Cap dependent translation contributes to resistance of myeloma cells to bortezomib

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Keywords: eIF4E, eIF2α, 4E-BP1, 4E-BP2, ERK, U266, MM.1S

Abbreviations: MM, multiple myeloma; UPR, unfolded protein response; BZ, bortezomib

Introduction

Multiple myeloma (MM) is characterized by excess of bone marrow plasma cells, osteolytic bone lesions, immunodeficiency and renal disease. Advances in the therapy of MM have led to an increase of survival time. Proteasome inhibitors, such as bortezomib (BZ), have been introduced to treat first relapsed or refractory MM, and more recently newly diagnosed patients. The combination therapy of thalidomide, bortezomib and dexamethasone has increased the time of progression-free survival (PFS) in patients with newly diagnosed multiple myeloma. In general, patients respond well to BZ. In spite of this, the disease remains incurable because patients develop resistance to BZ, with average life expectancies of 7–8 years from the diagnosis. The comprehension of the mechanism of action of BZ, and why myeloma cells are specifically sensitive to it, has therefore attracted the interest of several studies. In agreement with the pleiotropic role of the proteasome in cellular life, many causes have been linked to BZ induced toxicity. Yet, most of these causes do not fully explain the specific sensitivity of myeloma cells to proteasome inhibitors. Myeloma cells directly interact with bone marrow stromal cells. This interaction increases growth, survival, migration, and drug resistance of multiple myeloma cells. BZ reduces the adhesion of myeloma cells to bone marrow stromal cells, therefore reducing their viability. In addition, genetic and pharmacological evidences suggest that the activation of NFKB is critical for survival of mature B-cells. Activating mutations in the NFKB pathway are common in myeloma patients. BZ impairs the degradation of IKB, a negative regulator of NFKB, inducing downregulation of growth and anti-apoptotic signaling pathways. Other indirect mechanisms to explain BZ toxicity have been proposed. An attractive model to explain BZ effects is through the Unfolded Protein Response (UPR). In the UPR, accumulation of misfolded and undegraded proteins in the endoplasmic reticulum (ER) causes the induction of a three-branched response. Briefly, three ER transmembrane proteins, IRE1 kinase, ATF6 transcription factor and PERK kinase act as sensors of ER stress. The first two branches induce the expression of chaperone proteins and enzymes responsible for ER turnover.

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The latter branch blocks global translation by phosphorylation of eIF2α and activates translation-driven expression of genes necessary for the stress response. If the UPR is not effective, cells are driven to apoptosis. An obvious consequence of this model is that translation inhibitors should reduce the proteasome load, hence BZ-induced toxicity. Evidence for the presence of BZ-induced UPR in myeloma cells has been produced, but without discriminating between sublethal or lethal doses. The possibility that the translational load is not contributing to BZ-induced toxicity is that translation inhibitors should reduce the proteasome load, hence BZ-induced toxicity. Evidence for the presence of BZ-induced UPR in myeloma cells has been produced, but without discriminating between sublethal or lethal doses.

The treatment of MM cells with BZ is associated with proteasome inhibition, downregulation of growth and activation of apoptotic signaling pathways. We first validated the BZ effect on cell viability in two MM cell lines held as sensitive, MM.1S, or resistant, U266. We treated MM.1S and U266 with increasing BZ concentrations for 24 and 48 h. MTT assay was performed. Data are presented as percentage of untreated control. Calculated EC50 at 48 h were 11,93 ± 1.68 nM for MM.1S cells and 16,15 ± 1.81 nM for U266. Within 24 h of treatment, at the concentrations tested, U266 were resistant to BZ at concentrations up to 50 nM, whereas MM.1S were resistant to BZ at concentrations up to 50 nM, whereas MM.1S had an EC50 of 18.26 nM ± 1.68. We therefore decided to analyze the translational load in the presence of BZ-induced UPR in myeloma cells has been produced, but without discriminating between sublethal or lethal doses.

Bortezomib toxicity is not associated with proapoptotic eIF2α phosphorylation

Bortezomib toxicity is not associated with proapoptotic eIF2α phosphorylation. The latter branch blocks global translation by phosphorylation of eIF2α and activates translation-driven expression of genes necessary for the stress response. If the UPR is not effective, cells are driven to apoptosis. This model is attractive because myeloma cells produce large amounts of IgG, which make them potentially susceptible to UPR. An obvious consequence of this model is that translation inhibitors should reduce the proteasome load, hence BZ-induced toxicity. Evidence for the presence of BZ-induced UPR in myeloma cells has been produced, but without discriminating between sublethal or lethal doses. The possibility that the translational load is not contributing to BZ-induced toxicity is that translation inhibitors should reduce the proteasome load, hence BZ-induced toxicity. Evidence for the presence of BZ-induced UPR in myeloma cells has been produced, but without discriminating between sublethal or lethal doses.
Further accumulation of polyubiquitinated proteins was observed only in sensitive MM1.S cells (Fig. 1B). In agreement with the MTT data, cleaved caspase 3 and cleaved PARP were observed only in MM1.S cells (Fig. 1C). Thus, proteasome inhibition leads to similar short-term accumulation of poly-Ubiquitinated in both MM1.S and U266 cell. However, only the first undergoes an apoptotic response.

It has been proposed that the treatment of MM cells with proteasome inhibitors triggers the Unfolded Protein Response (UPR). In response to UPR, the PERK Kinase is activated by dimerization and phosphorylation. Once activated, PERK phosphorylates eIF2α resulting in translation attenuation. Therefore we investigated whether BZ had effects on eIF2α phosphorylation and on protein synthesis. We made these observations: first, the induction of eIF2α phosphorylation by BZ treatment was minimal, and present both in BZ-sensitive MM1.S cells and in BZ-insensitive U266 cells. Second, the basal level of eIF2α phosphorylation of myeloma cells was higher than in fibroblast (Fig. 1D). We conclude that the timing and extent of induction of eIF2α phosphorylation does not associate with BZ-induced death.

4E-BP1 dephosphorylation accompanies and accelerates bortezomib-induced death

Next, we assessed whether translation is affected by proteasome inhibition and if this correlates with induced toxicity. Briefly, the best-characterized pathway converging on translation is driven by mTORC1, which leads to the direct phosphorylation of 4E-BPs, and through S6K1 of rpS6. In general, rapid inhibition of mTORC1 by rapamycin or by mTOR blockers leads to the rapid dephosphorylation of both rpS6 and 4E-BP1. We assessed whether the mTORC1 pathway is affected by BZ. Surprisingly, BZ treatment affected phosphorylation of mTORC1 substrates only in BZ-sensitive cells. BZ treatment caused dephosphorylation of 4E-BP1, (Fig. 2A) in MM1.S sensitive cells, but not in U266 resistant cells. Next, we investigated the phosphorylation status of rpS6. The p70 ribosomal S6 kinases, directly regulated by mTOR, phosphorylate rpS6 on Ser-240 and Ser-244. The RAS/ERK pathway also regulates rpS6 phosphorylation independent of mTOR through the activation of p90 ribosomal S6K1 kinases that phosphorylate rpS6 on Ser-235 and Ser-236. Our data indicate that while 24 h BZ treatment affects 4E-BP1 phosphorylation, S6 phosphorylation is not compromised by BZ. Thus we hypothesize that mTORC1 activity was still present in BZ-treated cells. We pulled down mTORC1 complex from BZ-treated cells, in conditions of reduced in vivo phosphorylation of 4E-BP1. We found that BZ did not reduce mTORC1 kinase activity, at least in vitro (Fig. 2B). The data shown indicate a clear dephosphorylation of 4E-BP1 that is not accompanied by S6 dephosphorylation (Fig. 2A). The phosphorylation of rpS6 in Ser 235/236 may be explained by activation of the p90 ribosomal S6 Kinase (RSK) downstream of the Ras/ERK signaling cascade. Indeed, BZ induces ERK phosphorylation in a dose dependent manner (Supplementary Figure 1A). Since that 4E-BP is dephosphorylated upon BZ treatment in MM.1S, we analyzed whether BZ treatment causes an enrichment of 4E-BP1 bound to eIF4E. We evaluated cap complex assembly of initiation factors in both MM.1S and U266 treated with BZ. Data in Figure 2C show that BZ treatment causes an enrichment of 4E-BP1 bound to eIF4E at 24 h BZ treatment only in MM.1S. In agreement with the observed absence of effects of BZ treatment on 4E-BP1 phosphorylation in U266 cells, BZ did not affect 4E-BP1 binding to eIF4E in the same context (Fig. 2C). Next, we evaluated the relationship between BZ sensitivity and initiation of translation. Polysomal profiles demonstrated that BZ treatment caused an inhibition of initiation in sensitive MM.1S cells, but not in resistant U266 cells (Fig. 3A). To better address the effect of BZ on global translation we measured translational rate by methionine incorporation in MM.1S and U266 cells treated with BZ. Short BZ treatment does not affect global translation while long BZ treatment induces only in MM.1S cells a reduction of translation rate (Fig. 3B). These data suggest that translational load does not increase BZ toxicity, but rather that attenuation of cap dependent translation exacerbates BZ toxicity. Next, we directly tested the hypothesis. MM.1S cells have dephosphorylated 4E-BP1 upon BZ treatment, while 4E-BP1 remains phosphorylated in BZ insensitive U266. We infected U266 cells with retrovirus expressing non-phosphorylatable 4E-BP1 (4Ala) or empty control. We evaluated the efficiency of infection by western blotting (Fig. 4A). Next, we observed the viability of infected cells with the MTT assay. We found that...
4E-BP1–4Ala infected cells are, even if slightly, more sensitive to 48 h BZ treatment (Fig. 4B). Taken together data demonstrate that the translational load is not accelerating BZ-induced death, and suggest that the block of translation of specific mRNA accelerates BZ toxicity.

mTOR inhibition delays MM growth independently from bortezomib resistance

The data obtained suggest that the pharmacological inhibition of 4E-BP1 may act synergistically to BZ and/or be of value in treatment of MM. The most widely mTORC1 inhibitor is rapamycin; however, rapamycin treatment causes activation of a pro-survival feedback loop by ERK and Akt kinase activation. As previously shown, ERK is activated also by BZ treatment (Figure S1A). We examined the effect of ERK inhibition on MM viability, in conditions of BZ treatment. Data show that ERK inhibition in MM1.5 cells does not worsen BZ-mediated toxicity (Figure S1B), thus providing a rational for further analysis of mTOR inhibitors in myeloma cells. Next, we analyzed the effect of either mTORC1 inactivation by rapamycin or mTOR pharmacological blockade by PP242, singly or in combination with BZ. Briefly, we found that both i) mTORC1 inhibition by either rapamycin or PP242 decreased the survival of MM cells, ii) the effect of PP242 and rapamycin was independent from the one of BZ (Fig. 5A). Early trials with rapalogues have shown a limited response in myeloma cells. Thus, we examined the effects of PP242 on the growth of primary myeloma cells derived from patients. Since the interaction between stromal cells and myeloma cells is critical to their survival, the effect of mTORC1 blockers was examined on cocultured tumor and stromal cells. It must be emphasized that primary myeloma cells in culture do not proliferate vigorously. Of five patients cells tested, only one was found to partly respond to PP242, in conditions of BZ resistance (Fig. 5B). These data suggest that a subset of patients may respond to mTORC1 inhibition.

Variable levels of eIF4E and 4E-BP1/2 in myeloma patients

Determinants of sensitivity to mTORC1 inhibition are mutations in PI3K/RAS pathways or altered levels of 4E-BP1/2 vs. eIF4E. Specifically, higher levels of eIF4E vs 4E-BP1 confer rapamycin insensitivity and vice versa. We analyzed in myeloma patients (n = 122) the relative levels of 4E-BP1 and 4E-BP2 vs. eIF4E: Data show that approximately 15% of patients have higher relative levels of 4E-BP1 and 4E-BP2 (Fig. 6A–B).

Discussion

On the basis of available literature, we hypothesized that BZ induced an UPR response in myeloma cells, allowing us to identify eIF2α phosphorylation and uORF mRNAs critical for survival and resistance of cells. To address the problem, we set up conditions that allowed us to discriminate between BZ-induced toxicity and survival. In these conditions, we did not find evidence for eIF2α phosphorylation in BZ-induced lethality. This result is in apparent contrast with previous reports that analyzed the UPR in MM cells. However, here we focused on BZ concentrations able to induce cell death, thus ruling out a direct relationship between eIF2α phosphorylation and toxicity. Several groups have tried to explain the molecular bases of different individual responsiveness to bortezomib, exploiting human MM lines characterized by differential sensitivity. Nevertheless, the molecular basis of sensitivity or resistance to BZ among patients, remain largely unknown. We note that, MM1S cells have only 2-fold higher sensitivity than U266 to BZ. In the case of other drugs, such as for instance rapamycin, sensitive cells show about 100 fold difference vs. resistant cells in term of response to the drug. Sensitivity/resistance to BZ seems more a clinical concept than a genetically driven clear-cut difference. This said, U266 cells are considered by all means a good paradigm of BZ-resistant myeloma cells, whereas MM1S are considered sensitive. We found that, as in other tumor models, myeloma cells exhibit a sensitivity to translational inhibition, and a prosurvival activity of the mTORC1-eIF4E axis. Similarly to other tumor cell types, inhibition of mTORC1 may be beneficial to therapy. Mechanistic evidence demonstrates that the cytostatic effects of rapamycin, an highly specific mTORC1 inhibitor, is due to inhibition of eIF4F formation through
dephosphorylation of the eIF4E repressor 4E-BPs.\textsuperscript{39} Indeed, either decrease of eIF4E or increase of 4E-BP1 can bypass mTORC1 inhibition in vivo. Conversely, eIF4E increase or 4E-BP1 downregulation overcome rapamycin inhibition.\textsuperscript{34} We found that when BZ represses 4E-BP phosphorylation, in vivo, overexpression of not phosphorylatable 4E-BP1 worsens BZ toxicity. We also found an unexpected effect of ERK inhibition on BZ-treated cells. Rapamycin induces a feedback loop that activates ERK and can lead to resistance in other cancer cell types.\textsuperscript{28} However, in MM ERK inhibition does not increase BZ-induced toxicity, but reduces it. One unexpected observation is that BZ induces 4E-BP1 dephosphorylation, but not rpS6 dephosphorylation as rapamycin does. However, it is intriguing that kinase activity assays suggest that mTOR activity is not affected directly by BZ treatment. Thus, the dephosphorylation of 4E-BP1 may be due to specific phosphatase activities stimulated by BZ or other mechanisms such as mTOR and 4E-BP1 delocalization. A recent work suggests specificity of rapamycin sensitivity for substrate choice for mTORC1 kinase,\textsuperscript{39} possibly suggesting that BZ may act similarly. Alternatively, BZ may induce variations in steady-state levels of adaptors regulating mTORC1 specificity, in vivo. Future work is needed. We show that both mTORC1 and mTOR inhibition are effective in myeloma cell lines. Moreover, as shown in our and another study, the mTOR inhibitor PP242 has demonstrated efficacy against primary MM cells.\textsuperscript{40} The major effect of PP242 on tumor cells is the inhibition of cell proliferation.\textsuperscript{41} Primary myeloma cells grow poorly outside their bone marrow microenvironment. In spite of different conditions of culture (various cytokine combinations, various stroma substrates), primary MM cells cultured in vitro display a decline in growth and proliferation within three days of culture.\textsuperscript{42} This aspect limits the measurement of drug sensitivity in primary myeloma cells, especially for cytostatic agents like for PP242. Therefore, the inhibitory effect of PP242, in vitro in our conditions, is underestimated. Which would be the best way to employ mTORC1 inhibitors? In this sense, in agreement with a recent study, we found that blockade of mTOR is not synergistic with BZ treatment.\textsuperscript{43} Thus, mTOR inhibition may be useful in patients resistant to BZ. Data from this and other works would however suggest that patients that would benefit most from mTOR inhibition are without RAS mutations,\textsuperscript{32,44} and have high levels of 4E-BP, low eIF4E.\textsuperscript{35,36} These patients represent a subset of the whole MM population: it might be thus mandatory to identify them, before treatment. As a final remark, recent genome sequencing has unveiled new somatic mutations in myeloma cancer cells. Among them, it is curious to note that several of them are on factors associated with translational control, and that at least 50% patients have one mutation in one gene involved in protein synthesis.\textsuperscript{45} These data, together with our observations, may suggest that the translational machinery will be an attractive target for therapy in myeloma cells.

Materials and Methods

Cell culture and proliferation assay

The MM cell lines MM.1S and U266 cells were kindly provided by Dr Tonon. Cells were cultured in RPM1 1640 (Euroclone) supplemented with 10% FBS (fetal bovine serum; Gibco) 1% glutamine and a commercial antibiotic mix (Gibco). Cell proliferation was measured by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide]. Cells were plated in 96 well plate at a density of 20,000 cells per well. After treatment MTT solution was added and incubated for 3 h. The reaction product was quantified reading the absorbance at 570 nm using a microplate reader (Biorad).

Primary myeloma specimens and proliferation assay

Bone marrow (BM) samples for molecular studies were obtained during standard diagnostic procedures. Written informed consent was obtained from each patient. Plasma cells were purified from mononuclear BM cells obtained by Ficoll-Hypaque density gradient centrifugation using anti-CD138 micro beads on an AutoMacs Magnetic Cell Separator (MACS system, Miltenyi Biotec, Auburn, CA). The purity of positively selected plasma cells was assessed by flow cytometry and was\textsuperscript{3} 90% in all cases. For co-culture experiment HS-5 stromal cells were plated O/N in 96 well plates at a density of 500 cells per well. Primary Myeloma cells were plated at density of 10.000 cells/well on a layer of HS-5
stromal cells. Drugs were added at the concentration indicated and compared with DMSO treated controls. Cultures were then incubated for 24, 48 and 72 h in a 37 °C incubator with 5% of CO₂. MTT assay was performed. The experiment was done in triplicate measurement.

Drugs and reagents

The following antibodies were used: rabbit polyclonal anti-rpS6, anti-phospho-rpS6 (Ser235/236), anti-4E-BP1, anti-p44/42 MAPK (ERK1/2), anti-phospho-p44/42 MAPK-ERK 1/2 (Thr202/Tyr204), anti-eIF2α, anti-phospho-eIF2α (Ser51), anti-mTOR, (Cell signaling); mouse monoclonals anti-β actin (sigma), anti caspase -3 (Alexis), anti-PARP-1 (Millipore), anti-HA (Covance). Retrovirus pBABE-puro and 4E-BP1 4Ala were a gift of Dr. N. Sonenberg. Proteasome inhibitor bortezomib (BZ) was from Millennium Pharmaceuticals, Cambrige MA, mTOR inhibitor PP242, rapamycin and cycloheximide were from Sigma.

Western blot

MM cells were lysed with buffer containing 10 mM NaCl, 10 mM MgCl₂, 10 mM TRIS-HCl (pH 7.3), 1% Triton X-100, 1% sodium deoxycholate, 1 mM DTT, 5 mM NaF, 2 mM Na₃VO₄, 40 units/ml RNasin® (Promega, Milan, Italy) and protease inhibitor cocktail. The whole cell extract was clarified at 4 °C at 15,000 g for 10 min. The amount of recovered protein was quantified by the bicinchoninic acid (BCA) protein assay. Extracts were resolved on SDS-PAGE, transferred to Immobilon-P membranes (Millipore), and probed with appropriate antibodies. Equal amount of proteins was analyzed.

Kinase assay

Proteins from MM.1S cells were extracted in lysis buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA, 5 mM EGTA, 20 mM β-glycerophosphate and protease inhibitor cocktail) by freezing and thawing and clarified by centrifugation. Protein concentration was quantified by BCA. One mg of total extract protein was incubated with anti-mTOR antibody (1:100) for 2 h at 4 °C in constant rotation. Immunoprecipitation was performed with protein A for 30 min. Beads were washed one time with high-salt wash buffer (100 mM TRIS-HCl pH 7.4, 500 mM LiCl) and three times with kinase buffer (10 mM Hepes, 50 mM β-glycerophosphate, 150 mM NaCl). The beads were resuspended in kinase buffer. The kinase assay was performed by adding 10 µg GST-4E-BP1 recombinant protein or GST alone, 10 mM MnCl₂ and 4 µCi of γ-32P-ATP. The reaction was run at 30°C for 1 h and terminated by adding one volume of sample buffer. Samples were boiled 5 min, separated by SDS PAGE, transferred to Immobilon-P membranes (Millipore), and probed with anti-GST, anti-mTOR. Autoradiography was performed for one hour at room temperature.

m7GTP Cap column pull-down

Cells were collected by scraping, washed three times with cold PBS and pelleted by centrifugation. Cell pellets were resuspended in lysis buffer (50 mM TRIS-HCl at pH 7.4, 100 mM KCl, 1 mM EDTA, 1 mM EGTA, 20 mM NaF, 0.5 mM Na₃VO₄, 1% Triton X-100, and protease inhibitors), and incubated for 30 min at 4 °C. Cell debris were removed by centrifugation at 10,000 g for 10 min at 4 °C, and protein concentration was determined using the BCA (bicinchoninic acid) protein assay (Thermo scientific). Extract (300 µg) was incubated with 30 µL of m7GTP-agarose resin (GE Healthcare) for 1 h at 4 °C. The resin was washed three times with 1 mL of lysis buffer, boiled for 6 min in Laemmli buffer, and proteins were resolved by SDS-PAGE.

Polysomal profiles

Cells (40 x 10⁶) were collected and washed with cold PBS (phosphate saline buffer) with 10 µg/ml cycloheximide. Cells were resuspended in lysis buffer composed by 50 mM Tris–HCl pH 7.5, 100 mM NaCl, 30 mM MgCl₂, 0.1% NP-40, 100 µg/ml cycloheximide, 40 U/ml RNasin® (Promega), protease inhibitor cocktail (Sigma, St. Louis, MO). Lysed cells were left for 20 min at 4 °C, and lysates were then clarified by centrifugating at 18,000 g for 5 min at 4 °C. Supernatants were collected, and RNA concentration was quantified by reading Abs254. The
equivalent of 10 absorbance units at 254 nm were layered on to a 15–50% sucrose gradient in 50 mM Tris/acetate (pH 7.5), 50 mM NH₄Cl, 12 mM MgCl₂, and 1 mM DTT and centrifuged at 4 °C in a SW41Ti Beckman rotor for 3:30 h at 39000 rev./min. Samples were analyzed with BioLogic LP (BioRad) by reading the results are expressed as a means ± sd.

Retroviral infections

Retroviral constructs, pBABE empty vector and pBABE-HA-4E-BP1 (4Ala) were transfected into amphotrophic phoenix 293T packaging cells. After 48 h virus containing medium was collected. 10⁶ cells were plated in 24-well plate and incubated with virus supernatant and 8 mg/ml polybrene. Cells were spun at 1000 g for 2 h and then incubated for 3 h at 37 °C, 5% CO₂. Transduced cells were selected with puromycin 3 mg/ml for one week and further analyzed by immunoblot.

Gene expression profiling

Samples were analyzed with BioLogic LP (BioRad) by reading precipitation on glass microfiber filters (Whatman) and counted.

References

1. Kyle RA, Rajkumar SV. Multiple myeloma. N Engl J Med 2004; 351:1860-73; http://dx.doi.org/10.1056/NEJMra041875; PMID:15509819.
2. Cavo M, Pantani L, Petrucci MT, Patriarca F, Zamagni E, Donnarumma D, et al. Bortezomib-thalidomide-dexamethasone is superior to thalidomide-dexamethasone as consolidation therapy after autologous hematopoietic stem cell transplantation in patients with newly diagnosed multiple myeloma. Blood 2012; 120:9-19; PMID:22498745; DOI: 10.1182/blood-2012-02-408898.
3. Munshi NC, Anderson KC. New strategies in the treatment of multiple myeloma. Clin Cancer Res 2013; 19:3537-44; PMID:23515406; DOI: 10.1158/1078-0432.CCR-12-1881.
4. Sonneveld P, Goldschmidt H, Rosiñol L, Bladé J, Lahuerta JJ, Cavo M, Tacchetti P, Zamagni E, Atrai M, Lokhorst HM, et al. Bortezomib-based versus nonbortezomib-based induction treatment before autologous stem-cell transplantation in patients with previously untreated multiple myeloma: a meta-analysis of phase III randomized, controlled trials. J Clin Oncol 2013; 31:3279-87; http://dx.doi.org/10.1200/JCO.2012.48.4626; PMID:23897861.
5. Kumar SK, Rajkumar SV, Dispenziers A, Lucy MQ, Hayman SR, Buadi FK, Zeldenrust SR, Dingli D, Russell SJ, Lust JA, et al. Improved survival in multiple myeloma and the impact of novel therapies. Blood 2008; 111:2516-20; http://dx.doi.org/10.1182/blood-2007-11-106129; PMID:17975015.

Total RNA from CD138 positive cells was obtained from each sample by the RNeasy® kit (Qiagen, Valencia, CA) extraction procedure. To measure concentration and purity of RNA, a NanoDrop ND-1000 spectrophotometer was used (NanoDrop Technologies), purity of the extracted RNA was based on the 260/280 and the 260/230 O.D. ratios, as calculated and displayed by the NanoDrop spectrophotometer. Moreover, disposable RNA chips (Agilent RNA 6000 Nano LabChip kit) were used to determine the concentration and purity/ integrity of RNA samples using Agilent 2100 Bioanalyzer. Samples with at least 30 ng/uL RNA were labeled for gene expression profiling, using the Affymetrix Two-cycles Gene Chip microarray system (Affymetrix, Santa Clara, CA). cDNA synthesis, biotin-labeled target synthesis, HG U133 Plus 2.0 GeneChip arrays hybridization, staining, and scanning were performed according to the standard protocol supplied by Affymetrix. Microarray data were used to identify gene expression profile of 4E-BP1, 4E-BP2 and eIF4E in BM samples obtained from 122 newly diagnosed MM patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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18. Meister S, Schubert U, Neubert K, Herrmann K, Burger R, Gramatzki M, Hahn S, Schreiber S, Wilhelm S, Herrmann M, et al. Extensive immunoglobulin production sensitizes myeloma cells for proteasome inhibition. Cancer Res 2007; 67:1783-92; http://dx.doi.org/10.1158/0008-5472.CAN-06-2258; PMID:17308121.

19. Vincenz L, Jager R, O’Dwyer M, Samali A. Endoplasmic reticulum stress and the unfolded response: Targeting the achilles heel of multiple myeloma. Mol Cancer Ther 2013; 12:831-43; PMID:23729400; DOI: 10.1182/blood-2008-08-172734; 10.1182/blood.2008-08-172734.

20. Lee AH, Iwakoshi NN, Anderson KC, Glimcher LH. The dual PI3K and mTOR inhibitor NVP-BEZ235 exhibits anti-proliferative activity and overcomes mTORC1 phosphorylation-dependent feedback activation of Akt signaling through an IGFR-1-dependent mechanism. Oncogene 2007; 26:1932-40; http://dx.doi.org/10.1038/onc.2007.259; PMID:17001314.

21. Roux PP, Shahbazian D, Vu H, Holz MK, Cohen MS, Taubon J, Sonenberg N, Blenis J. RAS/ERK signaling promotes site-specific ribosomal protein S6 phosphorylation via RSK and stimulates cap-dependent translation. J Biol Chem 2007; 282:14956-64; http://dx.doi.org/10.1074/jbc.M700906200; PMID:17360704.

22. Carracedo A, Ma L, Tenya-Feldstein J, Rojo F, Salmena L, Alimonti A, Ega A, Sasaki AT, Thomas G, Kozma SC, et al. Inhibition of mTORC1 leads to MAPK pathway activation through a PI3K-dependent feedback loop in human cancer. J Clin Invest 2008; 118:3063-74; http://dx.doi.org/10.1172/JCI34579; PMID:18725988.

23. Wan X, Harkavy B, Shen N, Grohar P, Helman LJ. Rapamycin induces feedback activation of Akt signaling through an IGFR-1-dependent mechanism. Oncogene 2007; 26:1932-40; http://dx.doi.org/10.1038/onc.2007.259; PMID:17001314.

24. Proud CG. eIF2 and the control of cell physiology. Nat Rev Mol Cell Biol 2005; 16:3-12; PMID:16295476; DOI: 10.1038/nrm1539.

25. Di Nicolantonio F, Arena S, Tabernero J, Grosso S, Molinari F, Macarulla T, et al. Deregulation of the PI3K and KRAS signaling pathways in human cancer cells determines their response to everolimus. J Clin Oncol 2010; 12:263-72; PMID: 20345726; DOI: 10.1001/jama.2009.10593; 10.1001/jama.2009.10593.

26. Alain T, Morita M, Fonseca BD, Yanagiya A, Siddiqui ZA, Ruggero D, et al. Active-site inhibitors of mTOR target rapamycin-resistant outputs of mTORC1 and mTORC2. PLoS Biol 2009; 7:e38; PMID:19209957; DOI: 10.1371/journal.pbio.0010038.

27. Lee AH, Loo CK, Anderson KC, Glimcher LH. The dual PI3K and mTOR inhibitor NVP-BEZ235 exhibits anti-proliferative activity and overcomes mTORC1 phosphorylation-dependent feedback activation of Akt signaling through an IGFR-1-dependent mechanism. Oncogene 2007; 26:1932-40; http://dx.doi.org/10.1038/onc.2007.259; PMID:17001314.

28. Huang B, Frost P, Shi Y, Belanger E, Benavides A, Parvizshpor G, et al. Targeting TORC2 in multiple myeloma with a new mTOR kinase inhibitor. Blood 2010; 116:4560-8; PMID:20686120; DOI: 10.1182/blood-2010-05-285726; 10.1182/blood-2010-05-285726.

29. Feldman ME, Apol B, Uetaa O, Loewith R, Knight ZA, Ruggiero D, et al. Active-site inhibitors of mTOR target rapamycin-resistant outputs of mTORC1 and mTORC2. PLoS Biol 2009; 7:e38; PMID:19209957; DOI: 10.1371/journal.pbio.0010038.

30. Alain T, Morita M, Fonseca BD, Yanagiya A, Siddiqui ZA, Ruggero D, et al. Active-site inhibitors of mTOR target rapamycin-resistant outputs of mTORC1 and mTORC2. PLoS Biol 2009; 7:e38; PMID:19209957; DOI: 10.1371/journal.pbio.0010038.

31. Farag SS, Zhang S, Jansak BS, Wang X, Kraut E, Chan K, et al. Phase II trial of temsirolimus in patients with relapsed or refractory multiple myeloma. Leuk Res 2009; 33:1475-80; PMID:19261329; DOI: 10.1016/j.leukres.2009.01.039;

32. Haematologica 2012; 97:64-72; PMID: 21993678; DOI: 10.3324/haematol.2012.074872.

33. Zlei M, Egert S, Wider D, Ihorst G, Wasch R, Engelhardt M. Characterization of in vitro growth of multiple myeloma cells. Exp Hematol 2007; 35:1550-61; http://dx.doi.org/10.1016/j.exphem.2007.06.016.

34. Alain T, Morita M, Fonseca BD, Yanagiya A, Siddiqui ZA, Ruggero D, et al. Active-site inhibitors of mTOR target rapamycin-resistant outputs of mTORC1 and mTORC2. PLoS Biol 2009; 7:e38; PMID:19209957; DOI: 10.1371/journal.pbio.0010038.

35. Steinbrunn T, Stuhler T, Gartenholzer S, Rosenwald A, Moztok A, Unticker C, Einsele H, Chatterjee M, Bargou RC. Mutated RAS and constitutively activated Akl delineate distinct oncogenic pathways, which independently contribute to multiple myeloma cell survival. Blood 2011; 118:3660-70; http://dx.doi.org/10.1182/blood-2011-03-342934; PMID:22049583.

36. Chapman MA, Lawrence MS, Keis JS, Cibulskis K, Sougnez C, Schinzel AC, Harvie CL, Brunet JP, Ahmann GJ, Adli M, et al. Initial genome sequencing and analysis of multiple myeloma. Nature 2011; 471:467-72; http://dx.doi.org/10.1038/nature09837; PMID:21430775.