g/dL, respectively. If chemotherapy and/or interferon treatment had adverse effects on blood recovery, the aforementioned peripheral blood recovery was not required. PR was defined by a ≥50% reduction in M-component levels in the serum and urine by EP and a ≥50% reduction in the size of plasmacytomas (=long diameter × short diameter), if two dimensions were measurable, or a ≥30% reduction, if only one dimension was measurable, which was maintained for a minimum of 4 weeks without the emergence of new lesions. MR was defined as follows: (i) a 25–50% reduction in M-component levels in the serum and urine by EP, or a ≥50% reduction in M-component levels in the serum and urine by EP for <4-week duration; (ii) a 25–50% decrease in plasmacytoma size (=long diameter × short diameter), if two dimensions were measurable, or a ≥50% decrease in plasmacytoma size for <4-week duration (a 15–30% decrease, if only one dimension was measurable, or ≥30% decrease in plasmacytomas size for <4-week duration); and (iii) no emergence of new lesions for a minimum of 4 weeks. PD was defined as an increase in M-component levels and/or plasmacytomas or the emergence of new lesions. The remaining patients without new lesions for a minimum of 4 weeks were considered as NC, and SD was defined as MR plus NC. Because the response of patients ($n = 4$) who underwent ASCT between October 1995 and December 1996 was based on an institutional assessment, we excluded them from the pre-ASCT response assessment.

Statistical analysis. Continuous variables were analyzed using the Student *t* test, and categorical variables were analyzed using Fisher's exact test. The OS was calculated from the time of diagnosis or ASCT until the date of death, by any cause, or the date of last contact. Patients who could not be followed up were censored at the date of last contact. Survival curves were plotted according to the Kaplan–Meier method, and the log-rank test was used for comparisons among the groups. The Cox proportional hazard model was used to calculate the hazard ratios (HR) for each variable along with the 95% confidence interval (CI). A multivariate analysis was conducted by

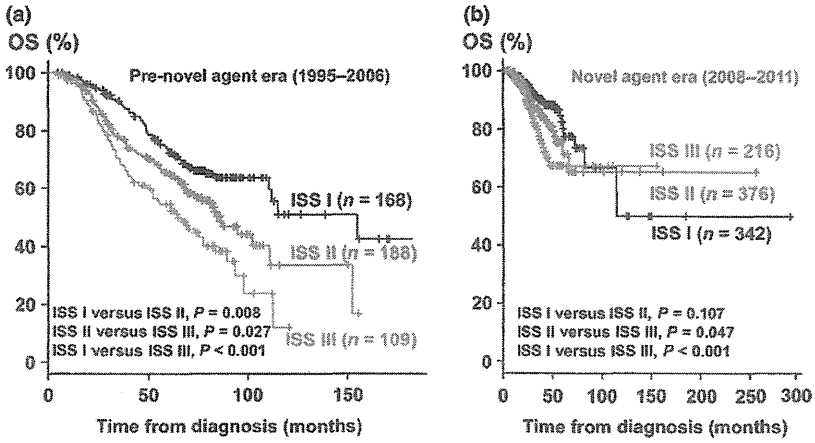

Fig. 2. The International Staging System (ISS) scores for patients who underwent autologous stem cell transplantation (ASCT) during the pre-novel (a) and novel agent eras (b). The overall survival (OS) was calculated from the time of diagnosis.

entering all variables that were associated with survival at a significance level of $P < 0.05$ into a Cox proportional hazard model. All statistical analyses were performed using the EZR software package (Saitama Medical Center/Jichi Medical University, Saitama, Japan)[3] along with a graphical user interface for the R software package (version 2.13.0; The R Foundation for Statistical Computing). A multivariate analysis was performed using the EZR software package (Saitama Medical Center/Jichi Medical University)[3] and SAS version 9.2 software (SAS Institute, Cary, NC, USA). *P*-values of <0.05 were considered significant in all analyses.

Results

The characteristics of patients before and after the approval of novel agents are shown in Table 1. There were no significant differences between the groups with regard to gender, performance status (PS) at ASCT, ISS categorization at diagnosis and myeloma type, except for age at ASCT, and planned post-ASCT therapy.

During the pre-novel agent era, 654 patients in Japan (359 men and 295 women) with a median age of 56 years (range: 22–70 years) underwent upfront single ASCT after Mel 200 treatment between October 1995 and December 2006. The median follow-up duration was 4.2 years with a 2-year OS rate of 82.0% (95% CI, 78.7–84.8), a 4-year OS rate of 64.7% (95% CI, 60.6–68.4) and the median survival was 6.3 years. During the novel agent era, 996 patients in Japan (577 men and 419 women) with a median age of 59 years (range: 18–73 years) underwent single ASCT after Mel 200 treatment between January 2008 and December 2011. The median follow-up duration was 1.6 years with a 2-year OS rate of

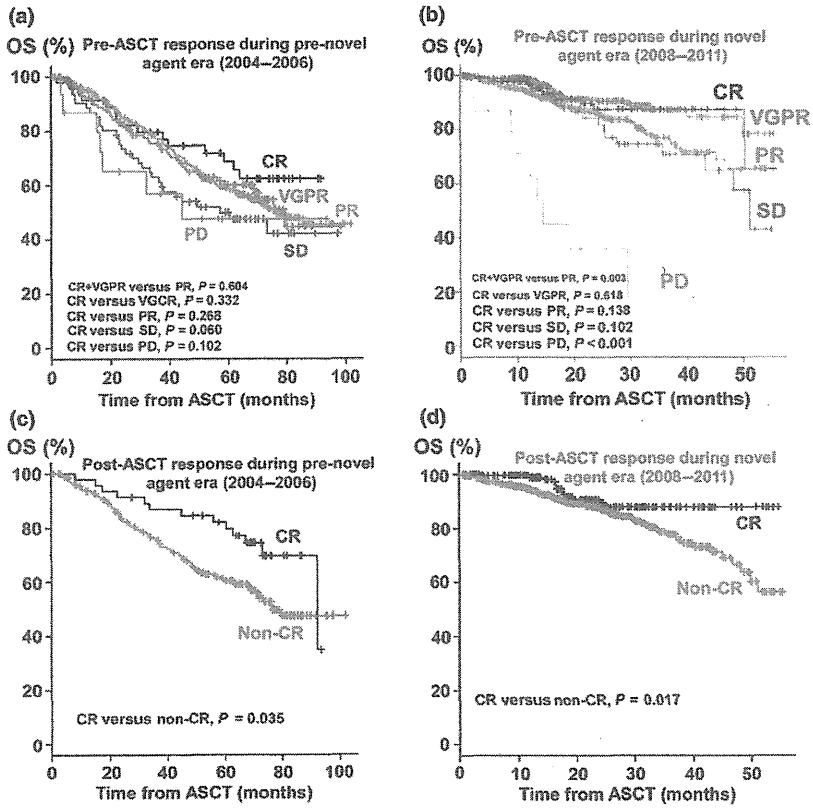

Fig. 3. Pre-autologous stem cell transplantation (ASCT) responses of patients who underwent ASCT during the pre-novel agent ([a] complete response [CR], 45 cases; very good partial response [VGPR], 119 cases; partial response [PR], 285 cases; stable disease [SD], 62 cases; progressive disease [PD], 16 cases) and novel agent eras ([b] CR, 123 cases; VGPR, 340 cases; PR, 434 cases; SD, 76 cases; PD, 15 cases). Post-ASCT responses of patients who underwent ASCT during the pre-novel agent ([c] CR, 45 cases; non-CR, 238 cases) and novel agent eras ([d] CR 182 cases; non-CR, 508 cases). Overall survival (OS) was calculated from the time of ASCT. These responses were analyzed using the data of pre-novel (January 2004–December 2006) and novel agent eras (January 2008–December 2011) based on the same response criteria.

86.9% (95% CI, 84.1–89.2). The OS during the novel agent era was significantly improved in comparison to the OS during the pre-novel agent era ($P = 0.019$; Fig. 1a). The factors associated with a superior OS were female gender ($P = 0.014$), a good PS ($P < 0.001$) and a low ISS score ($P < 0.001$; Table 2). Although the OS of female patients with MM significantly improved during the novel agent era ($P = 0.002$), the OS of male patients with MM did not ($P = 0.592$; Figs 1b,c,5). The median survival rates from the time of diagnosis for the ISS I ($n = 168$), II ($n = 188$) and III ($n = 109$) groups during the pre-novel agent era were 12.9, 7.2 and 5.4 years, respectively (Fig. 2a). The OS was significantly different when the ISS I group was compared with the ISS II ($P = 0.008$) and III ($P < 0.001$) groups and between the ISS II and III groups ($P = 0.027$). The 2-year OS rates from the time of diagnosis for the ISS I ($n = 342$), II ($n = 376$) and III ($n = 216$) groups during the novel agent era were 96%, 93% and 90%, respectively (Fig. 2b). In the ISS I group, the OS was significantly prolonged compared with the ISS III group ($P < 0.001$), but no significant differences were found between the ISS I and II groups ($P = 0.107$; Fig. 2b). The period from diagnosis to ASCT in the pre-novel agent era was 64–6079 days (median 213 days) and that in the novel agent era was 18–7201 days (median 218 days), and the difference between these groups was not significant ($P = 0.82$ by unpaired *t*-test; $P = 0.60$ by Mann–Whitney *U*-test). The pre-ASCT responses during the pre-novel agent era (January 2004–December 2006) were as follows: CR, 45 cases (8%); VGPR, 119 cases (22%); PR, 285 cases (53%); SD, 62 cases (12%); PD, 16 cases (3%); and no data, 10 cases (2%; Table 1). The 2-year OS rates for the CR, VGPR, PR, SD and PD groups were 82%, 82%, 85%, 73% and 65%, respectively. The median survival durations for the CR, VGPR, PR, SD and PD groups were not reached, 6.6, 6.4, 4.8, and 3.7 years, respectively (Fig. 3a). There were no significant differences in the OS between the CR group and the other response groups. The pre-ASCT responses during the novel agent era were as follows: CR, 123 cases (12%); VGPR, 340 cases (34%); PR, 434 cases (44%); SD, 76 cases (8%); PD, 15 cases (2%); and no data, eight cases (1%; Table 1). The 2-year OS rates for the CR, VGPR, PR, SD, and PD groups were 87%, 91%, 86%, 84% and 36%, respectively. There were no significant differences in the OS between the CR group and the other response groups, except between CR and PD ($P < 0.001$; Fig. 3b). The percentage of pre-ASCT CR + VGPR cases (463 of 988, 47%) during the novel agent era significantly increased in comparison with that during the pre-novel agent era (164 of 527, 31%; $P < 0.001$; Table 1), and there was a significant difference in the OS between the pre-ASCT CR + VGPR and PR groups during the novel agent era ($P = 0.003$; Fig. 3b). The post-ASCT CR rate during the novel agent era (182 of 690, 26%) also significantly increased compared with the pre-novel agent era rate (45 of 283, 16%; $P < 0.001$; Table 1). There were significant differences in the OS between the post-ASCT CR and non-CR groups during both the pre-novel and novel agent eras (Fig. 3c,d).

In a multivariate analysis, we analyzed the baseline factors that were significant in a univariate analysis; the post-ASCT response was excluded based on data unavailability for a large number of cases. The factors that were independently associated with superior OS were female gender ($P = 0.002$), PS of 0 or 1 ($P = 0.024$), ISS I versus II ($P = 0.046$) and III ($P < 0.001$), a pre-ASCT response better than or equal to PR ($P < 0.001$), and ASCT during the novel agent era ($P = 0.017$; Table 3). We classified patients into five categories on the basis of the number of prognostic factors (male gender, PS of 2, 3 or 4, ISS II or III, a pre-ASCT response less than PR, and ASCT during the pre-novel agent era). The numbers of patients with 0, 1, 2, 3, 4 and 5 prognostic factors were 251, 593, 394, 126, 17 and 1, respectively. We conducted Kaplan–Meier analysis according to the number of prognostic factors and revealed a clear OS stratification (Fig. 4). Only one patient displayed all five prognostic factors, and his OS was not shown. The patients who were included in this analysis underwent an ASCT during pre-novel (October 1995–December 2006) and novel agent (January 2008–December 2011) eras.

To further clarify the effects of novel agents across the various risk groups, we analyzed the differences in the OS of the groups before and during the novel agent era with respect to well-known prognostic factors (Fig. 5). In a comparison of the pre-novel and novel agent eras, the following factors were associated with a better OS: age ≤65 years ($P = 0.024$) at

Table 3. Univariate and multivariate analysis for survival

	Univariate analysis		Multivariate analysis	
	Hazard ratio (95% CI)	*P*-value	Hazard ratio (95% CI)	*P*-value
Age >65 *vs* ≤65 at ASCT	1.130 (0.713–1.791)	0.603	NA	
Male *vs* female	1.275 (1.049–1.550)	0.015	1.456 (1.155–1.837)	0.002
PS >1 *vs* 0 or 1 at ASCT	1.321 (1.142–1.528)	<0.001	1.477 (1.053–2.071)	0.024
ISS stage at diagnosis				
I	1.000	–	1.000	–
II	1.413 (1.079–1.852)	0.012	1.322 (1.005–1.739)	0.046
III	1.408 (1.220–1.624)	<0.001	1.840 (1.376–2.461)	<0.001
Pre-ASCT response				
CR/nCR/VGPR/PR *vs* SD/PD	1.206 (1.110–1.311)	<0.001	1.680 (1.240–2.277)	<0.001
Post-ASCT response				
Non-CR *vs* CR	1.939 (1.284–2.930)	0.002	NA	
ASCT during pre-novel agent era *vs* during novel agent era	1.310 (1.044–1.643)	0.020	1.366 (1.060–1.761)	0.017

The overall survival was calculated from the time of ASCT. The patients who were included in this analysis underwent an ASCT during pre-novel (October 1995–December 2006) and novel agent (January 2008–December 2011) eras. ASCT, autologous stem cell transplantation; CR, complete response; ISS, International Staging System; NA, not applicable; nCR, near complete response; PD, progressive disease; PR, partial response; PS, performance status; SD, stable disease; VGPR, very good partial response or better.

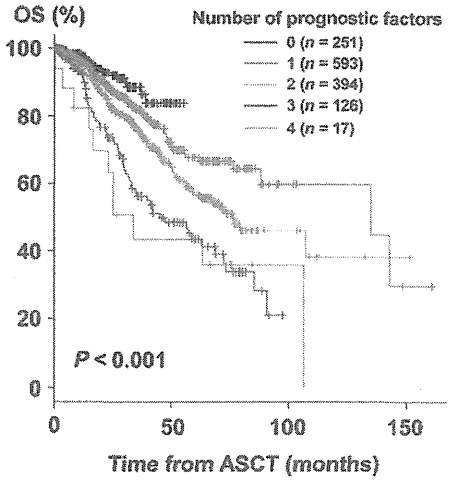

Fig. 4. Overall survival (OS) from the time of autologous stem cell transplantation (ASCT) according to the number of prognostic factors; male gender, performance status (PS) of 2, 3 or 4, the International Staging System (ISS) II or III, a pre-autologous stem cell transplantation (ASCT) response less than the partial response (PR), and ASCT during the pre-novel agent era. Only one patient displayed all five prognostic factors and his OS was not shown. The patients who were included in this analysis underwent an ASCT during pre-novel (October 1995–December 2006) and novel agent (January 2008–December 2011) eras.

ASCT, female gender ($P = 0.002$), PS of 0 or 1 ($P = 0.044$) at ASCT and ISS II ($P = 0.046$) at diagnosis.

Discussion

Novel agents have markedly changed therapies for MM. Thalidomide, lenalidomide and bortezomib were approved in the USA in 2006, 2006 and 2003, respectively, and were approved in many European Union member nations in 2008, 2007 and 2004, respectively. According to clinical studies performed in Europe and the USA in which novel agents had been introduced earlier than in Asian countries, significant improvements in responses and survival were observed in patients with MM who had been treated with novel agents.[4–6] However, few reports have described the outcomes of patients with MM who have been treated with novel agents in Asian countries. Our first aim was to provide the initial analysis of prognostic factors in a large cohort of newly diagnosed Japanese patients with MM who underwent single upfront ASCT during the novel agent era. OS significantly improved during the novel agent era and significant improvements in the 2-year OS were confirmed in patients with MM who were younger (≤65 years at ASCT; 82% *vs* 87%; $P = 0.024$), female (80% *vs* 90%; $P = 0.002$) and with a good PS (0 or 1 at ASCT; 83% *vs* 88%; $P = 0.044$; Fig. 5). These findings are consistent with those of previous reports.[6] Kastritis *et al.* demonstrate that the median OS in patients who began treatment after the introduction of novel agents increased by 12 months (48 *vs* 36 months; $P < 0.001$). This improvement was more pronounced in younger (≤70 years; 39 *vs* 74 months; $P < 0.001$) and female (36 *vs* 59 months; $P = 0.001$) patients but was less evident in older (>70 years; 26 *vs* 33 months; $P = 0.27$) and male patients (37.5 *vs* 40.5 months; $P = 0.062$).[6] Kumar *et al.*[4] report that in a larger cohort of 2981 newly diagnosed patients with myeloma, those who had been diagnosed in the previous decade experienced a 50% improvement in the OS (44.8 *vs* 29.9 months; $P < 0.001$). Furthermore, Costa *et al.*[7] also demonstrate by multivariate analysis using Center for International Blood and Marrow Transplant Research (CIBMTR) data that ASCT in the 2000–2004 cohort ($n = 6408$; HR = 0.77) or in the 2005–2010 cohort ($n = 11\,644$; HR = 0.68) were associated with lower risk of death compared with the 1995–1999 ($n = 2226$) cohort. Although we do not know the reason for a superior OS in female MM patients, Kristinsson *et al.*[8] and Kumar *et al.*[4] also show enhanced survival in female patients with MM using a total of 14 381 and 2981 patients, respectively. Landgren *et al.*[9] report that estrogen medication has been found to reduce the risk of developing MM among females, potentially due to the blocking effects on interleukin-6-mediated MM cell growth.[10]

Our second aim was to validate the ISS in Japanese patients with MM during the pre-novel and novel agent eras. Although our results demonstrate that the ISS could be used to stratify the OS of patients who underwent ASCT during the pre-novel agent era, we could not clearly stratify the prognosis of Japanese patients with MM in the ISS I and II groups who underwent upfront single ASCT during the novel agent era. In the pre-novel agent era, Nagura *et al.*[11] report that the ISS could stratify Japanese patients with MM who were treated with chemotherapy and ASCT. Kim *et al.*[12] also report that the ISS could predict the prognosis of Korean patients with MM who underwent ASCT as a first-line therapy during the pre-novel agent era. Furthermore, Kastritis *et al.*[6] report that the ISS was applicable in patients during the novel agent era. In contrast, Hari *et al.*[13] demonstrate using the CIBMTR data that the ISS III stage ($n = 449$) was associated with a higher risk

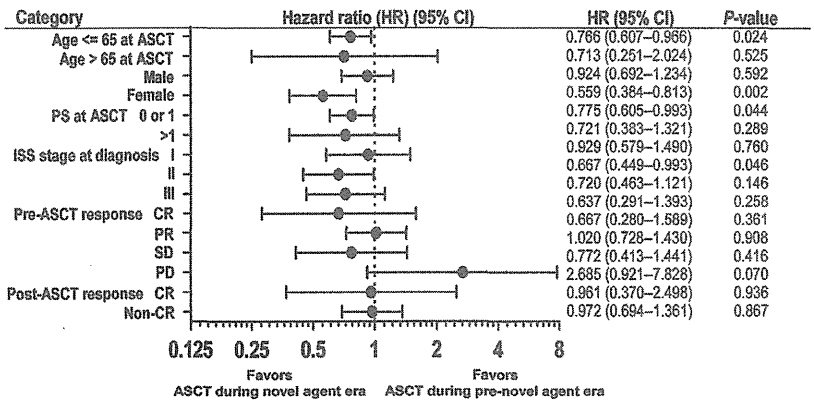

Fig. 5. Impact of autologous stem cell transplantation (ASCT) during the novel agent era on the overall survival (OS) from the time of ASCT in each stratified category. Effects of ASCT during the novel agent era are shown as forest plots. Circles on lines indicate hazard ratios compared with "ASCT during the pre-novel agent era," and horizontal lines represent the corresponding 95% confidence interval (CI). Pre-ASCT responses were analyzed using the data of pre-novel (January 2004–December 2006) and novel agent eras (January 2008–December 2011) based on the same response criteria. ISS, International Staging System; PS, performance status.

of mortality compared with the ISS II stage ($n = 230$; $P = 0.007$) but not the ISS II stage compared with the ISS I stage ($n = 50$; relative risk = 1.10, $P = 0.482$) in patients who received upfront ASCT for MM in the pre-novel agent era. Tan *et al.*[14] recently compared the OS of 221 patients with MM in Singapore who had been diagnosed from 2006 to 2009 (era 2), when an upfront bortezomib combination was approved for high-risk MM, with the OS of 262 patients who had been diagnosed from 2000 to 2005 (era 1), when bortezomib could only be administered upon relapse. The median OS was 4.2 years and was not reached in eras 1 and 2 ($P = 0.03$). The ISS retained its prognostic significance in era 1 ($P < 0.001$) but not in era 2 ($P = 0.07$), a finding that was consistent with our results. Iriuchishima *et al.*[15] also report the lack of a significant difference between the ISS stages among Japanese patients with MM in the novel agent era. The patients in the previous reports who received initial treatment other than single ASCT following high-dose melphalan (200 mg/m^2; Mel 200), such as tandem ASCT, melphalan <200 mg/m^2 or conventional chemotherapy, were included; therefore, it is of particular concern that this analysis was based on highly selected patients who underwent single ASCT following Mel 200. In the near future, novel prognosis models that include chromosomal and genetic data will be used to accurately predict the OS of patients with MM in place of the ISS.

Given the lack of available pre-ASCT induction regimen information in our database, we could not extract the data for patients with MM who actually received novel agents during the novel agent era for our analysis. The Kansai Myeloma Forum, a Japanese MM study group, reported that 95 cases received high-dose melphalan therapy with stem cell support from 2006 to 2013 and 83 of the 95 (87%) cases received at least one of the novel agents during their clinical courses.[16] According to that report, in clinical practice, from 2006, approximately 90% of all transplant-eligible Japanese patients with MM received therapy with the novel agents. Therefore, it is reasonable to assume that many of the patients who underwent ASCT between 2008 and 2011 were treated with novel agents. Bortezomib, thalidomide and lenalidomide were administered for refractory/relapse MM cases between December 2006 and September 2011; therefore, improvement in the OS of Japanese patients with MM during the novel agent era is probably due to the salvage therapy for insufficient response to pre-ASCT induction or relapse cases.

The findings in this article should be confirmed in prospective studies.

Acknowledgments

The authors would like to thank to Dr Eiichi Nagura of Chutoen General Medical Center, Kakegawa, Shizuoka, Japan for providing us with the OS data for Japanese patients with MM according to the ISS stage, and to Dr Kosei Matsue of Kameda Medical Center, Kamogawa, Japan and Dr Shinji Nakao of Kanazawa University, Kanazawa, Japan for the critical reading of the manuscript.

Disclosure Statement

The authors declare no conflicts of interest except that Drs Hiroyuki Takamatsu and Kazutaka Sunami have received honoraria fees from Celgene Corporation.

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