MAFb protein confers intrinsic resistance to proteasome inhibitors in multiple myeloma

Ya-Wei Qiang*, Shiqiao Ye, Yuhua Huang, Yu Chen, Frits Van Rhee, Joshua Epstein, Brian A. Walker, Gareth J. Morgan and Faith E. Davies

Abstract

Background: Multiple myeloma (MM) patients with t(14;20) have a poor prognosis and their outcome has not improved following the introduction of bortezomib (Bzb). The mechanism underlying the resistance to proteasome inhibitors (PIs) for this subset of patients is unknown.

Methods: IC50 of Bzb and carfilzomib (CFZ) in human myeloma cell lines (HMCLs) were established by MTT assay. Gene Expression profile (GEP) analysis was used to determine gene expression in primary myeloma cells. Immunoblotting analysis was performed for MAFb and caspase family proteins. Immunofluorescence staining was used to detect the location of MAFb protein in MM cells. Lentiviral infections were used to knock-down MAFb expression in two lines. Apoptosis detection by flow cytometry and western blot analysis was performed to determine the molecular mechanism MAFb confers resistance to proteasome inhibitors.

Results: We found high levels of MAFb protein in cell lines with t(14;20), in one line with t(6;20), in one with Igλ insertion into MAFb locus, and in primary plasma cells from MM patients with t(14;20). High MAFb protein levels correlated with higher IC50s of PIs in MM cells. Inhibition of GSK3β activity or treatment with Bzb or CFZ prevented MAFb protein degradation without affecting the corresponding mRNA level indicating a role for GSK3 and proteasome inhibitors in regulation of MAFb stability. Silencing MAFb restored sensitivity to Bzb and CFZ, and enhanced PIs-induced apoptosis and activation of caspase-3, −8, −9, PARP and lamin A/C suggesting that high expression of MAFb protein leads to insensitivity to proteasome inhibitors.

Conclusion: These results highlight the role of post-translational modification of MAFb in maintaining its protein level, and identify a mechanism by which proteasome inhibitors induced stabilization of MAFb confers resistance to proteasome inhibitors, and provide a rationale for the development of targeted therapeutic strategies for this subset of patients.

Keywords: MAF, Proteasome inhibitors, GSK3β, Caspases, Apoptosis, Myeloma, Drug resistance

Background

Multiple myeloma (MM) is a malignancy of terminally differentiated clonal plasma cells displaying significant molecular heterogeneity. The MF molecular subgroup defined by Gene Expression Profiling (GEP) at the University of Arkansas for Medical Sciences (UAMS) includes cases with overexpression of MAFb resulting from t(14;20) and C-MAF from t(14;16) [1]. Translocation t(14;20) occurs in approximately 1 to 2% of MM [2–4], and is associated with poor prognosis [3, 4] with the development of extramedullary disease [5] and primary plasma cell leukemia [6].

MAFb overexpression occurs as a result of t(14;20), in which genes at the 20q11 breakpoints are juxtaposed to IGH gene on 14q32 [4, 7, 8]. Additionally, high expression of the MAFb gene is also associated with translocation involving t(6;20) [7] or t(20;22) [9], or insertions of the 3’enhancers of Igλ or Igκ into the MAFb gene on 20q11 [10, 11]. Our previous work showed that t(4;14) and MF subgroup [including cases with a t(14;16) and t(14;20)] have an inferior survival [12, 13], and that the addition of Bortezomib (Bzb) to high dose melphalan
based regimens provided a survival advantage to patients with t(4;14), while not benefitting patients in the MF subgroup [14, 15]. Previously, we have demonstrated that regulation of C-MAF stability by GSK3 is responsible for resistance to proteasome inhibitors (PIs) in t(14;16) MM [16]. It is currently unclear if a similar molecular mechanism is the cause for proteasome inhibitor resistance in patients with high MAFb expression.

The MAFb gene, one member of MAF family, is a basic leucine zipper transcription factor [17]. The MAFb protein may function as a transactivator or transrepressor depending on the target sequence of MAF-responsive elements and interacting proteins, such as c-Fos and ETS-1 [18, 19]. Amongst the MAF family, MAFa and C-MAF display the strongest oncogenic activity, whereas MAFb is less effective in transforming cells [20, 21], although MAFb can induce transformation of embryonic fibroblasts, and transgenic mice with MAFb expressed in hematopoietic stem cells develop plasma cell neoplasia [22]. In myeloma, overexpression of MAFb in U266 cells also enhances proliferation [23]. A number of target genes regulated by MAFb are shared with C-MAF target genes, including Notch, CCND2, CCR1 and ITGB7 [23, 24]. Up-regulation of cyclin D2 enhances cell cycle progression in t(14;16) cases [24, 25] and a high proliferative index [13, 26], whereas upregulation of ITGB7 and CCR1 stimulates MM growth via enhancing MM cell adhesion to stromal cells and cytokine secretion [27, 28].

Here, we demonstrate that high MAFb protein expression is associated with resistance to proteasome inhibitors, and demonstrate that similar to C-MAF mediated proteasome inhibitor resistance, protein stabilization underlies MAFb resistance to proteasome inhibitors. The results provide a rationale for the development of targeted therapeutic strategies directed at the treatment of this poor performing molecular subgroup.

Methods

Cell lines and primary CD138+ plasma cells

Human multiple myeloma cell lines (HMCLs) MM1S was purchased from ATCC (Manassas, VA, Catalogue number: CRL-2974). Other HMCLs were kindly provided by Dr. Stuart Rudikoff, NCI, NIH and Dr. Michael Kuehl, NCI, NIH and described in detail previously [16, 29, 30]. Cells were cultured in growth medium as described previously [29]. CD138-expressing patient MM cells were isolated as previously described [16].

Gene expression analysis

MAFb gene expression was determined by GEP analysis of CD138-expressing MM cells from patients as described previously [1]. MAFb gene expression data set from primary CD138 expressing MM cell presented in this paper have been deposited in the NIH Gene Expression Omnibus (GEO; National Center for Biotechnology Information [NCBI], http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE2658 as described previously [1].

Silencing MAFb expression by short hairpin RNA

Knockdown of MAFb gene was performed in HMCLs expressing high levels of MAFb using a lentiviral expressing system [16]. Briefly, two sequences (5′-CCGGGCCCAGTCTT-3′ and 5′-CCGGCCCATGCTTGGCTTATACCTGCAAGACTGGCCTTTTTT-3′) specific to MAFb gene were engineered in lentiviral expressing system. A control oligonucleotide sequence not matching any sequence in the human genome (5′-CCGGTACAACAGCCACAACGTCTATCTCGAGATACACGTGGCCTGTTGATTTTTT-3′) was used as a control shRNA sequence (designated as shCon). Total RNA, isolated after 24, 48, or 72 h, was subjected to reverse transcription (RT)-PCR and qPCR to determine the degree of target gene silencing. Whole cell lysates were subjected to immunoblotting for MAFb protein measurement using a specific antibody.

MTT assay for cell proliferation

The proliferation of MM cells was determined by colorimetric 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay [31]. Briefly, MM cells (4×10^4/well) in 0.1 ml of IMDM medium with 10% FCS were cultured in flat-bottom, 96-well microliter wells. At the indicated time points, 10 ul of 5 mg/mL MTT was added to each well and incubated for 4 h at 37 °C, followed by adding 0.1 ml of 10% sodium dodecyl sulfate and incubation at 37 °C. Optical density was read on a Spectra Max340 Microplate Spectrophotometer (Molecular Devices) at 570 nm. Proteasome inhibitors including Bzb and Carfilzomib (CFZ) were used as described previously [32, 33].

Detection of apoptotic cells

Cells were cultured in growth media alone for 24 h and treated with or without serial concentrations of proteasome inhibitors for indicated time points. The apoptotic cells were stained following the manufacturer’s instructions and were analyzed by FACS Express 5 Flow Cytometry Professional software (De Novo Software).
Co-culture with bone marrow stromal cells

Co-culture of MM cells with human bone marrow stromal cells (HBMSCs) was performed to determine the effect of MAFb on MM apoptosis in the presence of the BM microenvironment as previously described [34]. MM cells were cultured on a bone marrow stromal layer in 6-well plates in the presence of serial concentrations of proteasome inhibitors (Bzb and CFZ) for 16 h. The MM cells were harvested and subjected to Annexin staining and apoptotic cells detected by flow cytometry analysis.

Immunoblotting

Proteins were isolated from cells treated with or without Bzb, CFZ, MG132, cycloheximide (CHX), SB216763 [34], and LiCl for indicated time points prior to harvesting. The concentration of protein in each sample was determined by BCA protein Assay. Equal concentrations of total protein (50 μl per lane) were separated by SDS-PAGE followed by electrophoretic transfer to Immobilon polyvinylidene difluoride membranes. The proteins in the membranes were detected by specific antibodies including Anti-MAFb, caspase-3, −6, −7, −8, and −9, PARP, lamin A/C antibodies [31]. The membranes were stripped and reblotting with anti-actin antibody for indicating protein loading.

Cycloheximide chase assay

For the study of MAFb half-life, cells were cultured in growth media in the presence of cycloheximide (CHX, 5 μg/ml, Sigma-Aldrich) for 0, 60, 120, 240 min and lysed at various time points. Cell lysate was subject to immunoblotting analysis. Protein loading was normalized by using anti-β-actin antibody. The autoradiographs were scanned and densitometry performed for quantification [33].

Immunofluorescence staining

Immunofluorescence staining was used to determine MAFb protein expression in cell nuclei using a MAFb antibody and DAPI as described previously [32]. Cells were incubated with an anti-MAFb antibody after being fixed with 3.7% formaldehyde in PBS. The slides were stained with Alexa Fluor 488-labeled secondary anti-mouse IgG antibody or Alexa 594-labeled secondary anti-rabbit IgG antibody for visualization. All images were captured under an Axioimager fluorescent microscope (Zeiss, Jena, Germany) and digitalized with Zeiss AxioCam MRc5 and Zeiss Axiovision software.

Real-time quantitative PCR

For quantitation of mRNA expression, total RNA was extracted from MM cell lines and cDNA was generated from 1μg RNA as described previously [33]. qPCR was performed using a QuantStudio™ 6 and 7 Flex Real-Time PCR Systems. The primers/probe sets including MAFb (Hs00271378_s1), ITGB7 (Hs01565750-m1) and CCR1 (Hs00174288-m1) were purchased from Life Technologies. The indicated genes were amplified using methods described in Additional file 1 and gene expression calculated using ΔΔCT analysis. [35, 36].

Statistical analysis

The statistical significance of differences between experimental groups was analyzed by Student t test using the Microsoft Excel software. A significant P value was less than 0.05 by 2-tailed test.

Results

Expression of MAFb in primary myeloma and HMCLs

We previously reported that patients in the MF subgroup including those with a t(14;20) have a poor survival and have not benefited from the addition of Bzb to their therapy [14, 15, 37]. We hypothesized that overexpression of MAFb protein confers intrinsic resistance of MM to proteasome inhibitors. To validate our hypothesis, we first assessed MAFb transcription in primary MM cells by examining MAFb mRNA from a panel of 803 patients belonging to several molecular subgroups using Affymetrix oligonucleotide microarrays. MAFb mRNA was highly expressed in 57 samples from patients in the MF subgroup, Fig. 1a. We next detected MAFb expression by q-RT-PCR in a panel of 32 HMCLs, Fig. 1b. The highest level of MAFb mRNA was seen in SACHI and EJM, both harboring t(14;20); intermediate levels of MAFb were seen in three cell lines; OPM-2 with t(4;14), XG2 with Ep enhancer of immunoglobulin light chain (IGL) inserted near MAFb in 20q11 [10] and L363 with t(6;20) [7]. Low MAFb mRNA levels were seen in four lines; LP1, ANBL6, H929 and Delta47, while no MAFb mRNA was detected in the remaining cell lines.

Consistent with the mRNA levels in t(14;20), immunoblotting analysis demonstrated abundance of MAFb protein in SACHI and EJM, OPM-2 and XG-2 cells, with a low level in L363 cells, Fig. 1c. In contrast, MAFb protein was not detected in MAFb mRNA negative HMCLs. Comparable high levels of MAFb mRNA and protein were seen in SACHI, and EJM cells. Surprisingly, despite lower levels of MAFb mRNA, a relative high level of MAFb protein was seen in OPM-2 cells which harbors a t(4;14).

As mRNA were detected in some cell lines with no detectable protein by immunoblotting, immunofluorescent analysis was used to validate these findings. Weak expression of MAFb proteins was detected in LP1, ANBL6, and H929 cells, with high MAFb expression in SACHI, EJM, OPM-2, XG-2 and L363, Fig. 1d. These results suggest that in MM, immunofluorescent detection of MAFb protein is more sensitive than immunoblotting analysis. We therefore extended our analysis to CD138 selected
MM cells using this sensitive method. We measured MAFb protein in CD138+ cells from 8 patients; two with t(14;20), two with t(4;14) and 4 belonging to other subgroups based on FISH analysis. The MAFb protein was highest in t(14;20), with intermediate levels in the two with t(4;14), and negative in the rest, Fig. 1e (data

Fig. 1 Expression of MAFb gene and protein in primary plasma cells and HMCLs. Affymetrix expression of MAFb mRNA was high in the GEP MF subgroup (a). Gene expression of 803 newly diagnosed MM patients was measured by U133 plus2.0 Affymetrix oligonucleotide microarray probe set 218559_s_at. The box plots indicate the middle of GEP signaling and dot lines with bars in black indicate quartiles. The cells were cultured in the growth mediate for 48 h and harvested for isolated RNA and MAFb mRNA in 27 HMCLs by qRT-PCR analysis as described in Material and methods (b). The cells were cultured in the growth mediate for 48 h and MAFb protein was measured in HMCLs by immunoblotting analysis described in Materials and Methods (c). MAFb protein in nuclei and cytoplasm of HMCLs (d) and MM PC from 4 patients (e) was determined with immunofluorescence staining with DAPI counterstaining. Images were taken with a fluorescence microscope with digital camera as described in supplemental data.
not shown). Taken together, these results indicate that MAFb protein abundance is likely correlated with the mRNA in t(14;20).

High MAFb protein associated with resistance to proteasome inhibitors

To investigate MAFb effects on the sensitivity of MM cells to proteasome inhibitors, we established the half-maximum inhibitory concentration (IC$_{50}$) of Bzb and CFZ for five MAFb protein positive HMCLs and a number of t(14;20) negative HMCLs including H929 by MTT assay for 48 h. The IC$_{50}$ of CFZ and Bzb for SACHI was 50- and 120-nM and, respectively, Fig. 2a. In contrast, treatment of H929 cells with 10-nM of Bzb or 6-nM of CFZ reached 50% of inhibition of cell proliferation, Fig. 1b. The IC$_{50}$ of Bzb and CFZ for SACHI cells were 12- and 8.2-fold higher than for H929 cells, respectively. These results indicate that SACHI cells are more resistant to PIs than H929. Similarly, a relatively high IC$_{50}$ for Bzb and CFZ was seen in EJM, Fig. 2c and XG2, Fig. 2d; respectively. The IC$_{50}$ values of Bzb and CFZ for L363 cells were at a midlevel, Fig. 2e. The IC$_{50}$ for Bzb and CFZ was 12- and 10-nM respectively, Fig. 2f, in OPM-2 cells despite similar high level of MAFb protein to SACHI and EJM. The mean IC$_{50}$ of Bzb (Fig. 2g) and CFZ (Fig. 2h) were significantly lower than those in cells, which lacked MAFb protein expression. The IC50s of Bzb and CFZ in each tested human MM cell lines are summarized at Additional file 1: Table S1. Taken together, these results suggest that myeloma cells with high MAFb protein due to translocation to 20q11 or Ig$\lambda$, or insertion on 20q11 are associated with resistance to proteasome inhibitors.

MAFb protein is a fast-degraded protein

To determine if protein degradation regulates the level of MAFb protein, we used cycloheximide (CHX) to inhibit new protein synthesis from mRNA and determined the protein half-life [38]. As shown in Fig. 3a, MAFb protein decreases in the presence of CHX in 5/5 HMCLs by 60 min, while the actin protein level remains stable for each cell line. The decay curves show that the time for half the amount of MAFb protein to be degraded (T1/2) for 4/5 cell lines was 75 min, and 65 min for L363 cells, Fig. 3b. The half-life of MAFb protein was shorter than beta-actin protein. These results suggest that stability of MAFb protein in MM cells is regulated by post-translational modification. The MAFb protein appeared to become phosphorylated as indicated by a gel mobility shift (a slow migrating band shifted from the main band) at OPM2, XG2 and L363, Fig. 1c and at 180-min for SACHI Fig. 3a.

Inhibition of GSK3 activity stabilizes MAFb protein.

Having demonstrated that protein degradation is responsible for MAFb protein levels in MM cells, we next sought to identify factors associated with regulating the stability of MAFb protein. Phosphorylation of MAF-A protein by GSK3 governs the degradation of the protein in other tissue as well as c-MAF in MM cells [16, 39]. To assess how the inhibition of GSK3 activity affects abundance of MAFb protein, we first used SB216763 to abrogate GSK3 activity. Treatment of HMCL with SB216763 led to blockage of degradation of MAFb protein, Fig. 3g. Significant stabilization of MAFb protein by SB216763 was observed by 120 min in SACHI, XG-2, L363, and OPM-2 cells, and by 180 min in EJM, Fig. 3h (h to l), indicating that activity of GSK3β is required for degradation of MAFb protein. To confirm that GSK3 activity affects MAFb stability, we treated the cell lines with LiCl, another GSK3beta inhibitor. Exposure to LiCl led to a stabilization of MAFb protein in all 5 MM cell lines within 120 min, (Additional file 1: Figure S1). These results confirm that GSK3 activity is required for degradation of MAFb protein in MM. Since MAPK regulates stability of MAF protein in other cell type [40], we determined if MAPK is involved in regulating stability of MAFb protein. To do so, we utilized U0126, which inhibits subsequent phosphorylation and activation of ERK by MEK [31]. Treatment of SACHI, EJM and XG-2 cells with U0126 did not affect MAFb protein levels (data not shown). We next looked at the effect of p38 and Jun N-terminal protein kinases on stabilization of MAFb protein by treatment of these cells with SB20350, a well-known, p38 inhibitor and SP6000125, a Jun N-terminal protein kinases, alone or in combination. Neither SB20350 alone, nor SP6000125 alone, nor combination of these two inhibitors together affect the level of MAFb protein, respectively (data not shown). Taken together, these results suggest that GSK3 activity regulates degradation of MAFb protein in MM cells independently of the MAPK pathway and p38 kinases.

Proteasome inhibitors stabilize MAFb protein

Having shown MAFb protein is regulated by GSK3 activity, we next sought to see if the proteasome inhibitors had an effect on the stability of MAFb protein. Treatment of SACHI cells with Bzb resulted in an increase in MAFb, Fig. 4a. An increase in MAFb was also observed following treatment with CFZ. A similar effect of Bzb and CFZ on MAFb protein was observed in XG-2, Fig. 4b, while the proteasome inhibitors had no effect on MAFb mRNA level by qPCR analysis (data not shown) suggesting that proteasome inhibitors prevent degradation of MAFb in a dose-dependent manner.

Since MAFb is a transcription factor [41], we determined the effect of proteasome inhibitors on localization of MAFb protein using immunofluorescent staining. Treatment of SACHI cells with 20-nM of Bzb and CFZ led to an increase of MAFb in the nucleus, Fig. 4c. Similar
Increases in MAFb in the nucleus and cytoplasm were seen in XG-2 and EJM in response to Bzb and CFZ, Fig 4d, while no increase MAFb protein was seen in OPM-2 cells (data not shown) although inhibition of GSK3 activity stabilized MAFb protein in this cell line. Taken together, these results suggest that blockade of proteasome activity by proteasome inhibitors prevents degradation of MAFb protein in the cells with t(14;20) or Igλ insertion, independent of mRNA regulation.
Knockdown MAFb expression restores sensitivity to proteasome inhibitors

To validate the ability of MAFb-mediated resistance to proteasome inhibitor, loss-of-function studies were undertaken using SACHI and EJM cells with robust MAFb expression. To reduce MAFb level, we used shRNA to reduce MAFb expression by 90% compared to cells infected with a scrambled shRNA (shCon), Fig. 5a. A decrease in MAFb protein level in shMAFb cell was observed with an associated decrease in nuclear expression, Fig. 5b and c. Upon MAFb knockdown, a reduction in expression of MAFb target genes, including CCR1, ITGB7 and CCND2 was evident, indicating a functional MAFb knockdown, Fig. 5d. We further explored the effect of MAFb knockdown on proliferation of SACHI cells, and found that proliferation in MAFb knockdown cells markedly reduced, Fig 5e. Furthermore, we evaluated the effect of knockdown of MAFb protein on sensitivity to proteasome inhibitors. In the presence of 200-, 300- and 400-nM of Bzb, the proliferation of shMAFb cells was significantly lower than those in shCon indicating knockdown of MAFb restores sensitivity to proteasome inhibitors. The effect of silencing MAFb on sensitivity to proteasome inhibitors was more obvious with CFZ, Fig. 5f. A similar increase in sensitivity to Bzb was seen in EJM upon knockdown of MAFb (data not shown). It should be noted that MM cell infected with lentivirus particle to expressing shCon or shMAFb lead to approximate 5% of cell undergoing apoptosis shCon and shMAF, compared with the cell without infected with lentivirus particle at the absence of treatment of proteasome inhibitors (data not shown). These results suggest that high expression of MAFb protein confers the intrinsic resistance of MM to proteasome inhibitors.

Silencing MAFb enhances proteasome inhibitor-induced apoptosis

The effect of knockdown of MAFb on proteasome inhibitors-induced apoptosis in shMAFb/SACHI and shCon/SACHI cells was determined using Annexin V staining and flow cytometry analysis. Increases in the
number of apoptotic cells were seen in shMAFb cells treated with Bzb, Fig. 6a and by CZF, Fig. 6b. These results indicate that silencing MAFb gene and protein enhances proteasome inhibitor-triggered apoptosis.

To see if the BM microenvironment affects the role of MAFb in proteasome inhibitor-mediated apoptosis, shMAFb and shCon cells were co-cultured with primary bone marrow stromal cells from myeloma patients.
Treatment of shMAFb cells with CFZ led to increases in apoptotic cells at the presence of co-culture with bone marrow stromal cells, Fig. 6c, suggesting that interaction with bone marrow microenvironment does not protect MM cells from proteasome inhibitor-induced apoptosis.

**Silencing MAFb enhances proteasome inhibitor-induced activation of caspases**

Since the caspase family plays an important role in apoptosis [42], we next determined whether specific caspases regulated proteasome inhibitor-induced apoptosis in MAFb knockdown cells. We used Western blot to analyze the inactive as well as the cleaved, active forms of caspases in the loss-of-function (shMAFb and shCon) cells exposed to Bzb and CFZ. The protein levels of the cleavage fragment of caspases-8, an initiator caspases that functions as an active executioner of the caspase pathway [42], in shMAFb/SACHI were higher than those in shCon/SACHI cells, Fig. 6d. An increase in of caspases-9, another initiator of caspases, was observed in shMAFb/SACHI cells, without Bzb treatment indicating that silencing MAFb itself induces MM cell apoptosis. Importantly, an obvious increase in the cleavage fragment of caspase 9 was seen at 20 min and maintained for 160 min after to Bzb treatment in shMAFb cells. These results indicate that Bzb-induced apoptosis is mediated via activation of caspases-8 and -9, and knockdown MAFb enhances Bzb-induced activation of caspases-8 and -9 in MM cells.

Because caspases-3, −6 and −7 proteins are substrates of active caspase-8 and -9 [42], we examined the cleavage products in both shMAFb and shCon cells. Increases in cleavage fragment of caspase 3 in shMAFb cells were seen in the cells following treatment with Bzb, Fig. 6e. In contrast, silencing MAFb did not affect activation of caspases-7 and -6 (middle panel and data not shown). These results indicate MAFb protein diminishes Bzb-induced activation of caspases-3 but not caspase-6 and -7.
Fig. 6 (See legend on next page.)
We next determine whether MAFb affects Bzb-induced activation of PARP, which is one of the major substrates of activated caspase-3 [43]. The cleavage of PARP facilitates cellular disassembly and serves as a hallmark of cells undergoing apoptosis [44]. Increases in the cleavage fragment of PARP were observed in shMAFb/SACHI cells. Moreover, the cleavage fragment of PARP was increased following treatment with Bzb. Thus, knockdown of MAFb promotes Bzb-induced activation of PARP.

Lamins are major factors in the structural organization and function of the nucleus and chromatin and cleavage of lamins results in nuclear deregulation and cell death [45]. We investigated the effect of MAF protein on activation of lamins in response to Bzb. An increase in cleavage fragments of lamin A/C in shMAFb cells was clearly seen with Bzb, Fig. 6d. Similar increases in cleavage fragments of caspases-3, PARP and lamin A/C were observed in shMAFb cells response to CFZ, Fig. 6e. Therefore, knockdown of MAFb was accompanied by increased activation of caspases – 8 and – 9, which in turn induced activation of caspase-3, and at the end resulting in the degradation of PARP and lamin A/C in response to the proteasome inhibitors.

Discussion
In this study, we demonstrate that cell lines with high levels of MAFb protein due to t(14;20) or Igλ insertion of 20q11 are resistant to Bzb and CFZ, and the lack of sensitivity of these cell lines to Bzb or CFZ is similar to HMCLs harboring a t(14;16) which have high C-MAF levels [16]. In contrast, HMCLs with t(4;14) or t(11;14) were more sensitive to Bzb and CFZ. This observation is consistent with clinical studies in which patients in the MF subgroup including patients with t(14;20)/MAFb [1] did not benefit from the addition of the Bzb in contrast to t(4;14) cases which gained a major advantage [14, 15, 37].

An assessment of the mechanisms by which t(14;20) patients with high MAFb expression are resistant to PIs is critical to understanding the biology of this subgroup and may provide a basis for targeted therapeutic approaches. The mechanisms that control the MAFb transcription in MM have been investigated by several laboratories [7, 8, 10, 11, 46]. It is well recognized that deregulation of MAFb transcription in t(14;20) patients results from juxtaposing the MAFb gene with the strong enhancer of the IgH locus, leading to overexpression of MAFb mRNA [7, 8]. Insertion of Igλ into MAFb locus is also associated with high transcription of MAFb in t(14;20) negative myeloma [10]. Vatsveen et al. reported that the cell line OH-2 that does not have an IGH translocation but has a complex translocation involving the IgK locus juxtaposed with both MYC and MAFb, expresses MAFb mRNA at a level that is higher than the three HMCLs SACHI, EJM and SKMM-1 that have t(14;20) [11]. Consistent with the high levels of MAFb mRNA, we found high levels of MAFb protein in SACHI and EJM. These results are in keeping with a study that shows MAFb protein in the nucleus of plasma cells in BM biopsies of MM patients with t(14;20) by immunohistochemistry [47]. When screening 32 MM cells lines, we found a high MAFb protein level in OPM-2 which harbors a t(4;14). This cell line has no cytogenetic abnormality involving in 20q11 or MAFb gene, suggesting an inconsistency between the gene transcriptional level and protein level of MAFb in t(4;14) cells. The high MAFb protein without comparable MAFb transcriptional levels suggests that post-transcriptional/translational regulation can govern the abundance of MAFb protein. In support our observation, Herath et al. have reported that MAFb and c-MAF are phosphorylated by GSK3eta in human MM cells [48].

Many proteins including transcriptional factors are known to be substrates of GSK3 [49]. Our results demonstrate that inhibition of GSK3 activity led to the accumulation of MAFb protein. Furthermore, both Bzb and CFZ induce stabilization of MAFb protein in a dose-dependent manner in MM cells with t(14;20) or t(6;20) or Igλ insertion in 20q, associated with accumulation of MAFb in nucleus and cytoplasm. Thus, PIs play a key role of regulating the stability of MAFb protein in MM cells. It should be noted that proteasome inhibitor-induced stabilization of MAFb protein did not occur in the OPM-2 cells with a t(4;14) despite high endogenous MAFb protein levels, and that inhibition of GSK3 activity prevented degradation of MAFb protein in OPM-2 cells. Indeed, the IC50 of Bzb and CFZ for OPM-2 and other cell lines with a t(4;14) were much lower than those with t(14;20), suggesting a difference in the degree of GSK3 activity between the t(14;20) and OPM-2 cells. In agreement with this hypothesis, several studies have reported that MM cells which harbor a deletion of PTEN have constitutive activation of AKT and
p70S6 kinase [50, 51], which in turn leads to constitutive phosphorylation and inactivation of GSK3 [52]. As a consequence, inactivity of GSK3 results in short survival time after diagnosis. Studies on MAFb gene expression by GEP analysis indicates that MF subgroup including high expression of MAFb together with C-MAF signatures belongs to high risk group [1] and associated with poor overall survival [12, 13] and patients in the MF subgroup associated with resistance to Bzb [14, 15].

Conclusions
In summary, high levels of MAFb protein in MM cells with a translocation involving q20 or Igλ insertion are associated with resistance to proteasome inhibitors. MAFb protein confers intrinsic resistance of MM cells to proteasome inhibitors via the abrogation of proteasome inhibitor-induced apoptosis and activation of the caspase family. The elucidation of the mechanism underlying MAFb-mediated resistance to proteasome inhibitors in myeloma with t(14;20) or Igλ insertion is important in the understanding of MM biology and may help in providing novel therapeutic approaches for targeting this rare subgroup of patients.

Additional file

Additional file 1: Supplementary data. Figure S1. described Exposure to LiCl, an inhibitor of GSK3beta led to a stabilization of MAFb protein in all 5 MM cell lines within 120 min. The half-maximum inhibitory concentration (IC50) of proteasome inhibitors, Bzb and CFZ in each tested human MM cell lines are summarized at Table S1. (DOCX 812 kb)

Abbreviations
Bzb: Bortezomib; COND2: CyclinD2; CCR: C-C Chemokine receptor type1; CFZ: Carfilzomib; GEP: Gene Expression Profiling; HBMSCs: Human bone marrow stromal cells; HMCLs: Human myeloma cell lines; Ig: Immunoglobulin; ITGB7: Integrin beta7; MM: Multiple myeloma; MTT: 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; Pts: Proteasome inhibitors

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Availability of data and materials
All data generated or analyzed during this study are included in this published article.

Authors’ contributions
YWQ conceptualized and designed the research, performed experiments, analyzed and interpreted the results, made figures, and wrote paper. SQY, YHH and YC performed experiments and analyzed data. FVR provided patient materials. JE helped design the experimental studies and edited the manuscript. BAW and GJM edited the manuscript. FED interested the results and wrote the manuscript. All authors have read and approved the manuscript.

Ethics approval and consent to participate
This study was approved by the review Board and Ethical Committee of University of Arkansas for Medical Sciences, and patients provided written informed consent to donate bone marrow for this this study.

Competing interests
The authors declare that they have no competing interests.

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