neuron action potential (SNAP) at her ulnar nerve and undetectable SNAP and CMAP at her tibial nerve, suggesting coexistence of demyelinations and axonal degenerations. These laboratory findings are summarized in Table 1. Pathological findings from her peripheral sural nerve biopsy represented axonal drop-out, bunch of myelin ovoid structures, uncompacted myelin lamellae, and widely spaced myelin as seen in cases of paraproteinemia associated neuropathies and/or POEMS syndrome (Fig. 1C). However, immunohistochemical examination showed the absence of IgM deposition on myelin sheaths. Although our examination could not detect any bone sclerotic lesions, (18)F-fluoro-deoxy-2-glucose positron emission tomography showed abnormal uptake into her sternum, ilium, spine, liver and spleen. The hepato-splenomegaly was shown in her abdominal computed tomography (CT) scan (Fig. 1D).

We diagnosed she suffered from an 'atypical' POEMS syndrome. The presenting clinical signs and findings met its diagnostic criteria [1]: 1) polyneuropathy, 2) M-proteinemia, 3) hepato-splenomegaly, 4) edema and hypertrichosis, and 5) elevation of plasma VEGF, but even without the evidence showing the bone sclerosis lesions (bearing two major and three minor criteria). Results of bone marrow biopsy, aspiration and flow cytometric analysis showing clonal infiltration of CD19+, CD20+, SmIg-λ+ lymphocytes support an association with WM, although POEMS syndrome is usually associated with plasma cell dyscrasia.

As her platelet counts decreased from 28.8 to $1.1\times10^4/\mu L$ at the hospital-day 16, the platelet transfusion and dexamethasone (24 mg) were administered. However, her platelets recovered transiently and her neurological findings did not improve at all, indicating dexamethasone alone was not effective. She got started with rituximab (375 mg/m^2, weekly administration for 8 cycles) and thalidomide (50 mg/day, daily oral administration) [5]. Soon after the second administration of rituximab, her numbness started to improve. Four weeks later, she could walk with assistance, and her grip strength was also getting recovered (right: 10.0 kg, left: 11.0 kg). The series of nerve conduction studies kept improving to almost normal value at 6 months after RTM treatment (Table 1). Hemoglobin and platelet counts also recovered while VEGF and sIL-2R decreased. Flow cytometry analysis of bone marrow sample revealed that CD19+, CD20+, SmIg-λ+ monoclonal lymphocytes decreased to 0.1%. The CT scan showed an amelioration of hepato-splenomegaly. These findings indicated substantial improvement in both POEMS syndrome and WM with the combination of thalidomide and rituximab (Table 1). The serum IgM level was getting less but still high (3606 mg/dL), as well as λ LC levels (κ: 5.83 mg/L, λ: 53.10 mg/L, κ/λ: 0.11). Finally she walked without any support and discharged from our hospital after 5 cycles of rituximab. Administration of rituximab was discontinued after 8 cycles while thalidomide was continued for 3 months after the last administration of rituximab, and IgM continued to decrease to as low as 1451 mg/dL. The patient no longer had numbness and was able to come back to work as a farmer.

3. Discussion

POEMS syndrome is a rare disorder characterized by polyneuropathy, organomegaly, endocrinopathy, M-protein and skin changes. It is associated with plasma cell dyscrasias, mostly accompanying IgG-λ or IgA-λ paraproteinemia [1,6]. There are limited numbers of case reports of POEMS syndrome associated with WM [2,3]. In a report from the Mayo Clinic among 99 patients with POEMS syndrome, only one single patient displayed IgM M-protein [7]. Recently, a serum VEGF is known as a useful diagnostic marker for POEMS syndrome [8]. This case filled 2 of major criteria: polyneuropathy and monoclonal plasma proliferative disorder, and also 3 of minor criteria: organomegaly, volume overload, and skin changes in clinical diagnostic criteria of POEMS syndrome [1,6]. Also VEGF, as a clinical marker, was elevated in her serum and supported diagnosis of POEMS syndrome. This vasodilating growth factor may cause skin changes, leg edema, hemangioma, and edematous change in the perineurium of peripheral nerves. In this case, initially serum VEGF level was high before treatment and then decreased, parallel to the recovery of the polyneuropathy symptoms, as association of VEGF to the pathogenesis of polyneuropathy can be suspected as previously reported.

Since POEMS syndrome mostly has an underlying plasma cell dyscrasia, treatments with such as melphalan and high-dose chemotherapy supported by stem cell transplantation are chosen as first line treatment. However, because WM appeared to be underlying this case, we utilized a combination of thalidomide and rituximab, based on previous reports showing the efficacy of thalidomide in POEMS syndrome [9,10] and rituximab in WM [11,12], respectively. Even though we considered a potential risk of neurotoxicity with a thalidomide therapy, high-dose chemotherapy supported by stem cell transplantation was not chosen because it is not recommended as a primary treatment for macroglobulinemia [6,11]. During the treatment with rituximab and thalidomide, her polyneuropathy dramatically improved both symptomatically and physiologically although the serum IgM and FLC concentrations remained high. On the initiation of the disease, her serum IgM concentration (1358 mg/dL) was less than that at the time of admission to our hospital, even when the patient was already suffering from severe numbness and ataxic paraplegia. From her clinical course, we considered that serum IgM might not directly contribute to the severity of the neuropathy. A nerve biopsy in POEMS syndrome has been reported to show uncompacted myelin lamellae without immunoglobulin fixation [13], which is compatible with our case. In addition, the serum auto-antibodies against peripheral nerve antigens (MAG, SGPG, and gangliosides) were negative. Therefore, factors other than auto-antibodies are likely to be involved in the development of polyneuropathy in this case.

The POEMS syndrome is known to have a λ light chain monoclonal antibody which is highly restricted to the Vλ1 subfamily in the immunoglobulin λ light chain variable region [14]. On the other hand, the κ light chain is preferentially present in WM (κ/λ ratio is 5:1) [8]. Our case had λ light chains, suggesting the importance of the λ light chain in the POEMS syndrome. However the role of the λ light chain in this case may be complicated, because our observation suggests an improvement of polyneuropathy despite IgM and FLC remaining in the serum. The detailed analysis of this unusual case may reveal important information regarding the pathogenesis of the 'atypical' POEMS syndrome.

In conclusion, we reported the first case of a POEMS syndrome associated with WM characterized by IgM-λ type monoclonal (M) protein and infiltration of CD20-positive lymphoplasmacytic cells. The combination therapy of rituximab and thalidomide effectively decreased CD20-positive lymphoplasmacytic cells and also improved her neurological symptoms. Our case suggested a new point of view for the diagnostic and therapeutic strategy of the 'atypical' POEMS syndrome.

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Clarithromycin attenuates autophagy in myeloma cells

MIKI NAKAMURA[1], YOSHITAKA KIKUKAWA[1], MOTOHIRO TAKEYA[2], HIROAKI MITSUYA[1] and HIROYUKI HATA[1]

[1]Department of Hematology, Kumamoto University Hospital; [2]Department of Cell Pathology, Graduate School of Medical Sciences, Kumamoto University, 1-1-1 Honjo, Kumamoto 860-8556, Japan

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Abstract. It has been reported that clarithromycin (CAM) augments the anti-tumor activity of thalidomide against multiple myeloma (MM) cells, while the mechanism remains unclear. A myeloma cell line or primary myeloma cells were treated with CAM. Autophagy was analyzed by morphological changes, LC3 expression and lysotracker staining. CAM induced vacuoles in the cytoplasm of MM cells which resembled autolysosomes. The manifestation of the CAM-induced vacuoles was blocked by treatment with PI3-kinase inhibitor. CAM induced an accumulation of LC3-II without affecting the mTOR or AKT pathways, eventually leading to cell death. CAM may halt the autophagy process after fusion of autophagosomes with lysosomes. This phenomenon may explain how CAM, combined with thalidomide, augments the cytotoxic effects of the latter on MM cells and suggests that modification of autophagy might represent a new approach for therapy of MM.

Introduction

Certain macrolide antibiotics are known to exert effects other than anti-bacterial activity, such as growth inhibition of certain cancer cells. Prolonged survival of lung cancer patients treated with macrolides, and experiments showing anti-tumor activity of CAM have been reported (1-5). In experimental settings, various effects of macrolides have been reported, e.g., anti-angiogenesis (6) and direct anti-tumor activity (7). Clarithromycin (CAM) is known to exert a potent anti-tumor effect on multiple myeloma cells, when combined with thalidomide or dexamethasone (8), despite there being some controversy at a setting of mono-therapy (9). Of note, high efficacy to myeloma of chemotherapeutic regimen combining CAM with lenalidomide, a derivative of thalidomide, has recently been reported (10).

Correspondence to: Dr Hiroyuki Hata, Department of Hematology, Kumamoto University Hospital, 1-1-1 Honjo, Kumamoto 860-8556, Japan
E-mail: hata@kumamoto-u.ac.jp

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In an attempt to clarify the mechanisms regulating the anti-tumor activity of CAM, we analyzed its direct effect on myeloma cells *in vitro*. We showed significant induction of vacuoles in human myeloma cell lines and primary myeloma cells by the treatment with CAM, which are related to morphological features of autophagy. Detailed analysis revealed that inhibition of autophagy process at late stage by CAM may contribute augmentation of anti-myeloma effect. We believe that this report is the first one showing a contribution of CAM to autophagy. Moreover, this finding may lead to a novel therapeutic approach by manipulating autophagy system, which has been proposed, but not yet clinically achieved (11).

Materials and methods

Cells and cell culture. The myeloma cell line 12PE (12), and primary myeloma cells purified by CD138-conjugated immuno-magnetic beads (Miltenvi Biotec, Auburn, CA, USA) were utilized. Clarithromycin (CAM) was obtained from Taisho-Toyama Pharmaceuticals (Tokyo, Japan) and dissolved in DMSO at a concentration of 10 mg/ml as a stock solution. Cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (Sigma, St. Louis, MO, USA) at 37°C under humidified conditions.

Analysis of autophagy. Morphology was analyzed either by May-Giemsa staining or electron microscopy. Lysosomes were stained with LysoTracker (Invitrogen, Carlsbad, CA, USA) and analyzed using fluorescent microscopy. Antibodies to LC3 were a kind gift of Dr T. Yoshimori (Department of Cellular Regulation, Research Institute for Microbial Diseases, Osaka University). Antibodies to phospho-AKT, AKT and p-70S6K1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Western blotting was performed by utilizing a Pre-cast Gel kit (Invitrogen), and membranes were visualized by ECL Plus detection kit (GE Healthcare Bio-Sciences, Uppsala, Sweden). 3-methyladenine, LY294002 and Bafilomycin A (BAF) were purchased from Sigma. Microscopic analysis for LysoTracker staining, a dye that stains acidic lysosomes, was performed with confocal laser scanning microscopy (Olympus, Tokyo, Japan).

Electron microscopy. Cell pellets were fixed with 1.0% glutaraldehyde for 60 min and postfixed with 1.0% osmium

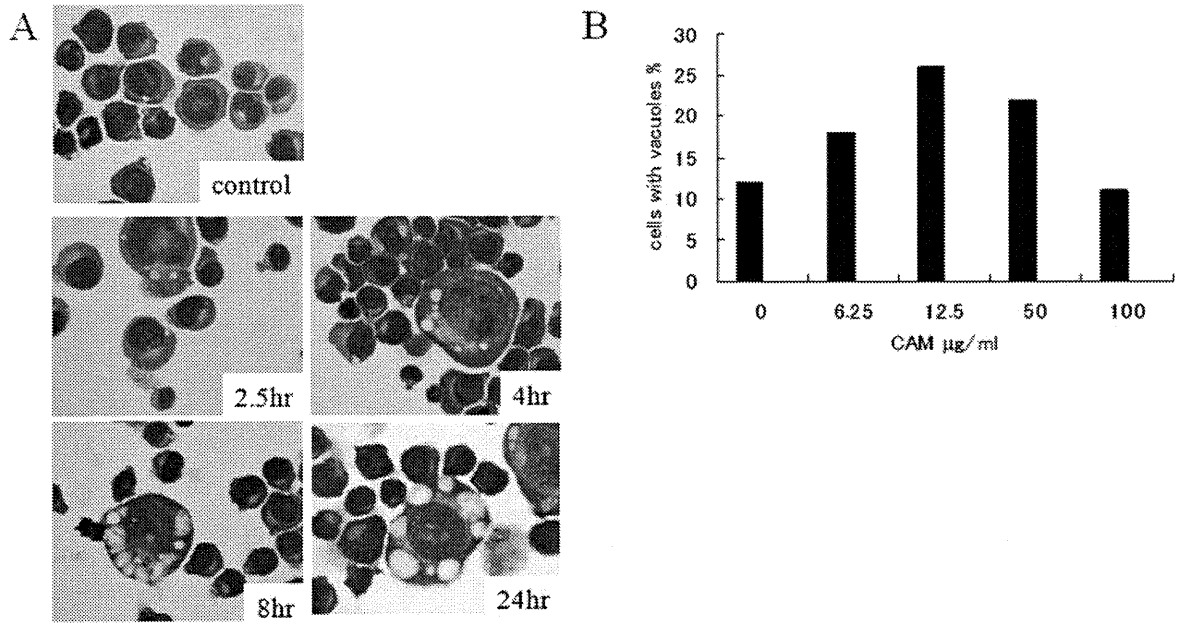

Figure 1. Induction of vacuoles in the cytoplasm of myeloma cells by CAM. (A) Time-dependent induction of vacuoles by CAM. Myeloma cell line 12PE was incubated with CAM at 50 μg/ml for the indicated periods. Significant induction of vacuoles was found in a time-dependent manner. (B) Dose-dependent induction of vacuoles by CAM. 12PE cells were treated with CAM at indicated concentrations for 18 h. Cells were stained with May-Giemsa staining solution on cytospin slides, and at least 300 cells were counted by microscopy. The proportions of cells with vacuoles are shown.

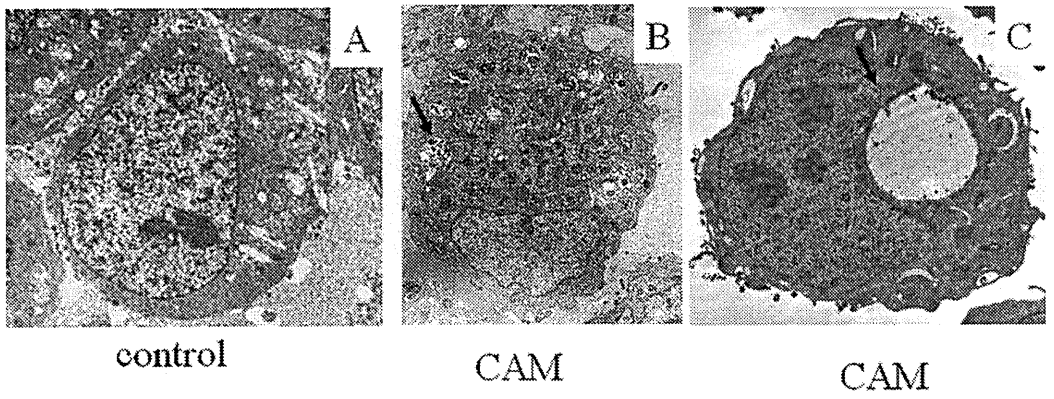

Figure 2. Electron microscopic analysis of 12PE cells treated with CAM at 50 μg/ml for 18 h. (A) Untreated cell; (B and C) CAM treated cells. Vacuoles with degenerated organelle considered to be autophagic vacuoles (B, arrow). Large vacuole with empty structure considered to be autolysosomes (C, arrow).

tetroxide for 30 min at 4°C. After dehydration in a graded ethanol series, the cells were embedded in epoxy resin. Ultrathin sections stained with uranyl acetate and lead citrate were observed using an H-7500 electron microscope (Tokyo, Japan).

Flow cytometry. Cells were stained with the Annexin V/PI staining kit (Medical and Biological Laboratory, Nagoya, Japan). Cells were then analyzed by Epics V flow cytometer (Coulter, Miami, FL, USA).

Results

Induction of vacuoles by CAM. The myeloma cell line 12PE was incubated with CAM at various concentrations for 18 h. Vacuolization was morphologically observed after treatment with CAM at a concentration of 50 μg/ml, and increased in a time-dependent manner with maximum vacuolization at 12.5 μg/ml which is potentially achievable concentration within cytoplasm (Fig. 1A). Quantification of vacuoles revealed that CAM increased vacuolization in a dose-dependent manner (Fig. 1B). Electron microscopy revealed that the small vacuoles represented vacuoles that contained degenerated organelles (Fig. 2B) and larger vacuoles containing no organelles (Fig. 2C) which are considered to be autophagosomes and autolysosomes, respectively. Primary purified myeloma cells cultured with CAM at a concentration of 10 μg/ml for 18 h showed similar vacuole formation to that found in cell lines (Fig. 3).

Co-localization of vacuoles with lysosomes. Electron microscopy suggested that small vacuoles are autophagosomes,

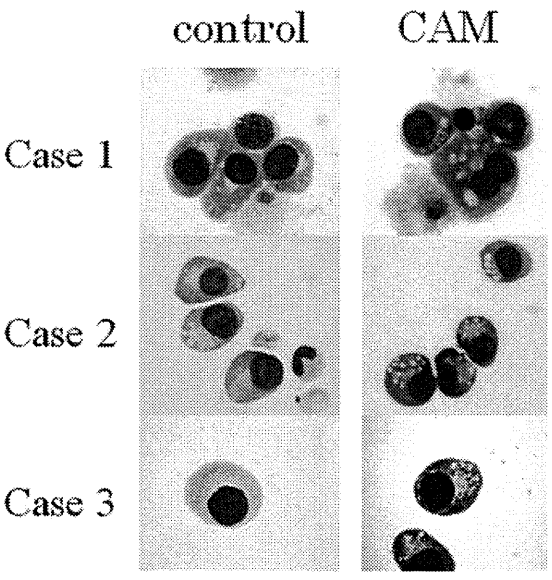

Figure 3. Induction of vacuoles by CAM in freshly isolated myeloma cells. Samples were obtained from three myeloma patients and purified by immunomagnetic beads. Cells were incubated with or without CAM at 10 μg/ml for 18 h. Cells were stained with May-Giemsa solution. Purified myeloma cells from all cases showed marked induction of vacuoles in the cytoplasm, as found in the myeloma cell line 12PE.

therefore, we hypothesized that large vacuoles observed by light microscopy may have been autolysosomes. To confirm this hypothesis, 12PE cells were treated with CAM and stained with LysoTracker, a dye that stains acidic lysosomes. As shown in Fig. 4, large vacuoles were stained red with LysoTracker, which clearly indicated that the vacuoles were acidic lysosomes. When cells were treated with CAM at increasing doses, vacuoles stained with LysoTracker accumulated in a dose-dependent manner (Fig. 5). This finding was also confirmed in primary purified myeloma cells (Fig. 6).

CAM induces accumulation of LC3-II. Taking these results together, it is suggested that CAM-induced vacuoles are autophagy-related vacuoles. To further examine this possibility, we analyzed processing of LC3-I to LC3-II, a hallmark of autophagy. We found that more accumulation of LC3-II by CAM as concentration of CAM increased (Fig. 7A). These results indicate either induction of autophagy or inhibition of autophagy at late stage by CAM. Subsequently, phosphorylated and total Akt were analyzed, since autophagy is initiated by dephosphorylation of Akt (13). However, phosphorylated Akt was not inhibited by CAM (Fig. 7B), suggesting that CAM may not induce autophagy through dephospholyration of AKT.

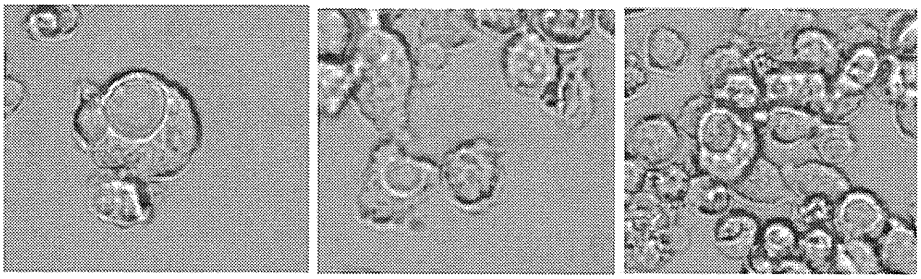

Figure 4. LysoTracker staining merged with phase contrast microscopy. 12PE cells were treated with CAM at 50 μg/ml for 18 h. Cells were then stained with LysoTracker for 15 min. Images of fluorescent microscopy and phase contrast microscopy were merged. Note that the red stain of LysoTracker co-localizes with large vacuoles.

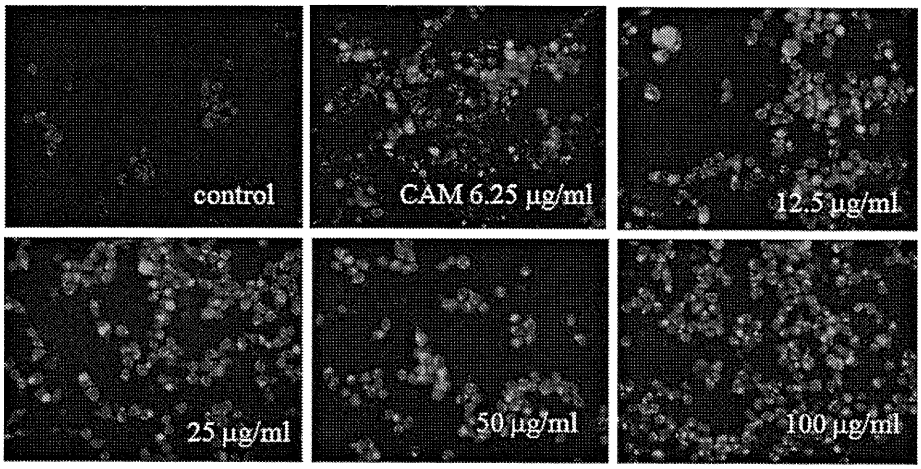

Figure 5. Induction of LysoTracker-positive vacuoles by CAM. 12PE cells were treated with CAM at indicated concentrations for 18 h. Vacuoles stained with LysoTracker accumulated by CAM in a dose-dependent manner.

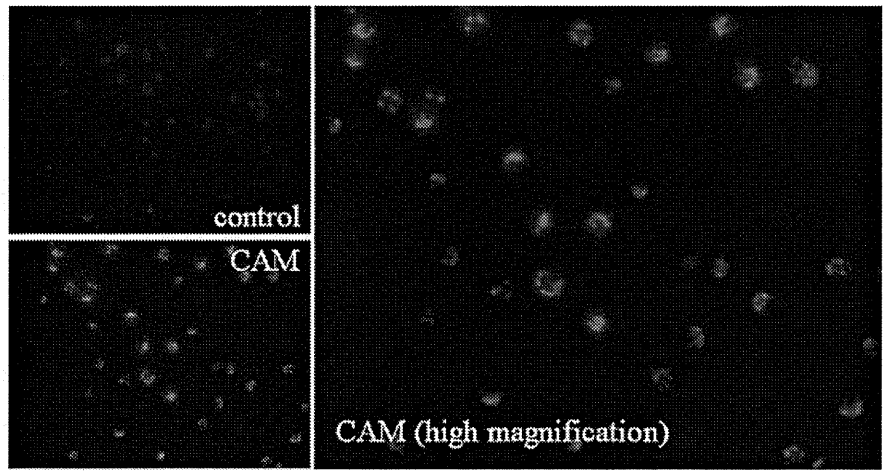

Figure 6. Induction of LysoTracker staining by CAM in freshly isolated myeloma cells. Myeloma cells from patient bone marrow were purified with immunomagnetic beads and incubated with CAM at 10 μg/ml for 18 h. Cells were then stained with LysoTracker. Induction of LysoTracker staining was clearly observed following treatment with CAM.

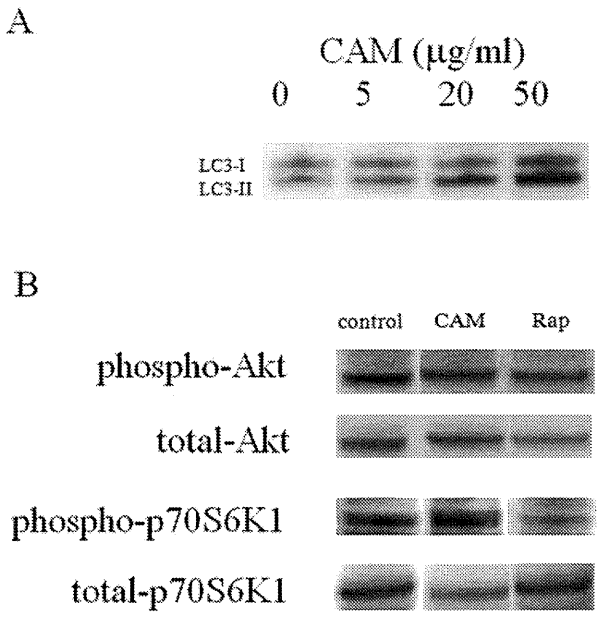

Figure 7. Induction of LC3-II and inhibition of phospho-p70S6K by CAM. 12PE cells were treated with CAM at 50 μg/ml for 18 h. Cell lysates were analyzed by Western blotting. (A) Dose-dependent induction of LC3-II was observed. (B) No reduction of phospho-p70S6K1 and phosphorylated AKT were found following treatment with CAM. Significant inhibition of phosho-P70S6K1 by rapamycin (Rap) at 1 μM is shown as a positive control.

Inhibition of vacuolization by 3-methyladenine. We further examined p70S6K1 since it locates downstream of mTOR, which is known to inhibit autophagy (14,15). There was no reduction of phospholyrated p70S6K by CAM (Fig. 7B) while rapamycin, an inhibitor of mTOR, markedly decreased phospho-p70S6K. These results suggest that CAM is not involved in regular autophagy initiation process mediated by mTOR inhibition.

We further treated cells with autophagy inhibitor 3-methyl-adenine (3MA), an inhibitor of autophagy-initiation, in combination with CAM. Interestingly, vacuole formation by CAM was clearly inhibited by 3MA, as shown by morphological analysis (Fig. 8A) and LysoTracker staining (Fig. 8B), suggesting that CAM-induced vacuole formation locates downstream of autophagy initiation.

Induction of cytotoxicity by CAM. Since the combination of CAM and thalidomide is reported to be effective for myeloma (8,10), we considered that CAM alone might affect cell viability. To analyze this possibility, 12PE cells were treated with CAM and analyzed by Annexin V/PI staining. As shown in Fig. 9, CAM induced necrotic cell death or late apoptosis, which is shown by double positivity for Annexin V and PI. Since CAM induced vacuolization of cytoplasm, which is distinct from fragmentation of nucleus, and no significant induction of caspase 3 by CAM (data not shown), CAM induced necrotic cell death rather than apoptosis.

Discussion

Autophagy is a phenomenon that starts with autophagosome formation and leads to fusion of autophagosomes with lysosomes (16). It is recognized that the initiation of autophagy is induced by nutrient starvation. Although a direct and simple method for identifying autophagy has not been established, specific morphology under the electron microscope and processing of LC3-I to LC3-II are well recognized as features of autophagy (17). As these processes are controlled by 3MA (18), inhibition of vacuoles by 3MA also confirms that vacuoles are related to autophagy.

As we found induction of small vacuolization following treatment with CAM that led to large vacuole formation we investigated the possible relation of autophagy with CAM, and found that CAM indeed induced autophagic vacuoles, which was proven by morphological examination with electron microscopy and detection of LC3-II. Induction of autophagic vacuoles by CAM was further confirmed by treating cells with 3MA. It is understood that autophagy is accompanied by inhibition of the phospho-AKT (13) and inhibits phosphorylation of mTOR, as rapamycin induces autophagy by inhibiting mTOR (19). However, our results

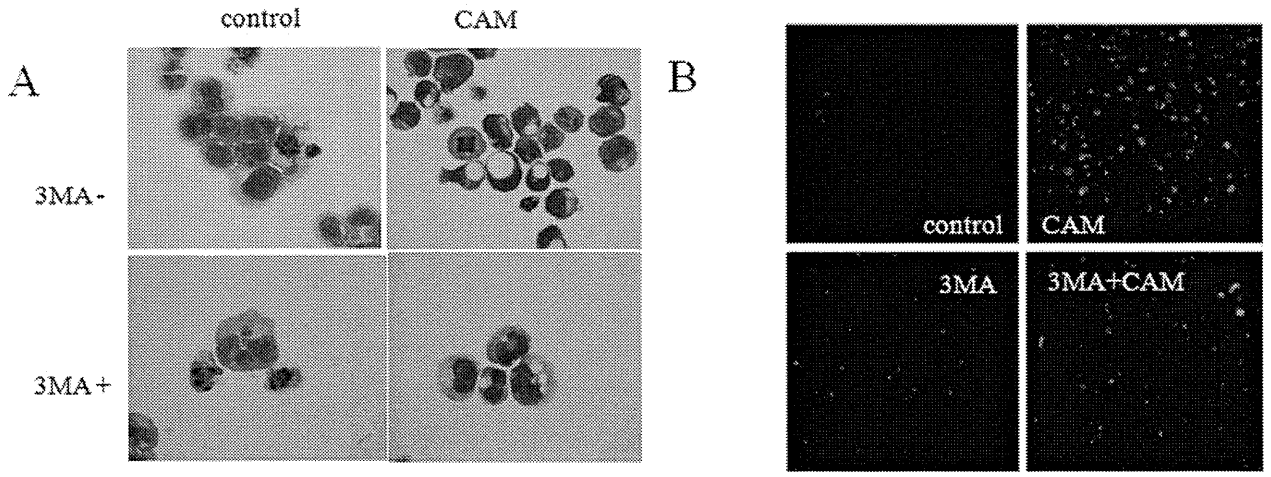

Figure 8. Inhibition of CAM-induced vacuole formation by 3-methyladenine (3MA). 12PE cells were treated with CAM at 50 μg/ml with or without 3MA. (A) May-Giemsa staining. (B) LysoTracker staining. In the presence of 3MA, there was significant loss of vacuoles.

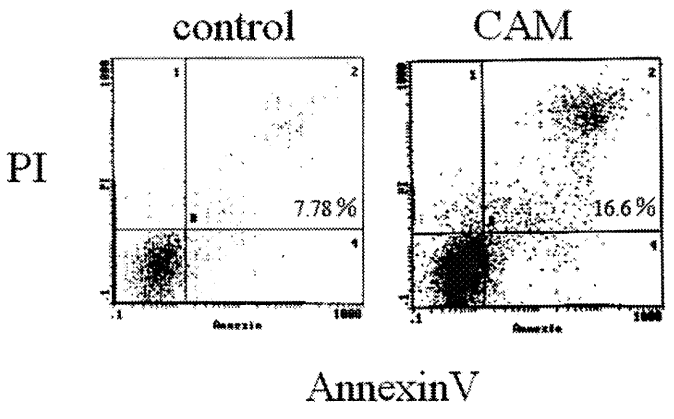

Figure 9. Induction of cell death by CAM. 12PE cells were treated with CAM at 50 μg/ml for 18 h. Cells were then stained with Annexin V/PI and analyzed with flow cytometry. Induction of cell death, which is characterized by both Annexin V- and PI-positive cells, was found in CAM-treated cells (right panel).

showed that CAM did not inhibit phosphorylation of AKT nor p70S6K, indicating that CAM does not likely induce autophagy.

Surprisingly, there was accumulation of large vacuoles in a time-dependent manner in the presence of CAM, even 18 h after its addition. This finding might be explained by potent and continuous inhibition of autophagy by CAM at late stage of autophagy process. As we found marked accumulation of LC3-II by the treatment with bafilomycin (data not shown), CAM may exert similar effect although the point of inhibition may be different (Fig. 10). Based on the fact that both bafilomycin and CAM belong to macrolides, inhibition of autophagy process by CAM may not be an unexpected phenomenon. It is hypothesized that accumulation of autolysosomes is needed for CAM to encapsulate foreign microorganisms into autophagosomes, since autophagy is not only an energy-recycling process, but also a self-defense process, as shown with *Streptococcus* (20). This hypothesis may fit our findings, because CAM is an active agent against

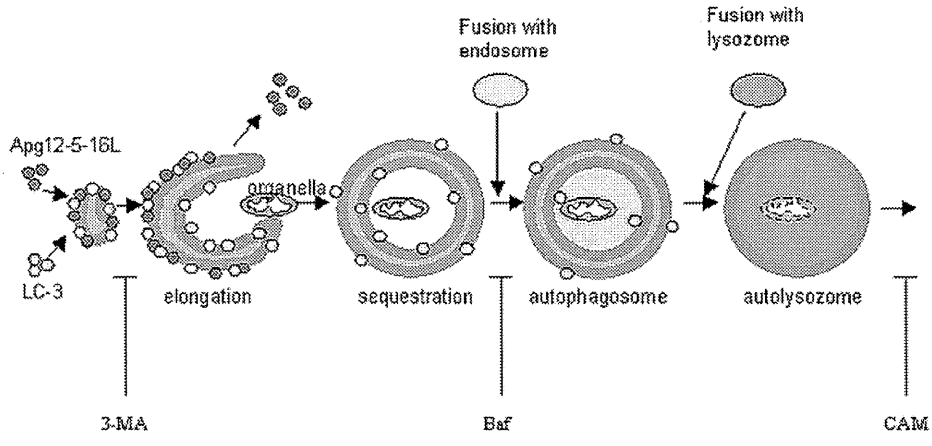

Figure 10. A hypothesis explaining inhibition of autophagy process by CAM. The autophagy process is inhibited by 3-methyladenine (3-MA) and bafilomycin (Baf) at early point and fusion with autophagosomes, respectively. Our results suggest that CAM may inhibit the process at late stage of autophagy.

intracellular micro-orgasms such as *Mycobacterium*. Despite the fact that we found accumulation of autophagic vacuoles by CAM in tumor cells, a similar phenomenon may exist in normal immune cells, such as macrophages. Further evaluation of the hypothesis requires an experimental system that utilizes microorganisms and macrophages.

We found a cytotoxic effect of CAM in myeloma cells. This effect is distinct from apoptosis in terms of characteristics in morphology, flow cytometry analysis and activation of caspases. We consider that CAM-induced cell death might be from inhibition of autophagy. It is hypothesized that autophagy might be constitutively active in myeloma cells because of their hypoxic circumstances in the bone marrow where myeloma cells grow *in vivo* as Azad *et al* reported (21). Therefore, autophagy should play an important role in myeloma cells under various stresses (22). CAM may disrupt this rescue pathway thus resulting in cell death *in vivo* as we found vacuole formations in primary MM cells. This concept has been reported by Ito *et al*, showing inhibition of autophagy results various anti-tumor effects in malignant tumors (23,24).

We found that CAM induced various phenomenon at concentration from 6 to 50 μg/ml. Pharmacokinetic analysis showed concentration of CAM in serum is approximately 1 μg/ml (25) while it concentrates >10 times in cytoplasm (26), indicating that our *in vitro* experiments were appropriate.

We believe that this report is the first showing induction of autophagy by CAM, which is a well-known and world-wide utilized orally taken antibiotic. Despite our findings only referring to myeloma cells, a similar phenomenon may be found in various tumor cells, which show a survival benefit following treatment with CAM, such as lung cancer cells (1-5,27,28). In this context, unveiling of mechanisms regulating autophagy by CAM should be important not only for understanding the biology of cancer cells but also extending survival of cancer patients.

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ORIGINAL ARTICLE

Bortezomib plus dexamethasone for relapsed or treatment refractory multiple myeloma: the collaborative study at six institutes in Kyoto and Osaka

Tsutomu Kobayashi · Junya Kuroda · Kazuho Shimura · Teruaki Akaogi · Eri Kawata · Miki Kiyota · Takashi Tanaka · Yuri Kamitsuji · Satoshi Murakami · Mayumi Hatsuse · Akira Okano · Toshiki Iwai · Satomi Ueda · Masahiko Koshida · Hitoji Uchiyama · Yosuke Matsumoto · Hiroto Kaneko · Nobuhiko Uoshima · Yutaka Ueda · Yutaka Kobayashi · Chihiro Shimazaki · Shigeo Horiike · Masafumi Taniwaki

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Abstract We conducted a retrospective collaborative investigation of bortezomib (Bor) plus dexamethasone (Dex) therapy (BD Tx) for 88 relapsed or refractory (Rel/Ref) MM patients at six institutes. One cycle BD Tx comprised of Bor (1.3 mg/m^2/day) on days 1, 4, 8 and 11, and Dex on days 1, 2, 4, 5, 8, 9, 11 and 12, every 21 days, and the mean number of BD Tx cycles was 3. The overall response rate was 66.9%, the median overall survival (OS) was 510 days, and the median progression-free survival (PFS) was 113 days. Attainment of partial response (PR) with the first course of BD Tx associated with the longer OS and PFS and late good responder, while no patient who did not achieve PR with the first cycle attained better than very good PR (VGPR) with the subsequent BD Tx. Patient age of less than 64 years old also associated with the longer OS and PFS. In addition, both an earlier disease stage and Dex dosage had a significant impact on OS, while the attainment of VGPR within 2 cycles had a significantly longer PFS. Earlier BD Tx courses may be predictive for the subsequent therapeutic pathway of Rel/Ref MM.

Keywords Bortezomib · Multiple myeloma · Relapse

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T. Kobayashi · J. Kuroda (✉) · K. Shimura · M. Kiyota · H. Uchiyama · Y. Matsumoto · S. Horiike · M. Taniwaki
Division of Hematology and Oncology, Department of Medicine, Kyoto Prefectural University of Medicine, 465, Kajii-cho, Kamigyo-ku, Kyoto 602-8566, Japan
e-mail: junkuro@koto.kpu-m.ac.jp

K. Shimura · H. Kaneko
Department of Hematology, Aiseikai Yamashina Hospital, Kyoto, Japan

T. Akaogi · E. Kawata · M. Kiyota · T. Tanaka · Y. Kobayashi
Department of Hematology, Kyoto Second Red Cross Hospital, Kyoto, Japan

Y. Kamitsuji · N. Uoshima
Department of Hematology, Matsushita Memorial Hospital, Osaka, Japan

S. Murakami · M. Hatsuse · A. Okano · C. Shimazaki
Department of Hematology, Kyoto Social Insurance Hospital, Kyoto, Japan

T. Iwai · S. Ueda · M. Koshida · Y. Ueda
Department of Hematology, Kyoto First Red Cross Hospital, Kyoto, Japan

1 Introduction

Multiple myeloma (MM) has remained an incurable disease, however, recent advances in novel anti-myeloma agents, such as the immunomodulatory agents (IMiDs) thalidomide (Thal) and lenalidomide, or bortezomib (Bor), an inhibitor for 26S proteasome, have significantly prolonged overall survival (OS) and progression-free survival (PFS) [1, 2]. The efficacy and safety of Bor alone or in combination with other anti-myeloma agents for relapsed or refractory (Rel/Ref) MM has been repeatedly reported in various clinical trials, such as the APEX trial, the SUMMIT trial, or the CREST study [3–11], and these have established BD therapy (Tx) as one of the most promising second-line therapeutic approaches for Rel/Ref MM. Despite the favorable efficacy of BD Tx for Rel/Ref MM, factors predictive for response and the long term effect have not been clearly identified. For this reason, we

retrospectively investigated the efficacy, safety and predictive factors for response to BD Tx by 88 Rel/Ref MM patients in a collaborative study of six independent institutes in Kyoto and Osaka, Japan.

2 Materials and methods

2.1 Patients and institutions

A total of 88 Rel/Ref MM patients were treated with BD at six independent institutions, Kyoto Prefectural University of Medicine, Kyoto First and Second Red Cross Hospitals, Matsushita Memorial Hospital, Kyoto Social Insurance Hospital and Aiseikai Yamashina Hospital between October 2003 and July 2009. At least one cycle of BD Tx was administered to all 88 patients, 2 cycles to 69 patients, 3 cycles to 45 patients, 4 cycles to 32 patients, and 5 or more cycles to 24 patients with a median follow-up of 264.5 days. Data for disease stage evaluation according to the International Staging System (ISS) was available for 83 patients, involvement of the chromosomal deletion of 13q (13q−) was assessed by FISH study in 79 patients, while type of paraprotein, Dex dosage, and treatment history for Thal, high-dose chemotherapy with autologous stem cell transplantation (auto-SCT), allogeneic-SCT (allo-SCT) and others, were evaluable for all patients.

2.2 Treatment schedule

Bor (1.3 mg/m^2/day) was intravenously administered on days 1, 4, 8 and 11, and Dex (20 or 40 mg/day) was administered on days 1, 2, 4, 5, 8, 9, 11 and 12, with a 10-day rest period every 21 days. A dose reduction/interruption of Bor (1.0, or 0.7 mg/m^2) or treatment discontinuation was carried out along with the dose-modification guideline [12, 13]. The dose of Dex was reduced to 2–16 mg/day in 38 patients those previously experienced ≥Grade 3 corticosteroid-related AEs, such as fluid retention, hyperglycemia, psychiatric disorders, or corticosteroid withdrawal symptoms. Also, the administration of Bor on day 11 and of Dex on days 11 and 12 of each cycle could be omitted at the doctor's discretion, based on AEs and patients' condition. The resultant median number of treatment cycles was 3 (range 1–30 cycles). In case the discontinuation of BD Tx either by disease progression or the intolerance, post-BD Tx therapy was not specified and was performed for individual cases according to their own clinical histories. The prophylactic administration of oral acyclovir (ACV), 200 mg/day, for varicella zoster virus (VZV) infection was allowed by doctors' discretion.

2.3 Response criteria

The International Myeloma Working Group (IMWG) criteria were used for the assessment of treatment response [14]. Data for OS and PFS had been analyzed by 31 July 2009. PFS was defined as the period from the date of BD Tx initiation to the date of the first assessment of disease progression or the date of death for patients without disease progression. OS was calculated from the initiation of BD Tx to the last date of patient follow-up or the date of death from any cause. AEs were graded according to the Common Terminology Criteria for Adverse Events (CTCAE), version 4.0.

2.4 Statistical analysis

Fisher's exact test was utilized for univariate analysis to assess the effects of categorical factors on response rate. PFS and OS were estimated with the Kaplan–Meier method. Factors in BD Tx suspected of having an effect on OS and PFS were analyzed by means of univariate analyses using log-rank tests. Factors assessed in this study include patients' age, type of paraprotein, disease stage according to ISS, presence of 13q-, Dex dosage, prior treatment histories, such as Thal, auto-SCT, or allo-SCT, achievement of partial response (PR) at the first course of BD Tx and achievement of very good PR (VGPR) or better within the initial 2 courses of BD Tx. The confidence interval was 95% for all analyses and $p < 0.05$ was considered statistically significant.

3 Results

3.1 Patients' background

Patients' background is summarized in Table 1. At the initiation of BD Tx, 14 patients were 16–54 years old, 21 were 55–64 years old, 36 were 65–74 years old and 17 were over 75 years old, and the median patient age was 68. Of the evaluable patients, 85.5% were classified as over stage II, and 40.5% possessed 13q−. The median daily dose of Dex was 20 mg/day (range 2–40 mg/day). All patients were treated with at least one prior chemotherapeutic regimen other than BD Tx. Twenty-six patients had a prior history of Thal treatment, 23 patients had received high-dose melphalan therapy supported by auto-SCT, and seven had received allo-SCT.

3.2 Efficacy of BD Tx

BD Tx induced complete response (CR) in one, VGPR in 21, PR in 37 and stable disease (SD) in 25 patients, but

failed with disease progression without any improvement in four patients. Overall ORR (CR + VGPR + PR) was 67.0% (Supplementary Table 1). The median OS was 510 days, and the estimated OS at 2 years was 41.4%, while the corresponding values for PFS were 113 days and 14.4%. Thirty-eight patients died during the observation period (Fig. 1).

3.3 Assessment of factors in BD Tx for Rel/Ref MM with an effect on OS and PFS

The effects of patients' background on ORR for BD Tx are summarized in Table 2. ORR for patients with IgG-type paraprotein was significantly worse than that for patients with non-IgG-type paraprotein (53.3 vs. 79.1%, $p = 0.02$), although their clinical background did not differ largely (Supplementary Table 2). ORR for patients over 75 years old was significantly worse than that for patients less than 74 years old, while ORR for patients 55–64 years old was significantly better than that for other age brackets. The median number of cycles of BD Tx was the same for the different age groups. On the other hand, ORR for BD Tx was not influenced by ISS stage, the presence of 13q-, prior treatment with Thal, auto-SCT, or allo-SCT.

Next, we assessed the factors associated with OS and PFS for BD Tx. Besides the factors analyzed for ORR described above, we also investigated the impact of the response to the first two cycles or less of BD Tx on OS and PFS (Table 3). Patient age of less than 64 years old and attainment of better than PR with the first course of BD Tx had significantly favorable effects on both OS and PFS in our series. Specifically, patients less than 64 years old showed longer median OS (912 vs. 296 days, $p < 0.05$) and median PFS (180 vs. 108 days, $p = 0.04$) (Fig. 2a). Also, the achievement of better than PR with one cycle of BD Tx was associated with longer median OS (1164 vs. 296 days, $p = 0.03$) and median PFS (180 vs. 100 days, $p = 0.03$) (Fig. 2b). In addition, both an earlier disease stage (ISS I or II) and Dex dosage over 20 mg/body/day had a significantly favorable impact on OS (Fig. 2c, d). The median OS periods for patients administered with Dex ≥20 and <20 mg/day were 561 and 274 days, respectively ($p = 0.03$). Fourteen patients attained VGPR or better within 2 cycles of BD Tx, and those who attained VGPR or better within 2 cycles showed a significantly longer PFS (median PFS 395 vs. 110 days, $p < 0.05$), and also tended to show a longer OS, although the difference was not statistically significant. Median OS was not achieved for patients who attained VGPR or better within 2 cycles, whereas it was 481 days for the rest of the patients (Fig. 2e).

Table 1 Patient characteristics ($n = 88$)

Median age, years (range)	68 (16–85)
Sex, male/female (%)	52/36 (59.1/40.9)
Type of paraprotein *n* (%)	
IgG	45 (51.1)
IgA	25 (28.4)
Others	18 (20.5)
ISS stage *n* (%)	
I	12 (14.5)
II	30 (36.1)
III	41 (49.4)
Deletion of 13q+/− (%)	32/47 (40.5/59.5)
Dexamethasone dosage (mg/day) *n* (%)	
20–40	48 (54.5)
2–16	38 (43.2)
Data missing	2 (2.3)
Type of prior treatment regimens *n* (%)	
Thalidomide	26 (29.5)
Autologous stem cell transplant	23 (26.1)
Allogeneic stem cell transplant	7 (8.0)

3.4 Impact of response to the first course of BD Tx on subsequent clinical courses

We investigated whether response to the first course of BD therapy also predisposed the subsequent clinical courses (Fig. 3). Of the 43 patients (48.9%) who attained PR or

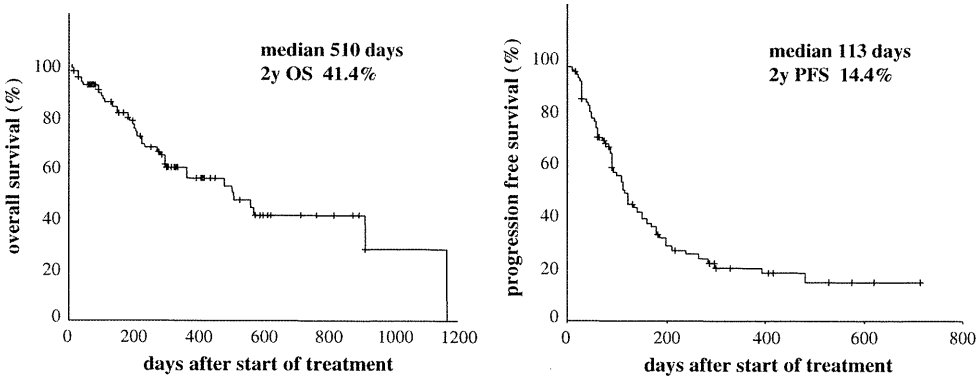


Fig. 1 Overall survival (OS) (*left*) and progression-free survival (*PFS*) (*right*) for 88 relapsed/refractory (Rel/Ref) multiple myeloma (MM) patients treated with bortezomib plus dexamethazone therapy (BD Tx) analyzed with the Kaplan–Meier method

better by the first cycle of BD Tx, the best responses to the complete BD Tx were CR for one, VGPR for 18 and PR for 24. Twenty-nine patients discontinued BD Tx within the first five courses mainly because of AEs (55.2%). Other reasons for discontinuation were disease progression (10.3%) and a change of treatment to high-dose chemotherapy with auto-SCT (10.3%). Of the 45 patients who did not attain PR for the first cycle of BD Tx, none attained CR or VGPR and only 15 attained PR even with continuation of BD Tx. Thirty-two discontinued BD Tx within five cycles, and the major reason for discontinuation in this cohort was disease progression (56.3%).

Table 2 Patients' background and ORR/OS/PFS

	n	ORR (%)	Median OS (day)	Median PFS (day)
Age				
–54	14	64.3	912	161
55–64	21	81.0	1164	180
65–74	36	63.9	481	110
75–	17	52.9	197	92
M-sprotein				
IgG	45	53.3	510	101
IgA	25	84.0	Not achieved	116
Others	18	72.2	481	180
ISS				
I	12	66.7	912	198
II	30	70.0	573	150
III	41	63.4	301	90
13q−				
+	32	65.6	364	120
−	47	68.1	510	110
Thal				
+	26	61.5	481	113
−	62	66.1	502	113
Auto-SCT				
+	23	73.9	573	171
−	65	61.5	363	110
Allo-SCT				
+	7	57.1	912	71
−	81	65.4	502	116

3.5 AEs

AEs which occurred during BD Tx are summarized in Table 4. The AEs with a major grade 3–4 were thrombocytopenia (48.9%), peripheral neuropathy (PN) (25.0%) and infection (25.0%). Two patients were affected by Bor-related acute pulmonary complications [15, 16]. PN was the AE most often leading to discontinuation of BD Tx. As far as we retrospectively analyzed, no patient who was given prophylactic ACV was complicated by VZV infection.

4 Discussion

The question of which factors are predictive for favorable treatment outcomes with BD Tx has been controversial [10, 17, 18]. In our study, achievement of PR with the first course of BD Tx and patients' age of less than 64 years old were shown to have significantly favorable impacts on both OS and PFS. In addition, the achievement of VGPR or CR within two cycles of BD Tx resulted in significantly longer PFS and also tended to improve OS. These results suggest that an early response to BD Tx for Rel/Ref MM is crucial for a favorable outcome for subsequent clinical courses. In our series, 44.2% of the patients who attained better than PR with the first course of BD Tx eventually achieved CR or VGPR as the best response, leading to longer OS and PFS, while none of the patients who did not achieve PR during the first course of BD Tx achieved VGPR even with continuation of BD Tx. Moreover, only 10.3% of the former group discontinued BD Tx because of disease progression during the observation period, while 56.3% of the latter group experienced

Table 3 Impact of patients' background and treatment response for OS and PFS (univariate analysis)

		OS (*p* value)	PFS (*p* value)
Age	≤64 vs. ≥65	0.0010	0.0404
M-protein	IgG vs. non-IgG	0.7454	0.0714
ISS	III vs. I or II	0.0345	0.1865
13q−	+ vs. −	0.9415	0.9692
Dex dosage (mg/day)	≥20 vs. <20	0.0324	0.5734
Piror thalidomide	+ vs. −	0.2153	0.4384
≥PR by 1st course BD Tx	+ vs. −	0.0264	0.0274
≥VGPR within 2 courses BD Tx	+ vs. −	0.0958	0.0107
Prior HDCT/Autologous-SCT	+ vs. −	0.1940	0.6241
Prior Allogeneic-SCT	+ vs. −	0.4921	0.7257

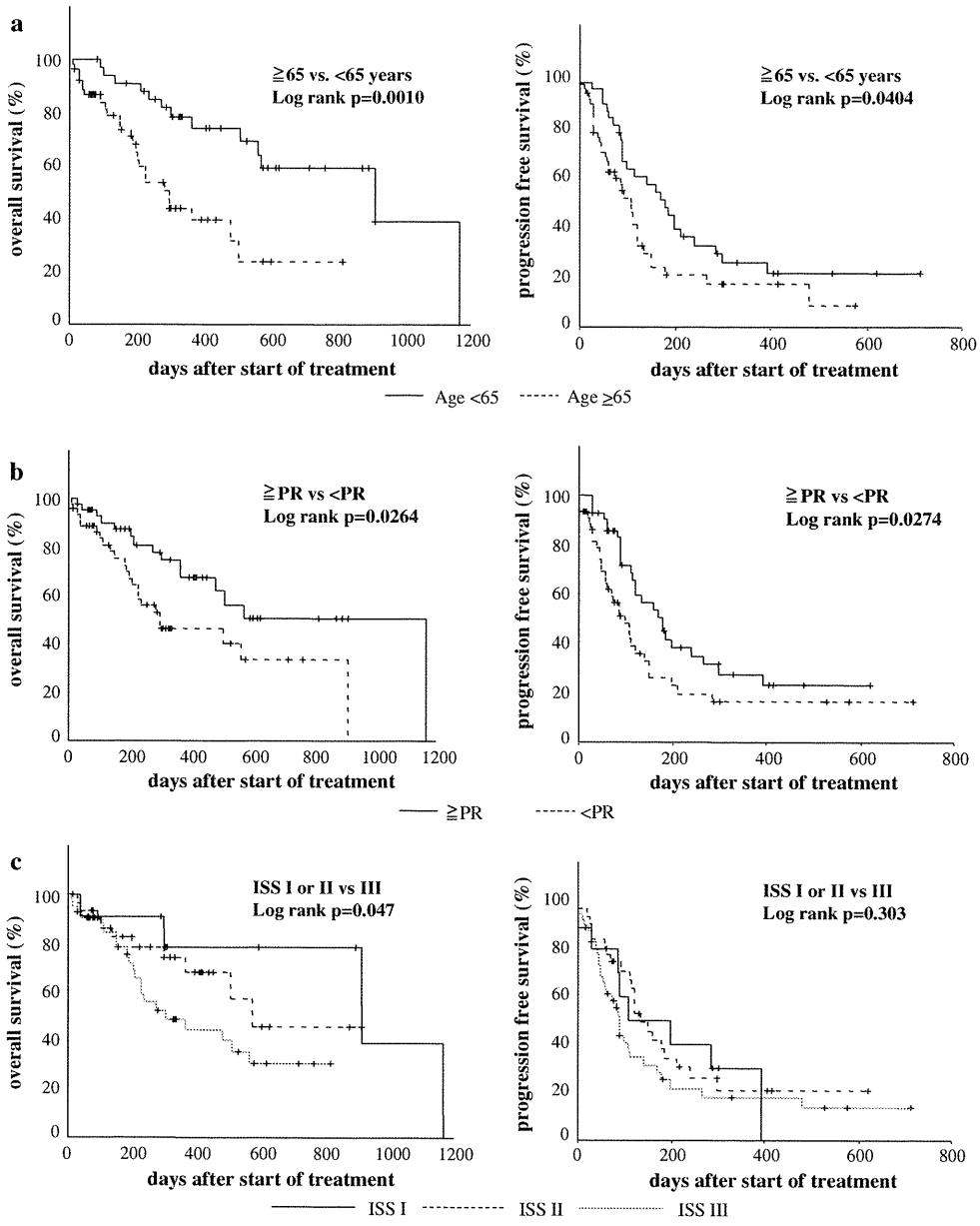


Fig. 2 Impacts of variables on OS and PFS for BD Tx. **a** OS (*left*) and PFS (*right*) for patients over 65 years old (*dotted line*) and less than 64 years old (*solid line*). **b** OS (*left*) and PFS (*right*) for patients who attained PR or better (*solid line*) and less than SD (*dotted line*) in response to the first cycle of BD Tx. **c** OS (*left*) and PFS (*right*) for patients with ISS stage I (*solid line*), II (*dotted line*), or III (*thin line*). **d** OS (*left*) and PFS (*right*) for patients administrated with Dex ≥20 mg/day (*dotted line*) and with Dex <20 mg/day (*solid line*). **e** Impact of attainment of VGPR (*solid line*) and less than SD (*dotted line*) within two cycles of BD Tx on OS (*left*) and PFS (*right*)

disease progression even with the continuation of BD Tx. These results indicate that, although there seem to be no predictive factors before the start of BD Tx, careful observation of the response to the first course may indicate the future clinical course for Rel/Ref MM. If PR is not achievable with the first BD Tx, an early change to an alternative therapeutic approach should be at least considered. Dex dosage was also found to be predictive for OS with BD Tx, in accordance with previous studies those showed the effectiveness of Dex over 20 mg in BD combination. Also, although disease stage according to ISS was predictive for OS with BD Tx, the direct impact of ISS-determined disease stage on the outcome for BD Tx remains questionable, since ISS was basically defined on the basis of OS. In fact, ORR did not differ among patients in our study with different disease stages. Although previous studies demonstrated that patients with prior Thal exposure had worse outcome when treated with bortezomib [18, 19], this was not true in the present study. Finally, we also analyzed the impact of therapies prior to BD in 38 patients whose precise clinical records were available. We found no statistical differences in both OS and PFS among patients with different number of prior regimen in this population (Supplementary Fig. 1). In addition, neither the number of chemotherapy regimens prior to BD Tx nor the duration from initial therapy to BD Tx significantly influence on the outcome of BD therapy in this population (data not shown).

Fig. 2 continued

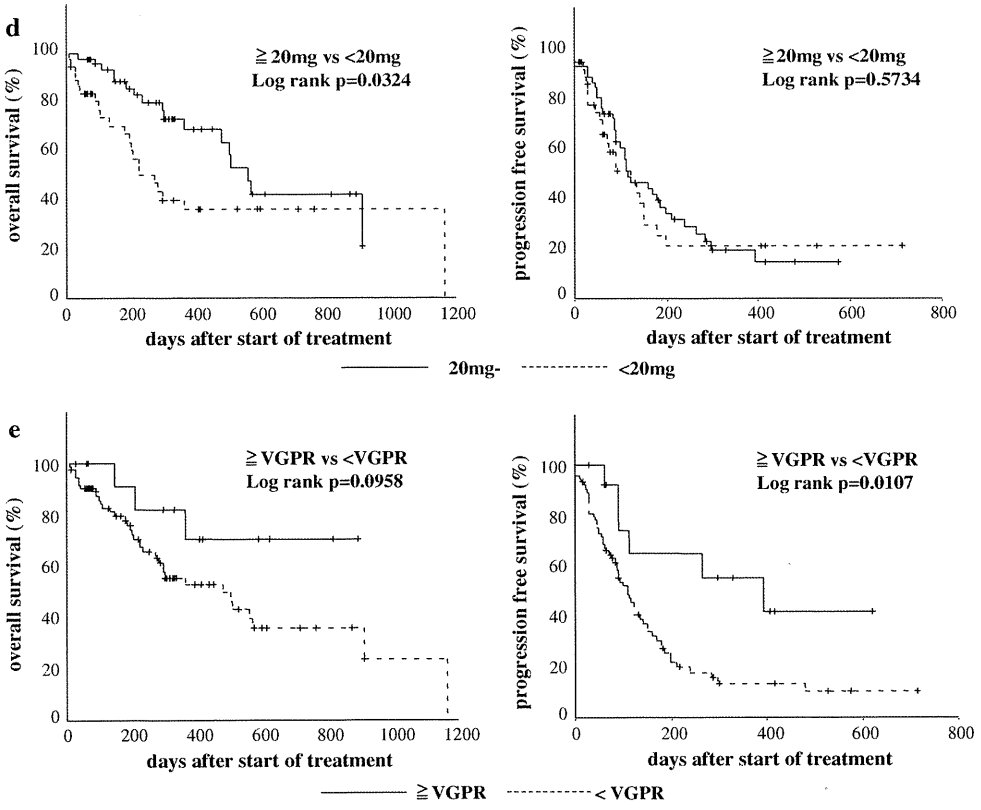
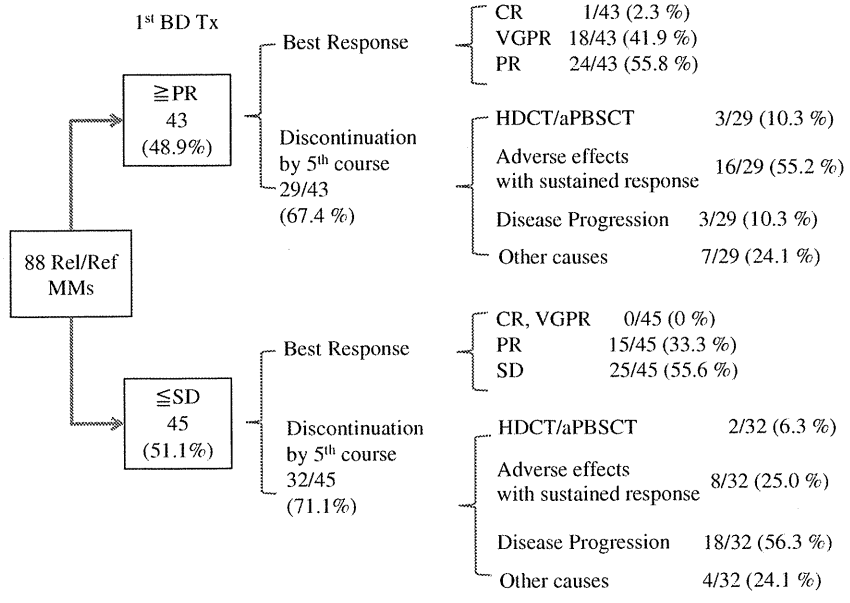


Fig. 3 Impact of the response to the first course BD Tx on the subsequent clinical course. HDCT/aPBSCT: high-dose chemotherapy with autologous PBSCT. Other factors include the cessation of BD Tx due to disease stabilization, change to treatment for other diseases, patient's decision, or poor compliance



In this study, ORR for BD Tx for Rel/Ref MM patients was 66.9%. While this result was better than outcomes reported by various previous studies with ORR ranging from 33 to 50%, the CR rate in our study was greatly inferior to previously reported CRs of 4–13% [4, 5, 20]. One explanation for the difference in CR rates between our study and previous trials may be that the median number of treatment cycles for patients enrolled in this study was less than those used in previous clinical trials. For instance, the APEX trials in which the median number of BD Tx cycles was six, which is double that for our study, demonstrated that the therapy of longer duration led to improvement in ORR, and that the best response of CR occurred on or after cycle 8 in 22% of the patients. Similarly, approximately

Table 4 Major adverse events occurring in BD Tx

Adverse event, *n* (%)	Grade 1–2	Grade 3–4	Total
Diarrhea	18 (20.5)	7 (8.0)	25 (28.4)
Constipation	21 (23.9)	9 (10.2)	30 (34.1)
Ileus	0 (0.0)	3 (3.4)	3 (3.4)
Fatigue	17 (19.3)	6 (6.8)	23 (26.1)
Peripheral neuropathy	29 (33.0)	22 (25.0)	51 (58.0)
Hyponatremia	18 (20.5)	5 (5.7)	23 (26.1)
Rash	5 (5.7)	0 (0.0)	5 (5.7)
Fever	6 (6.8)	0 (0.0)	6 (6.8)
Pneumonitis	0 (0.0)	2 (2.3)	2 (2.3)
Sepsis	2 (2.3)	4 (4.5)	6 (6.8)
VZV infection	5 (5.7)	7 (8.0)	12 (13.6)
Infection, others	8 (9.1)	18 (20.5)	26 (29.5)
Neutropenia	8 (9.1)	14 (15.9)	22 (25.0)
Anemia	18 (20.5)	15 (17.0)	33 (37.5)
Thrombocytopenia	14 (15.9)	43 (48.9)	57 (64.8)

($n = 88$)

20% of the patients who responded attained maximal M-protein reduction during cycle 8 or later [4]. These results suggest that continuation of BD Tx is crucial for the achievement of CR or best response. The shorter treatment for our patients was due to the cessation of BD Tx within a relatively short period because of somewhat higher incidence and severer degree of AEs, especially PN, in our series. Indeed, 61 of the 88 patients in our study discontinued BD Tx within five cycles, and AEs accounted for discontinuation by 39.3% of them. Specifically, of the 43 patients who attained PR or better with the first BD Tx but discontinued within five cycles of BD Tx 55.2% did so because of AEs. Among various AEs, Bor-induced PN (BIPN) was the major cause for discontinuation of BD Tx. In our series, 25% patients experienced Grades 3–4 (G3/4) BIPN. The incidence of G3/4 BIPN seemed relatively higher than previous studies with Bor in Western countries, while was quite similar with the result in the previous Japanese study by Ohguchi et al. [21]. It is conceivable that the degree and the nature of BIPN differ among different races, and further study is needed. Although BIPN is generally considered to be reversible after the cessation of drug administration [13], this was not always the case for several of our patients, whose symptoms continued or even got worse after the discontinuation of Bor. There is an urgent need for a less toxic treatment protocol and a more effective prophylactic strategy for BIPN. Combined together, patients who achieved PR or better with the first course BD Tx are likely to be good candidates for the repeated BD Tx, otherwise, BD Tx may result in increase of its AEs with less or no clinical benefits.

In conclusion, our study demonstrated that BD Tx is effective for Rel/Ref MM, and also indicated that the first course response to BD Tx is associated with both OS and PFS in Rel/Ref MM. In addition, the attainment of VGPR or CR within two cycles of BD Tx was found to be associated with longer PFS. These results suggest that a good response during the earlier BD Tx courses may be predictive for the subsequent therapeutic pathway of Rel/Ref MM.

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ORIGINAL ARTICLE

Downregulated plasma miR-92a levels have clinical impact on multiple myeloma and related disorders

S Yoshizawa[1], JH Ohyashiki[2], M Ohyashiki[1], T Umezu[3], K Suzuki[4], A Inagaki[5], S Iida[5] and K Ohyashiki[1,3]

Recent studies have demonstrated that one-third of known microRNAs (miRNAs) are stably detectable in plasma. Therefore, we assessed plasma miRNAs to investigate the dynamics of oncomir 17-92a, which is highly expressed in multiple myeloma (MM) patients. The plasma miR-92a level in symptomatic MM patients was significantly downregulated compared with normal subjects ($P<0.0001$), regardless of immunoglobulin subtypes or disease stage at diagnosis. In contrast, miR-92a levels in peripheral blood $CD8^+$ or $CD4^+$ cells from MM patients were lower than those of normal subjects, and the miR-92a levels of the cells tended to correlate with plasma miR-92a levels. The plasma miR-92a level in the complete remission group became normalized, whereas the partial response (PR) and very good PR groups did not reach the normal range. In smoldering MM, the plasma miR-92a level did not show a significant difference compared with normal subjects. Our findings suggest that measurement of the plasma miR-92a level in MM patients could be useful for initiation of chemotherapy and monitoring disease status, and the level may represent, in part, the T-cell immunity status of these patients.

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Keywords: multiple myeloma; plasma miR-92a; T lymphocytes;

INTRODUCTION

MicroRNAs (miRNAs) consist of approximately 18–22-nucleotide non-coding RNA molecules that regulate posttranscriptional gene expression by degradation or repression of mRNA molecules.[1-3] Individual miRNAs can target multiple mRNAs and control transcription in approximately one-third of human genes. Recent studies have shown that these miRNAs are closely involved in cell differentiation, proliferation, apoptosis or oncogenesis.[1-3] In various human cancer, there is evidence of the alteration of tumor tissue-specific miRNA.[4-6] A 2008 study demonstrated that miRNAs stably exist in serum and plasma,[7,8] and a recent advance revealed the presence of circulating miRNAs within lipoprotein (known as microvesicles), which may help to protect the miRNAs from RNase-dependent degradation.[9,10] Moreover, some extracellular circulating miRNAs in blood plasma are independent of exosomes and are bound to Ago2 protein, resulting in strong nuclease/proteinase resistance,[11] thus indicating a possible role of circulating miRNAs in healthy subjects and disease condition.[7,8] The biological relevance of circulating miRNAs remains unclear, although they may have an important role in cancer metastasis or neo-angiogenesis. Therefore, circulating miRNAs are thought to be possible diagnostic or prognostic biomarkers of human diseases.[12-14]

It is well-known that multiple myeloma (MM) cells have a high expression level of the miR-17-92a cluster,[15] whereas plasma miR-92a levels in acute lymphoid leukemia or non-Hodgkin's lymphoma are extremely downregulated.[16-18] Downregulation of miRNAs, let-7a and miR-16 in myelodysplastic syndromes also has been reported, and their levels were significantly associated with progression-free survival and overall survival, suggesting that certain miRNAs in plasma can serve as noninvasive biomarker in hematologic malignancies.[19] To gain more insight into the clinical relevance of plasma miR-92a expression, we evaluated plasma miR-92a levels in MM patients at various phases and in patients with related disorders. In addition, we examined cellular miR-92a levels in circulating lymphocytes obtained from untreated MM patients and compared them with the plasma miR-92a level to ascertain the possible association between immunological condition and plasma miR-92a expression.

MATERIALS AND METHODS

Patients and samples

We evaluated peripheral blood obtained from 168 patients with monoclonal gammopathies: 138 with symptomatic MM, 8 with smoldering MM (SMM) and 22 with monoclonal gammopathy of undetermined significance (MGUS). The diagnosis of monoclonal gammopathy was based on the definition of the International Myeloma Working Group using the level of serum M-protein, proportion of plasma cells in bone marrow and the presence of end-organ damage.[20] At the time of plasma collection, the disease status of the 138 patients with symptomatic MM was as follows (Table 1): 62 newly diagnosed, 8 complete remission (CR), 11 very good partial response (VGPR), 15 partial response (PR), 14 stable disease and 28 progressive disease. None of the 62 newly diagnosed MM patients had del(13q) anomaly, where the miR-17-92a located, according to the standard cytogenetic technique. Of the eight SMM patients, two had received cytotoxic therapy because of an increase in M-protein ($\geqslant 5$ g/dl). No MGUS patients had received chemotherapy before plasma collection. We analyzed plasma from 113 normal individuals and isolated lymphocytes from 21 healthy volunteers as the control. These samples were handled similarly to those obtained from MM patients.

[1]Department of Hematology, Tokyo Medical University, Tokyo, Japan; [2]Institute of Medical Science, Tokyo Medical University, Tokyo, Japan; [3]Department of Molecular Science, Tokyo Medical University, Tokyo, Japan; [4]Department of Hematology, Japanese Red Cross Medical Center, Tokyo, Japan and [5]Department of Medical Oncology and Immunology, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan. Correspondence: Professor K Ohyashiki, Department of Hematology, Tokyo Medical University, 6-7-1 Nishi-shinjuku, Shinjuku-ku, Tokyo 160-0023, Japan.
E-mail: ohyashik@rr.iij4u.or.jp
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Table 1. Plasma miR-92a/miR-638 expression levels in monoclonal gammopathies

	Number of subjects	*Median*	*Mean*	*Standard error*	*Minimum*	*Maximam*	*95% CI*
Normal	113	1.244	1.388	0.06993	0.2154	3.58	1.249-1.527
Symptomatic MM at diagnosis	62	0.05202	0.1008	0.01899	0.000045	0.8586	0.06285-0.1388
Myeloma subtype							
Ig G	26	0.06107	0.121	0.03378	0.000045	0.8586	0.05146-0.1906
Ig A	12	0.1508	0.05435	0.1901	0.006992	3.127	0.3020-1.089
Ig D	3	0.1667	0.2353	0.1423	0.0304	0.5087	−0.3769-0.8475
Bence-Jones protein	14	0.03866	0.0973	0.04252	0.004289	0.6177	0.005448-0.1892
Non-secretory myeloma	4	0.06367	0.07089	0.02974	0.01013	0.1461	−0.02376-0.1655
Plasma cell leukemia	3	0.02628	0.03341	0.01985	0.003151	0.07081	−0.05201-0.1188
Disease status of MM							
Newly diagnosed	62	0.05202	0.1008	0.01899	0.000045	0.8586	0.06285-0.1388
CR	8	1.494	1.602	0.354	0.2285	3.643	0.7653-2.439
VGPR	11	0.2176	0.3591	0.09528	0.04138	1.028	0.1468-0.5714
PR	15	0.8179	0.7967	0.2018	0.1216	1.979	0.5409-1.052
Stable disease	14	0.3404	0.4251	0.2634	0.003508	0.9965	0.2479-0.6022
Progressive disease	28	0.06335	0.2018	0.04978	0.003319	0.8586	0.09962-0.3039
SMM	8	0.7943	1.188	0.4791	0.09087	5.242	0.3002-1.348
MGUS	22	0.4055	0.6403	0.1309	0.007652	2.428	0.3681-0.9124

Abbreviations: CI, confidence interval; CR, complete remission; MGUS, monoclonal gammopathy of undermined significance; MM, multiple myeloma; PR, partial response; SMM, smoldering MM; VGPR, very good partial response. Disease status of multiple myeloma was categorized by International Myeloma Workshop Consensus criteria.[20]

This study was approved by the institutional review board of Tokyo Medical University (no. 930, approved 24 June 2008). Written informed consent was obtained from all the participants prior to the collection of specimens according to the Declaration of Helsinki.

TaqMan low-density array screening

Total RNA was isolated with the mirVana PARIS kit (Ambion, Austin, TX, USA). Plasma samples from five patients or five healthy individuals were mixed evenly, and 500 μl of mixed plasma was diluted with 500 μl of binding solution. After a 5-min incubation, 1 μl of 1 nM ath-miR-159 (Hokkaido System Science, Hokkaido, Japan) was added to each aliquot, followed by vortexing for 30 s and incubation on ice for 10 min. Subsequent phenol extraction and filter cartridge work was performed according to the manufacturer's instructions. In all, 3 μl of RNA solution from the 50-μl elute was used as an input in each reverse transcription (RT) reaction. The RT reaction and pre-amplification step were set up according to the manufacturer's recommendations. miRNAs were reverse transcribed with the Megaplex Primer Pools (Human Pools A v2.1; Applied Biosystems, Foster City, CA, USA). RT reaction products from the plasma sample were further amplified with Megaplex PreAmp Primers (Primers A v2.1). The expression profile of the miRNAs was determined with the Human TaqMan miRNA Arrays A (Applied Biosystems). Quantitative RT-PCR was performed on an Applied Biosystems 7900HT thermocycler according to the manufacturer's recommended program. With the use of SDS2.2 software and DataAssist (Applied Biosystems), the expression of plasma miRNAs was calculated based on their Ct values normalized by those of ath-miR-159, which was spiked in each plasma sample.

miR-92a quantitative RT-PCR

Total RNA in cells was isolated with an miRNeasy Mini Kit (Qiagen, Germantown, MD, USA), and RNA in plasma was extracted as reported previously.[17,18] miRNAs were quantified with TaqMan MicroRNA Assays (Applied Biosystems) with modification and miRNA-specific stem-loop primers (has-miR-92a, 000431; has-miR-638, 001582; Applied Biosystems), as we reported previously.[17,18] The plasma miR-92a expression was normalized to miR-638 expression, and the cellular miR-92a expression levels were normalized to RNU6B (001093; Applied Biosystems). We have never detected U6B in a plasma sample, although U6B is commonly used as an internal standard for miRNA expression analysis in cells. To normalize the expression level of miR-92a in plasma, we compared plasma miR-92a expression using an external standard, cel-miR-39 (Hokkaido System Science), as well as an internal standard, miR-638, which is stably detected in all samples. The results using miR-638 are compatible with those using cel-miR-39 (data not shown). We therefore used miR-638 as a reference in each plasma sample, as previously reported.[17,18]

Lymphocyte separation

The $CD4^+$ or $CD8^+$ T-cell fractions were separated with an isolation kit for humans (Miltenyi Biotec, Bergisch Gladbach, Germany) and AutoMACS Pro Separator (Miltenyi Biotec), according to the supplier's instruction, and stored at −80 °C until use.

Statistical analysis

GraphPad 5.0 software (GraphPad Software Inc, San Diego, CA, USA) was used for statistical analysis. The Mann-Whitney test was used to determine statistical significance between two groups and one-way ANOVA for three or more groups. We also used the Chi-square test and Student's *t*-test, when appropriate. *P*-values <0.05 were considered to indicate statistically significant differences.

RESULTS

Identification of differentially expressed plasma miRNAs in MM patients and healthy volunteers

Plasma miRNA expression was initially screened using the TaqMan low-density array system. Of the 381 miRNAs represented on the well plates, 331 miRNAs were not detected after the 35 PCR cycles. Among the remaining 50 miRNAs, upregulated and downregulated miRNAs in MM plasma samples (expression level in the sample was 4-fold greater or lower than that of healthy volunteers) were selected. We then evaluated the rank order of expression of each miRNA by ΔCt value among all detected miRNAs.

Most of the miRNAs were downregulated in plasma samples obtained from MM patients (Figure 1a). The most striking difference of expression was found in miR-223. In addition, members of the miR-17-92a cluster (miR-19b, miR-17, miR-92a, miR-20a and miR-19a) were significantly decreased in MM

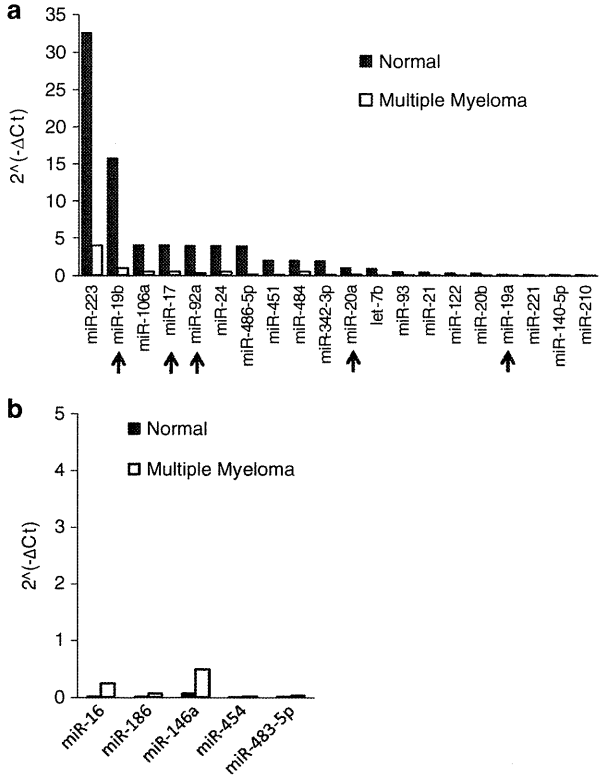

Figure 1. Identification of expressed plasma miRNAs in MM. (**a**) Downregulated miRNAs in MM (open bars). The expression level in the sample was 4-fold lower than that of normal controls (solid bars). Arrows indicate miR-17-92a polycistronic cluster. (**b**) Upregulated miRNAs in MM (open bars). The expression level in the sample was 4-fold higher than that of normal controls (solid bars).

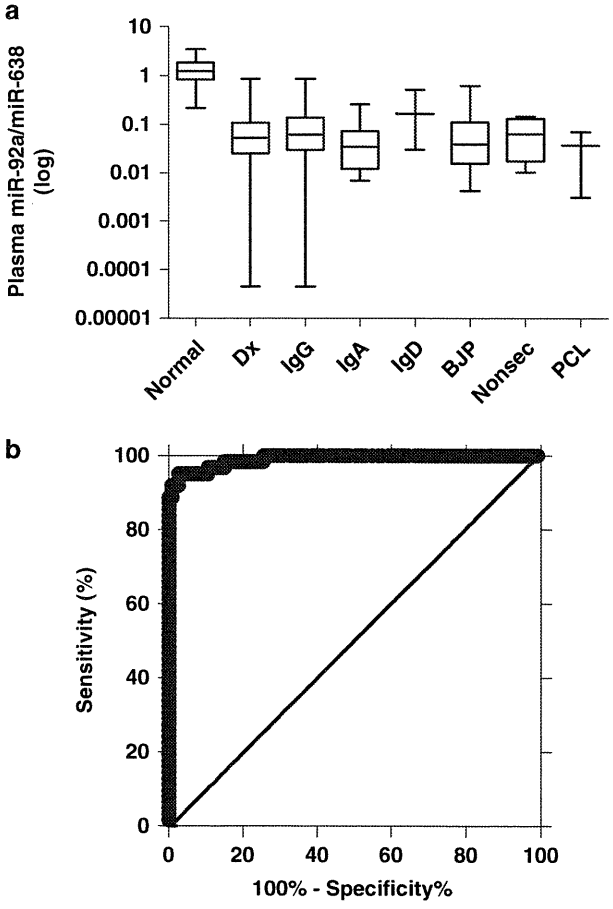

Figure 2. Plasma miR-92a values in MM and receiver operating characteristic curve. (**a**) Plasma miR-92a level (miR-92a/miR-638) in patients with MM at the time of diagnosis. A significant downregulated plasma miR-92a level is notable in MM at diagnosis (MM-Dx) ($P<0.0001$). No particular difference in plasma miR-92a level is evident among immunoglobulin subtypes, Bence-Jones protein type or non-secretory MM by one-way ANOVA. Bars indicate minimum to maximum plasma miR-92a levels and boxes indicate 95% confidence interval (CI). (**b**) The cut-off level of plasma miR-92a/miR-638 in all MM at diagnosis is 0.2593. The sensitivity is 91.94% (95% CI: 82.17–97.33%) and specificity is 99.12% (95% CI: 95.17–99.98%).

samples. Although we found several upregulated miRNAs in MM samples, the Ct values were generally low in both MM samples and normal controls (Figure 1b). We therefore focused on downregulation of the miR-17-92a cluster, rather than the single miRNA (that is, miR-223), because the expression of miR-17-92a cluster is essential in the lymphoid ontogeny and measured the expression level of miR-92a in a large series of patients.

Plasma miR-92a level of symptomatic MM

Plasma miR-92a levels were significantly lower in newly diagnosed MM patients ($n=62$) compared with healthy controls (Student's *t*-test: $P<0.0001$); the level of plasma miR-92a in symptomatic MM patients was $<10\%$ compared with normal subjects (mean ± standard error 0.1008 ± 0.01899 vs 1.388 ± 0.06993; Table 1). Among the MM patients, there were no significant differences in the plasma miR-92a levels, irrespective of MM subtype ($P=0.4472$) (Figure 2a), light chain type ($P=0.4413$) (Supplementary Figure 1A) or International Staging System staging ($P=0.1955$) (Supplementary Figure 1B). The presence of anemia ($P=0.0990$), bone lesion ($P=0.6701$), renal damage ($P=0.4258$) or hypercalcemia ($P=0.1989$) did not affect plasma miR-92a level at MM diagnosis (Supplementary Figure 2AD, Supplementary Table 1). Plasma miR-92a level was not correlated with beta2 microglobulin elevation ($P=0.3675$), albumin level ($P=0.0693$), elevated lactate dehydrogenase ($P=0.4863$) or performance status ($P=0.9850$) (Supplementary Table 1).

The receiver operating characteristic curve was generated by comparing the patients' ΔCt values with those of healthy volunteers. The analysis showed that miR-92a was a good marker of newly diagnosed MM (AUC = 0.9810), indicating 91.94% sensitivity (95% confidence interval: 82.17–97.33%) and 99.12% specificity (95% confidence interval: 95.17–99.98%) when the cut-off levels of plasma miR-92a in MM patients at diagnosis could be achieved (that is, 0.2593) (Figure 2b).

Plasma miR-92a level in the various states of MM

We next compared the plasma miR-92a level in MM patients in various clinical states (Table 1). Patients in CR ($n=8$) had a significant increase in plasma miR-92a compared with those at diagnosis ($P<0.0001$), and seven out of the eight patients were normalized ($\geqslant 0.2593$ cut-off level). Of the PR patients ($n=15$), a significant increase in plasma miR-92a was notable ($P<0.0001$), but these patients still had low plasma miR-92a compared with normal subjects ($P=0.0033$). Although patients with stable disease ($n=14$) had a partially normalized plasma miR-92a level, three patients had an extremely low level, similar to those of patients with newly diagnosed MM. In progressive disease patients ($n=28$), the plasma miR-92a level again downregulated and no significant difference was notable compared with newly diagnosed MM (Figure 3a).

Plasma miR-92a levels of MGUS and SMM patients

We also measured plasma miR-92a levels in 8 patients with SMM and 22 with MGUS (Table 1). Compared with normal subjects, patients with SMM showed no significant differences in plasma miR-92a levels ($P=0.4642$), but they had elevated levels compared with MM patients ($P=0.0496$) (Figure 3b). The plasma miR-92a level was significantly decreased in MGUS patients compared with normal subjects ($P<0.0001$), but they had significantly higher plasma miR-92a levels compared with MM patients ($P=0.0005$). No difference in plasma miR-92a levels was noted between SMM and MGUS patients ($P=0.2959$). The plasma miR-92a levels did not correlate with duration between the time of diagnosis of MGUS and the measurement of levels in this study (data not shown).

miR-92a level in separated lymphocytes

We then compared miR-92a levels in T lymphocytes obtained from healthy subjects ($n=21$) with those of MM patients at diagnosis ($n=6$) or at CR/VGPR status ($n=5$). The miR-92a level in $CD4^+$ or $CD8^+$ T lymphocytes in MM patients at diagnosis was significantly lower compared with that of healthy subjects ($CD4^+$: $P=0.0178$; $CD8^+$: $P=0.0092$) (Figure 4a). Comparison of miR-92a of $CD4^+$ and $CD8^+$ T lymphocytes showed a roughly linear correlation, and most MM patients in CR/VGPR status had an increased level (Figure 4b). Although no apparent correlation was evident in normal subjects, MM patients with low levels of plasma miR-92a had low levels of miR-92a expression in $CD4^+$ (Figure 4c) and $CD8^+$ T lymphocytes (Figure 4d), and three out of the five MM patients with CR/VGPR had normal levels of plasma and cellular miR-92a.

DISCUSSION

The use of plasma miRNAs as potential biomarkers is a growing research area,[12-14] and this is the first study to look at plasma miRNAs in MM. Our findings indicate the possible clinical utility of plasma miRNA level in MM patients. The recent introduction of new agents has improved the response rate and survival in patients with MM.[21] In MM treatment, the decision to start chemotherapy usually depends on the presence of clinical symptoms with high evidence level, so-called CRAB (hypercalcemia, renal failure, anemia, bone lesion) symptoms.[22] Moreover, the therapeutic target point in MM patients has been proposed,[23] and practical guidelines for the therapeutic strategy have been helpful in clinical decision-making for MM treatment.[24,25] However, there are some MM patients for whom the timing and combination of chemotherapy are difficult to decide,[26] although high-risk MM patients can be classified.[27,28] Because, decisions regarding chemotherapy are occasionally difficult in non-secretory or asymptomatic MM patients, a novel diagnostic marker is urgently required, in addition to the conventional diagnostic tool.[21] In the current study, we demonstrated the downregulation of plasma miR-92a in symptomatic MM patients, irrespective of the presence or absence of renal damage or M-protein, suggesting that the measurement of the plasma miR-92a could be helpful for deciding when to initiate chemotherapy.

Chromosomal abnormalities and molecular alterations in MM cells have been extensively investigated, and some of them are currently incorporated into the risk analysis.[23,27,29] In addition, pathogenesis of extracellular circumstances, including angiogenesis,[30] in MM patients is an important issue not only for understanding the biology of myeloma but therapeutic strategies as well.[21] In myeloma cells, overexpression of the miR-17-92a has been noted in the transformation from MGUS to MM.[15] Therefore, miR-17-92a expression is thought to correlate with tumor burden in MM patients. In contrast, we found no difference in plasma miR-92a levels between MGUS and SMM patients. Because the plasma miR-92a level was significantly different between patients with SMM

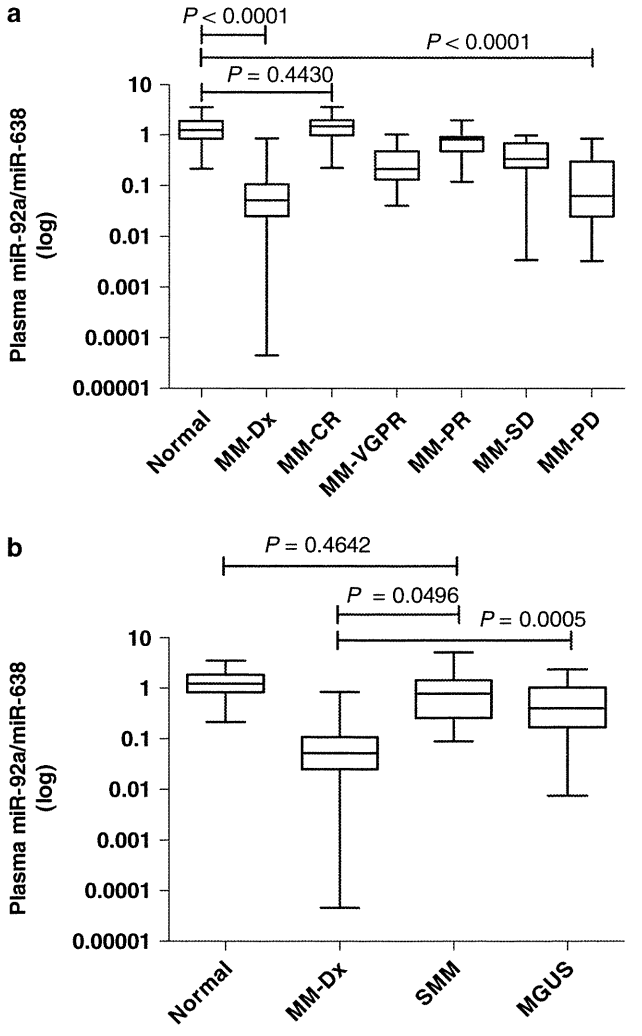

Figure 3. Plasma miR-92a expression in MM at various clinical phases and SMM or MGUS. (**a**) The downregulated plasma miR-92a level at myeloma diagnosis (MM-Dx) was normalized in CR (MM-CR) and was partially normalized at VGPR (MM-VGPR) or PR (MM-PR) phase. (**b**) Patients with SMM had a higher plasma miR-92a level than that of symptomatic myeloma ($P=0.0496$), but they had low levels compared with normal subjects. Although some patients with MGUS had low levels of plasma miR-92a, most MGUS patients had plasma miR-92a levels similar to those with SMM. Bars indicate minimum to maximum plasma miR-92a levels and boxes indicate 95% CI.

and symptomatic myeloma, the level of plasma miR-92a level could reflect the pathological condition of patients rather than the tumor burden of myeloma cells in the body. Moreover, normalization of the plasma miR-92a level after obtaining CR suggests that the plasma miR-92a level might serve as an indicator for therapeutic response. The number of treated patients studied was small, however, and further research is needed to clarify this possibility.

Studies have shown that some circulating miRNAs in cancer-bearing patients are tumor-derived,[7] and thus such miRNAs might be used as biomarkers of cancer.[12-14] The downregulation of plasma miR-92a has been recognized in patients with hematologic neoplasia, including acute leukemia,[16,17] non-Hodgkin's lymphoma[18] and hepatocellular carcinoma,[31] as well as in patients with cardiovascular diseases.[32] Because miR-17-92a is essential in the development and ontogeny of the lymphoid system,[33,34] we examined the correlation between miR-92a levels in separated lymphocytes and plasma. The plasma miR-92a levels was