BioMed Research International

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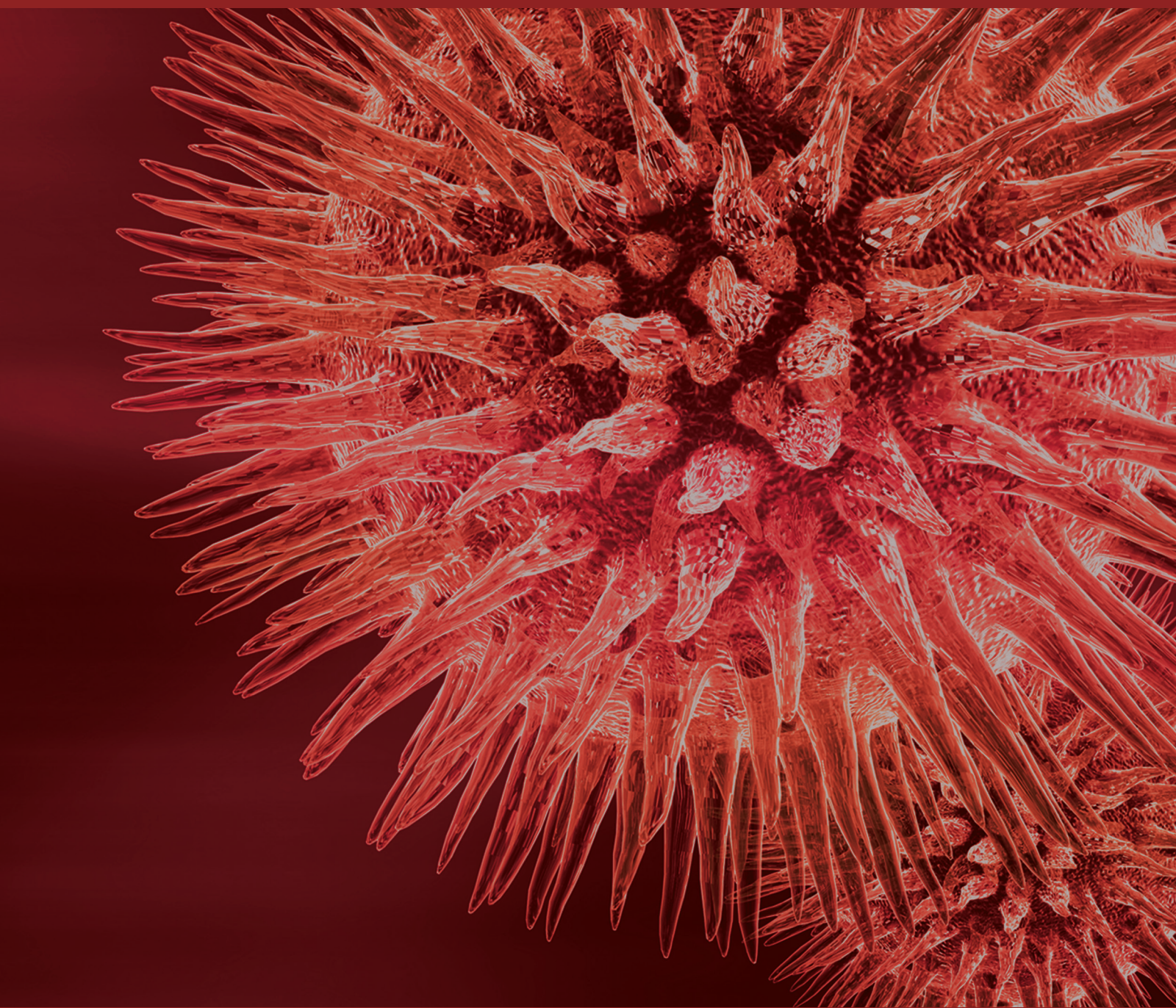
Guest Editors: Mariateresa Fulciniti, Nicola Amodio, Michele Cea, Patricia Maiso, and Abdel Kareem Azab



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Editorial

Biological Insights into Myeloma and Other B Cell Malignancies

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Multiple myeloma (MM) is a hematologic cancer characterized by the accumulation of malignant plasma cells in the bone marrow, which causes bone destruction and marrow failure [1]. Despite the fact that clinical introduction of novel drugs has led to a dramatic improvement of disease, MM ultimately relapses and remains an incurable disease [2]. Consistently, research in this field has dramatically grown, as demonstrated by the increased rate of publications during the last decade. Original research and review articles published in this special issue focus on identification of new targets and pathways for prognostic and therapeutic application in myeloma, as well as validation of novel antitumor agents.

(1) Diagnosis, Monitoring, and Prognosis of MM. Extensive gene expression profile analysis has provided interesting insight into the disease biology, and its correlation with clinical outcome has opened a new direction for risk stratification as well as novel target and therapy identification. Prognostic models such as the Durie-Salmon staging system and International Staging System (ISS) are available and account for the disease burden [3]; however, the original analysis of ISS system did not include Chinese patients' data and take into account the recent introduction of novel agents. The data provided in the manuscript by J. Lu et al. represent the first multicenter retrospective study analyzing ISS value in a large number of unselected Chinese myeloma patients. The results demonstrate that ISS still has prognostic value in Chinese patients with MM, but not in patients receiving bortezomib-based therapy. Therefore, further studies are needed to develop more suitable and robust stratification systems to predict prognosis and optimize treatment strategy early during the course of the disease.

Minimal residual disease (MRD) has emerged as one of the most relevant prognostic factors in MM and should be included in a new definition of complete response (CR) [4]. In the manuscript by M. Fulciniti et al., the authors reviewed current definition of deep response in MM, advantages and limitations of current MRD assessment assays, and clinical evidences for MRD monitoring as a prognostic tool for therapeutic decisions in MM, providing the rationale for the use of MRD assessment in the evolving MM clinical paradigm.

(2) Role of the Bone Marrow Milieu. The bone marrow (BM) microenvironment plays a crucial role in MM pathogenesis. MM cells reside in and dynamically interact with various subsets of cells in the BM including mesenchymal cells (MSCs), osteoclasts (OCLs), osteoblasts (OBLs), and vascular endothelial cells, which support growth and survival of the tumor cells and lead to development of drug resistance [5]. These cells not only physically interact with MM cells but also secrete growth and/or antiapoptotic factors. The cross talk between MM cells with bone marrow stromal cells or other cellular components is finely tuned by a plethora of cytokines,

growth-factor, and other molecules which can be released either by MM cells or by cells of the BM microenvironment. Several studies have demonstrated deregulation of the cellular and humoral components of the BM in MM including increased OCL activity and inhibited OBL activity, and both are involved in the pathophysiology of the bone lesions in MM [6].

The review by F. Accardi et al. summarizes the preclinical and clinical evidence on the effects of bortezomib and other new Proteasome Inhibitors (PIs) on myeloma bone disease. Osteoclastic formation and activity are inhibited by PIs, mainly through the blockade of RANKL signaling pathway in the osteoclast progenitors. However, the more significant impact of the bone remodeling by this class of drugs is the capacity to stimulate either the osteogenic differentiation of MSC or the osteoblastic function, leading to the consequent bone formation with a considerable anabolic effect. Osteocytes are also possible targets of PIs with a stimulatory effect on their viability. The preclinical evidence, thus, is confirmed in MM patients treated with bortezomib and more recently with carfilzomib. An improvement of the bone remodeling markers was observed in the patients treated with PIs. The histomorphometric data in MM patients treated with bortezomib prominently indicated that PIs can stimulate the bone formation process and induce the bone regeneration process. Bone healing, as well as an increase in the BMD, has also been reported in some of the patients treated with bortezomib. Overall, the literature data support the use of these drugs to restore bone integrity in MM patients.

MM cells are cradled within the BM microenvironment by an array of adhesive interactions between the BM extracellular matrix (ECM) components and a variety of adhesion molecules on the surface of MM cells, which results in the "cell adhesion-mediated drug resistance" (CAM-DR) thought to be one of the major mechanisms by which MM cells escape the cytotoxic effects of therapeutic agents [7]. MM is characterized by continuous spread of cancer cells at different sites of the bone marrow, through continuous cell trafficking including adhesion of myeloma cells to vascular wall. This process requires the presence of P-selectin on the endothelium and stroma, as well as PSGL-1 on tumor cells. PSGL-1 was previously suggested as a novel target for immunotherapy in MM using small molecule inhibitor which demonstrated sensitization of MM cells to therapy, controlling tumor growth and dissemination. However, poor pharmacokinetic profile with a very short half-life may hold up further usage of this drug in MM. The study by B. Muz et al. demonstrates that inhibition of P-selectin/PSGL-1 axis using humanized monoclonal antibodies is a promising approach for the treatment of MM with high efficacy in inhibition of P-selectin/PSGL-1 interactions and sensitization of MM cells to therapy, along with favorable pharmacokinetics.

Angiogenesis is fundamental to tumor growth and spread in many hematological disorders, particularly MM [8]. The angiogenic potential of MM is regulated by several pro- and antiangiogenesis cytokines produced by myeloma cells and other cell types in the tumor microenvironment. The study by T. Valković et al. determines the plasma levels of monocyte chemotactic protein-1 (MCP-1), as well as its possible association with angiogenesis, in 45 newly diagnosed MM patients and 24 healthy controls. The manuscript reveals a positive association between plasma MCP-1 levels, angiogenesis, and clinical features in patients with MM. However, additional prospective studies with a respectable number of patients should be performed to authenticate these results and establish MCP-1 as a possible target of active treatment.

(3) Genetic and Epigenetic Abnormalities. MM cells accumulate various genetic and epigenetic abnormalities that drive the malignant phenotype and confer distinct biologic sequelae and disease outcomes [9].

miRNA are important transcriptome modifiers that play important role in myeloma tumor progression, survival, and development of drug resistance [10]. In their review, Raimondi et al. discuss the role of endogenous noncoding RNAs as microRNAs as a novel class of regulators of the intercellular communication between MM cells and other cells of the BM milieu, focusing on the therapeutic potential of experimental strategies aimed at modulating microRNA levels in MM cells and capable of overcoming the tumor-promoting BM microenvironment.

Moreover, S.-F. Lin and W.-C. Yang review molecular mechanisms underlying MM development, progression, and resistance to treatment, further highlighting the relevance of microRNAs as well as immune dysfunctions and conventional or novel therapies targeting such vulnerabilities. Overall, authors advocate that understanding the genomic landscape of MM deserves more attention in the prospect of developing personalized and effective targeted therapies that overcome resistance to currently used anti-MM drugs.

In addition to microRNAs, dysregulation of transcription factors (TFs) features prominently in the biology of MM and B cell malignancies. TFs are the downstream effectors of signaling pathways within cells, which receive growth and other signals from the microenvironment; they also regulate cellular homeostasis as well as cell survival and proliferation. On this basis, mutations in TF genes or dysregulation of TFs' expression could play a significant role in cancer pathogenesis and drug resistance. The TF EBF1 is the master regulator of the specification, development, and maintenance of the B-lymphoid lineage, and perturbations of EBF1 expression and/or functions have been associated with the development of B cell malignancies [11]. In this regard, M. Mesuraca et al. have reviewed the role of two zinc finger proteins, namely, ZNF423 and ZNF521, as potent inhibitors of EBF1 and as likely contributing to the development of B cell leukaemia diseases.

Genome instability, defined by higher rate of genomic changes acquisition per cell division compared to normal cells, represents a prominent feature of MM cells [12]. M. Cea et al. provide a comprehensive overview of the current knowledge of genomic instability in MM both in terms of its contribution to disease development and progression and in terms of possible relevance as therapeutic target. The paper describes mechanisms by which genetic aberrations give rise to multiple pathogenic events required for myelomagenesis and concludes with a discussion of the clinical applications of these findings in MM patients.

Altogether, the data presented in this special issue may contribute to increasing our current knowledge of the biology of MM and B cell malignancies.

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Research Article

Plasma Levels of Monocyte Chemotactic Protein-1 Are Associated with Clinical Features and Angiogenesis in Patients with Multiple Myeloma

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The aim of this pilot study was to determine the plasma levels of monocyte chemotactic protein-1 (MCP-1) and possible associations with angiogenesis and the main clinical features of untreated patients with multiple myeloma (MM). ELISA was used to determine plasma MCP-1 levels in 45 newly diagnosed MM patients and 24 healthy controls. The blood vessels were highlighted by immunohistochemical staining, and computer-assisted image analysis was used for more objective and accurate determination of two parameters of angiogenesis: microvessel density (MVD) and total vascular area (TVA). The plasma levels of MCP-1 were compared to these parameters and the presence of anemia, renal dysfunction, and bone lesions. A significant positive correlation was found between plasma MCP-1 concentrations and TVA ($p = 0.02$). The MCP-1 levels were significantly higher in MM patients with evident bone lesions ($p = 0.01$), renal dysfunction ($p = 0.02$), or anemia ($p = 0.04$). Therefore, our preliminary results found a positive association between plasma MCP-1 levels, angiogenesis (expressed as TVA), and clinical features in patients with MM. However, additional prospective studies with a respectable number of patients should be performed to authenticate these results and establish MCP-1 as a possible target of active treatment.

1. Introduction

Multiple myeloma (MM) represents a common hematological neoplasm characterized by monoclonal expansion of plasma cells within the bone marrow, production of monoclonal immunoglobulins, and tissue impairment. The unpredictable biological behavior of this neoplasm reflects complex interactions between plasma cells and other components of the bone marrow microenvironment. Despite great improvements in therapy and significant prolongation of life expectancy, MM remains an incurable disease [1].

The limited success achieved by targeting only myeloma cells in conventional and/or high-dose chemotherapy highlights the importance of understanding the role of the bone marrow microenvironment and its specific contribution to myelomagenesis. In MM, the microenvironment is composed of clonal plasma cells, extracellular matrix proteins, bone marrow stromal cells, inflammatory cells, and microvessels. Substantial evidence indicates that interactions between these components play a key role in the proliferation and survival of myeloma cells, angiogenic and osteoclastogenic processes, and the development of drug resistance, which all lead to disease progression [2]. The antimyeloma activity of proteasome inhibitors (bortezomib, carfilzomib) and immunomodulatory drugs (thalidomide, lenalidomide, and pomalidomide) is based on their capacity to disrupt these pathophysiological processes [3, 4].

Angiogenesis is fundamental to tumor growth and spread in many hematological disorders, particularly MM [5]. The angiogenic potential of MM is regulated by a plethora of

proangiogenesis and antiangiogenesis cytokines produced by myeloma cells and other cell types in the tumor microenvironment [6].

Among the many biologically active factors produced by the MM microenvironment are chemokines and their receptors, which participate in cell homing, attraction of leukocytes, tumor growth, and bone destruction [7, 8]. One of the CC chemokines secreted by MM cells is monocyte chemotactic protein-1 (MCP-1), which acts as a potent chemoattractant for monocytes, basophils, eosinophils, endothelial cells, a subset of T lymphocytes, and myeloma cells through its CCR2 receptor [9, 10]. In addition, MCP-1 is the first CC chemokine reported to play a direct role in tumor angiogenesis [11]. However, no studies have yet explored associations between plasma MCP-1 levels, angiogenesis, and the main clinical features in newly diagnosed, untreated myeloma patients, such as anemia, renal dysfunction, and bone disease, which was the aim of the present pilot study.

2. Methods

2.1. Patients. We retrospectively analyzed 45 newly diagnosed, previously untreated myeloma patients (22 males, 23 females; median age 69 years; age range 44–86 years) and 24 age-matched healthy individuals as a control group (12 males, 12 females; median age 67 years; age range 35–83 years). Diagnoses were established at the Department of Hematology, Clinical Centre Rijeka, between 2010 and 2012 according to the International Myeloma Working Group Criteria [12]. The main characteristics of the patients are summarized in Table 1.

The clinical parameters at the time of diagnosis were anemia (hemoglobin 20 g/L below the lower limit of normal, defined as 138 g/L for men and 119 g/L for women), renal dysfunction (serum creatinine level above the upper limit of normal, defined as 117 μmol/L for men and 96 μmol/L for women), and bone disease (the presence of any lytic lesion or severe osteopenia with compressive fractures on standard bone radiographs). The study was approved by the local ethics committees.

2.2. Immunohistochemistry. Bone marrow biopsies (BMBs) from our myeloma patients were fixed in Schaffer fixative for 24 hours and decalcinated in osteodec (Bio-Optica, Milan, Italy) for 4-5 hours. Sections were stained with hematoxylin-eosin, Giemsa, periodic acid Schiff (PAS), Prussian blue, and Gomori's stain for reticulin fibers. Sections of paraffin embedded BMB samples were processed for immunohistochemical analysis in a Dako Autostainer Plus (DakoCytomation Colorado, Fort Collins, CO, USA) according to the manufacturer's protocol using the Envision procedure (DAKO EnVision FLEX, High pH KIT K801021, Glostrup, Denmark). Samples were routinely immunohistochemically stained with anti-CD138 (clone MI15, m7228, DAKO Glostrup, Denmark), Ig kappa (number 40191, DAKO, Glostrup, Denmark), and Ig lambda (number 40193, DAKO, Glostrup, Denmark) antibodies for detection of the monoclonal antibody anti-CD34 Class II (m7165 clone QBEnd10, DAKO, Glostrup, Denmark), which was used to highlight endothelial cells.

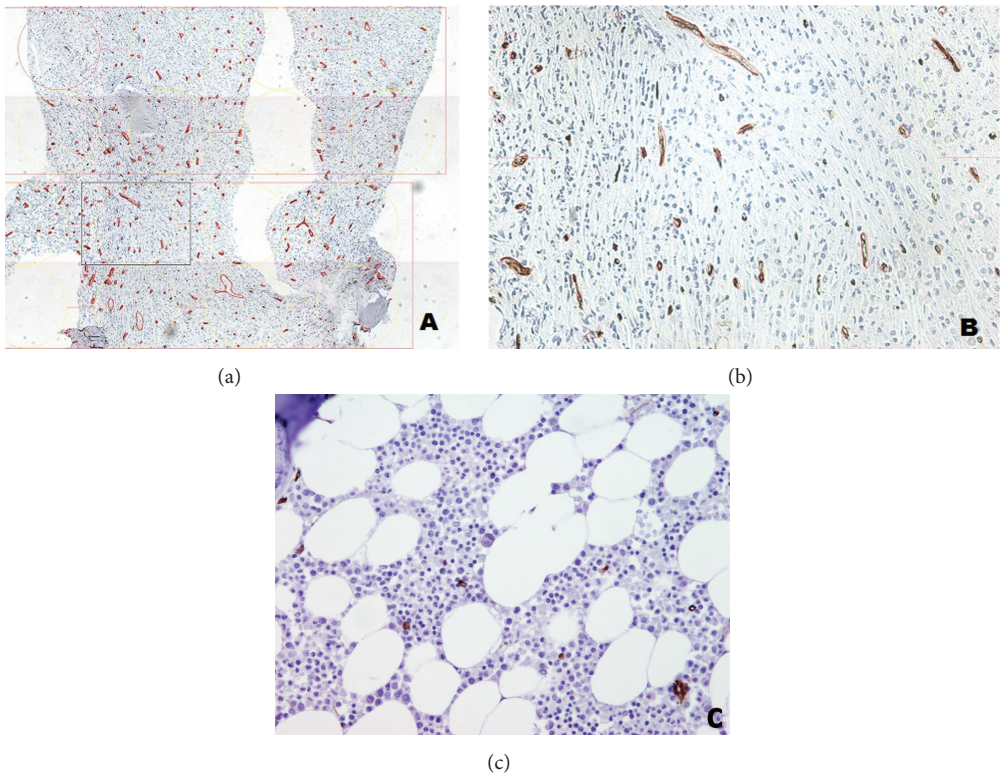
Table 1: Clinical features of patients with multiple myeloma (MM) and healthy volunteers.

Clinical features	Patients with MM ($N = 45$)	Healthy controls ($N = 24$)
Age and sex distribution	Cases	Cases
Male	22	12
Female	23	12
Age (years)	Median 69	Median 67
	Range 44–86	Range 35–83
Plasma cell percentage		
Median	69	
Range	15–97	
Durie-Salmon stage	Cases	
I	8	
II	9	
III	28	
Renal dysfunction	Cases	
Yes	16	
No	28	
Anemia	Cases	
Yes	35	
No	9	
Bone disease	Cases	
Yes	31	
No	14	

Renal dysfunction = serum creatinine level above the upper limit of normal; anemia = hemoglobin value 20 g/L below the lower limit of normal; bone disease = presence of any lytic lesion or severe osteopenia with compressive fractures on standard bone radiographs.

Epitope retrieval was achieved by immersing slides in Tris-EDTA buffer (pH 9.0) and boiling for 15 minutes in a water bath at 97°C. The slides were then incubated with CD34 monoclonal antibody at 1 : 100 dilution for 30 minutes at room temperature. For negative controls, a limited number of cases were stained by substituting primary antibody with buffer solution (DAKO).

2.3. Evaluation of Immunostaining. All slides stained with anti-CD34 were scanned and analyzed using the Alphelys Spot Browser 2 integrated system and software-controlled (Alphelys Spot Browser 2.4.4., France) stage positioning of a Nikon Eclipse 50i microscope mounted with a 1360 × 1024 resolution Microvision CFW-1310C digital camera as described previously [13]. Computer-assisted image analysis (CIA) was used for a more objective and accurate determination of angiogenic parameters. Briefly, during digital image analysis, the software detected objects of interest based on pixel color properties (wavelength, intensity, and saturation), grouping, and morphometry (size and shape). These measurements were used to calculate the average number of microvessels per 1 mm^2, referred to as the microvessel density (MVD), and the percentage of microvessel area in the total section area or the total vascular area (TVA) (Figures 1(a) and 1(b)).



(a) (b)

(c)

Figure 1: Computer-assisted image analysis (CIA) of the average number of microvessels per 1 mm^2, referred to as the microvessel density (MVD) as shown on (a), and the percentage of microvessel area in the total section area, or the total vascular area (TVA) on (b). For comparison normal bone marrow (×100) stained with CD34 is attached on (c).

2.4. Measurement of MCP-1 in Plasma. The concentration of MCP-1 was measured in plasma samples by enzyme-linked immunoassay (ELISA; Quantikine R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. These assays employ the quantitative sandwich immunoassay technique. The optical density of each well was measured using the microplate reader set at 450 nm. The concentration of MCP-1 in each plasma sample was calculated from standard curves and reported in picograms per milliliter.

2.5. Statistical Analysis. Statistical analyses were performed using MedCalc for Windows, version 12.2.1.0 (MedCalc Software, Ostend, Belgium). The distribution of data was tested for normality using the Kolmogorov-Smirnov test. The Mann-Whitney U test was used to assess whether MCP-1 plasma concentrations differed significantly between categories: patients with bone lesions versus patients without bone lesions, patients with renal dysfunction versus patients without renal dysfunction, and patients with anemia versus patients without anemia. Correlations between MCP-1 and angiogenic parameters (MVD and TVA) were studied using the Pearson correlation. Statistical differences with $p < 0.05$ were considered significant.

3. Results

MCP-1 was detected in plasma samples from all patients and healthy controls, and no significant differences were found between MM patients (median 105.6 pg/mL, range 8.3–299.5 pg/mL) and healthy controls (median 103.5 pg/mL, range 69.5–175.2 pg/mL; $p = 0.83$). Plasma MCP-1 levels were significantly higher in patients with renal dysfunction (median 120.3 pg/mL, range 84.7–299.5 pg/mL) in comparison with patients who had no renal impairment (median 91.5 pg/mL, range 8.3–277.4 pg/mL; $p = 0.02$; Figure 2). Likewise, plasma MCP-1 levels were higher in patients with anemia (median 109.5 pg/mL, range 32.1–299.5 pg/mL) in comparison with patients who had normal hemoglobin values (median 78.9 pg/mL, range 8.3–170.2 pg/mL; $p = 0.04$; Figure 3). Furthermore, patients with evident bone lesions had significantly higher concentrations of plasma MCP-1 compared to patients without the presence of any lytic lesion or severe osteopenia with compressive fractures on standard bone radiographs (median 110.3 pg/mL, range 32.1–299.5 pg/mL versus median 86.4 pg/mL, range 8.3–138.9 pg/mL; $p = 0.01$; Figure 4 and Table 2).

Angiogenic parameters for the patient cohort were as follows: median MVD was 179 (range 42–685) and median TVA was 2.09% (range 0.41%–17.3%). Comparison of the plasma MCP-1 levels and angiogenic parameters in patient BMBs yielded the following results: there was a significant positive correlation between plasma MCP-1 concentrations and TVA ($r = 0.347$, $p = 0.02$), but no significant correlation was found regarding MCP-1 and MVD ($r = 0.207$, $p = 0.18$; Table 3).

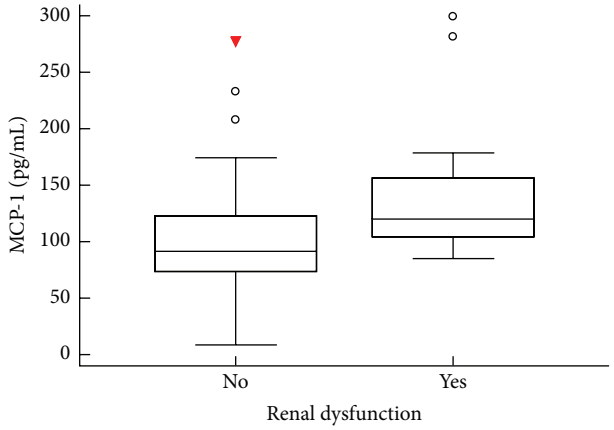


Figure 2: Comparison of plasma MCP-1 levels between patients who had normal creatinine values and those with renal dysfunction. The plasma concentration of MCP-1 was significantly higher in patients with renal dysfunction ($p = 0.02$, Mann-Whitney U test). The bars indicate the 75th and 25th percentiles, and the line in each box represents the median.

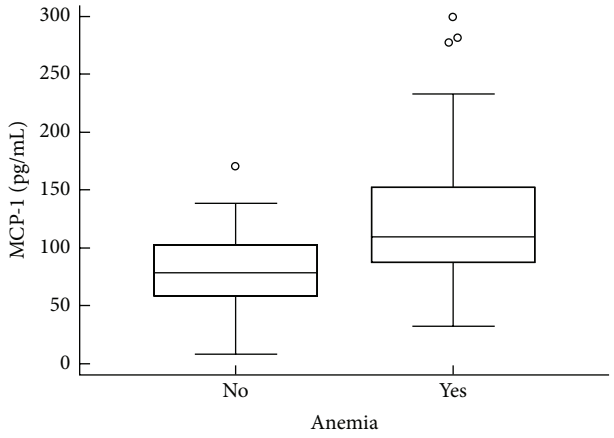


Figure 3: Comparison of plasma MCP-1 levels between patients without anemia and patients with anemia. The plasma concentration of MCP-1 was significantly higher in patients with anemia ($p = 0.04$, Mann-Whitney U test). The bars indicate the 75th and 25th percentiles, and the line in each box represents the median.

4. Discussion

Our current research is a continuation of the series of pilot studies attempting to identify potentially important cytokines influencing MM. Our previous preliminary results indicated a positive association between plasma levels of OPN, bone destruction, and tumor burden, suggesting that OPN can be a useful biomarker for monitoring bone disease and tumor mass [14].

Bone marrow angiogenesis increases with disease progression across the spectrum of plasma cell dyscrasias [15, 16]. Furthermore, parameters of angiogenesis have been established as adverse prognostic factors for MM survival [17–19], correlating with other prognosticators [15, 16, 20, 21]. Novel agents against MM, such as proteasome inhibitors

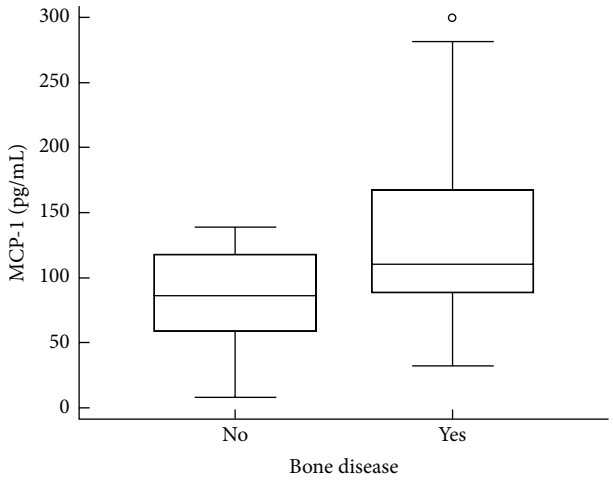


Figure 4: Comparison of plasma MCP-1 levels between patients with bone disease and those without bone lesions. The plasma concentration of MCP-1 was significantly higher in patients with bone disease ($p = 0.01$, Mann-Whitney U test). The bars indicate the 75th and 25th percentiles, and the line in each box represents the median.

Table 2: Measured plasma MCP-1 levels based on clinical parameters in patients with MM.

Clinical feature	MCP-1 (pg/mL)	
	Median	Range
Renal dysfunction		
No	91.5	8.3–277.4
Yes	120.3	84.7–299.5
p value	**0.02**	
Anemia		
No	78.9	8.3–170.2
Yes	109.5	32.1–299.5
p value	**0.04**	
Bone disease		
No	86.4	8.3–138.9
Yes	110.3	32.1–299.5
p value	**0.01**	

p values are based on the Mann-Whitney U test.
Renal dysfunction = serum creatinine level above the upper limit of normal; anemia = hemoglobin value 20 g/L below the lower limit of normal; bone disease = presence of any lytic lesion or severe osteopenia with compressive fractures on standard bone radiographs.

Table 3: Correlation between plasma MCP-1 levels and analyzed parameters of angiogenesis in MM patients.

		Parameter of angiogenesis	
		MVD	TVA
MCP-1 (pg/mL)	r^1	0.207	0.347
	p^1	0.18	0.02

[1]Pearson correlation.
MVD = total count of microvessels per 1 mm^2; TVA = total area occupied by microvessels (as percentage of total section area).

or immunomodulatory drugs that significantly improve the response to therapy and survival, exhibit marked antiangiogenic effects. However, we do not yet know which of these numerous soluble factors play central roles in the regulation of angiogenesis. Salcedo et al. demonstrated that MCP-1 may directly induce blood vessel formation *in vivo*, which can be inhibited using neutralizing antibodies against MCP-1 [11]. Niu et al. showed that MCP-1 promotes angiogenesis *via* a transcription factor, MCP-1-induced protein [22]. To the best of our knowledge, the current pilot study is the first study concerning possible correlations between plasma levels of MCP, parameters of angiogenesis, and clinical manifestations in patients with MM. The presence of MCP-1 in plasma samples from all patients and healthy controls implicates this chemokine in physiological and pathological processes. Even though the patients had slightly higher concentrations of MCP-1 than controls, the difference was not significant. This finding can be attributed to the small number of samples. Until now, only a few studies of MM have explored both MVD and TVA. Rana et al. found a significant correlation between MVD and TVA, both of which correlated with other examined histological features associated with prognosis and residual disease in myeloma patients [23]. Bhatti et al. also demonstrated a good correlation between MVD and TVA; "complete responders" had significantly less angiogenesis than "nonresponders," but only MVD was a good predictor of a complete response in patients with MM, particularly when the analysis was performed using a computerized image analyzer [24]. Tzenou et al. evaluated angiogenesis in trephine biopsy specimens from 36 patients with Waldenström's macroglobulinemia. Only TVA, not MVD, significantly correlated with time to first therapy and overall survival [25]. Our results show for the first time a positive correlation between plasma MCP-1 levels and angiogenesis in myeloma patients, as patients with higher plasma MCP-1 levels had significantly higher TVA in BMBs, whereas MVD failed to show a significant association with chemokine concentrations. The reason for this finding is questionable. Of course, it may be a consequence of the rather small number of patients; therefore, this result should be retested in a larger sample. However, it is doubtful whether these two angiogenic parameters actually provide the same biological information. The magnitude of MVD is determined mainly by the number of small blood vessels, many of which still do not have a formed vascular lumen. On the other hand, TVA is defined as the percentage of microvessel area in the total section area, meaning that the presence of larger vessels with formed vascular lumen increases the value of TVA more than the number of small blood vessels. Further investigations are needed to fully clarify this issue.

Our preliminary results demonstrated a significant association between plasma MCP-1 levels and the main clinical features of MM. Namely, patients with higher chemokine levels exhibited more severe bone disease, renal impairment, and anemia. The association between increased plasma level of MCP-1 and creatinine concentration should be taken with caution because it may be the result of decreased renal clearance of this chemokine in patients with renal failure.

We hypothesized that, in myeloma patients, proinflammatory cytokine tumor necrosis factor-alpha (TNF-α) upregulates the production of interleukin-6 (IL-6), which then mediates increased secretion of MCP-1. These three biologically active factors produced by myeloma cells and other cellular elements of the bone marrow microenvironment can influence the activity and survival of osteoclasts and osteoblasts and increase inflammatory processes in the bone marrow and kidneys, leading to anemia and renal disease. The role of TNF-α and IL-6 in the progression of myeloma cell growth, survival, angiogenesis, and osteoclastogenesis and the inhibition of osteoblast activity is well established, as well as their adverse prognostic significance in this hematological neoplasm [8, 26–30]. Lee et al. reported that the level of IL-6 in bone marrow aspirates from myeloma patients positively correlates with the level of TNF-α, and these cytokines correlated with poor prognostic factors and short overall survival [30]. Lee et al. also demonstrated that IL-6 secretion is regulated by TNF-α via the JAK/STAT pathway in U266 myeloma cells [30]. Arendt et al. found that IL-6 induces MCP-1 expression in myeloma cells, suggesting a new mechanism by which IL-6 may contribute to disease pathogenesis [31]. Johrer et al. reported that transendothelial migration of myeloma cells is increased by TNF-α via TNF receptor 2 and autocrine upregulation of MCP-1, demonstrating again the possible mutual relationship between these cytokines and chemokines [32]. Speaking of bone disease in MM, Liu et al. showed that MM cells increase the production of MCP-1 by bone marrow stromal cells, which then enhances osteoclast formation [33]. Although the current study has limitations, such as the small sample size, missing complete clinical data for one patient, and retrospective design, that limit any strong conclusions, our preliminary results indicate a positive association between plasma MCP-1 levels, angiogenic parameters, and clinical features in patients with MM.

5. Conclusion

The current pilot study found a positive association between plasma MCP-1 levels, angiogenesis (expressed as TVA), and the main clinical features of MM (i.e., bone disease, renal dysfunction, and anemia) in newly diagnosed MM patients. However, additional prospective studies with a respectable number of patients should be performed to authenticate the angiogenic potential of MCP-1 and its biological value as a biomarker for monitoring bone or renal disease in patients with MM. The plasma levels of TNF-α and IL-6 should also be evaluated parallel to MCP-1.

Ethical Approval

Ethical approval was received from the Ethics Committees of the Rijeka University Hospital Centre and School of Medicine, University of Rijeka.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper. The authors have no

support from any organization for the submitted work and no financial relationships with any organization that might have an interest to the submitted work or any other relationships or activities that could appear to have influenced the submitted work.

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Review Article

MicroRNAs: Novel Crossroads between Myeloma Cells and the Bone Marrow Microenvironment

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Multiple myeloma (MM) is a hematologic malignancy of differentiated plasma cells that accumulate in the bone marrow, where a complex microenvironment made by different cell types supports proliferation, survival, and drug resistance of tumor cells. MicroRNAs (miRNAs) are short non-coding RNAs that regulate gene expression at posttranscriptional level. Emerging evidence indicates that miRNAs are aberrantly expressed or functionally deregulated in MM cells as the result of multiple genetic or epigenetic mechanisms and that also the tumor microenvironment regulates MM cell functions by miRNAs. Consistently, modulation of miRNA levels in MM cells has been demonstrated to impair their functional interaction with the bone marrow microenvironment and to produce significant antitumor activity even able to overcome the protective bone marrow *milieu*. This review will describe the most recent findings on miRNA function in the context of MM bone marrow microenvironment, focusing on the therapeutic potential of miRNA-based approaches.

1. Introduction

Multiple myeloma (MM) is a complex hematologic malignancy, driven by several genetic and epigenetic alterations. It is characterized by high infiltration and accumulation in the bone marrow (BM) of malignant plasma cells (PCs), which secrete a monoclonal protein detectable in the blood and/or urine [1]. After non-Hodgkin lymphoma, MM represents the second most common hematologic disease accounting for more than 10% of all hematologic cancers and 2% of annual cancer-related deaths [2]. MM is often preceded by premalignant conditions including monoclonal gammopathy of undetermined significance (MGUS), indolent multiple myeloma (IMM), and smoldering multiple myeloma (SMM). MM, which can also occur *de novo*, is a subsequent, late-stage of this progression [1]. Diagnostic criteria of symptomatic myeloma include the presence of at least 10% MM cells in the BM and of monoclonal protein in serum and/or urine, along with MM-related end-organ or tissue damage (including hypercalcemia, renal dysfunction, anemia, immunodeficiency, and bone destruction) [1]. Although extensive preclinical research [3] has provided the basis for the clinical introduction of novel therapeutics such as immunomodulatory agents (thalidomide, lenalidomide, and pomalidomide) or proteasome inhibitors (bortezomib, carfilzomib), which have significantly improved the response rate and overall survival of MM patients, MM still remains an incurable disease [4, 5].

It is noteworthy that MM cells home to and dynamically interact with the BM, which provides a survival and drug-resistance framework by direct interaction of MM cells with bone marrow stromal cells (BMSCs) and extracellular

matrix (ECM) components [6]. Indeed, PC trafficking in and out from the BM is responsible for the progression of the disease to new BM sites [7]. The BM microenvironment (BMM) is highly heterogeneous and contains several cell types, including osteoclasts (OCs), osteoblasts (OBs), and endothelial, inflammatory, immune, and BM-derived stromal cells, originating from normal cells but becoming altered during tumor progression; in addition, the BM niche is also composed of a non-cellular compartment including the ECM and several signalling molecules, composed of cytokines, chemokines, and growth factors [8–11].

The identification of molecules regulating the cross-talk between MM cells and the BMM represents a challenging area of research in order to unveil the BM-related mechanisms promoting MM development and possibly to identify more effective targets for therapeutic intervention.

MicroRNAs (miRNAs) have gained increasing attention in MM research [12] since they have been found deregulated in MM cells and can target many oncogenes or tumor suppressor genes, thus affecting MM growth *in vitro* and *in vivo* [13–16]. miRNAs are the most abundant class of small RNAs (22–25 nucleotides in length) in animals. They represent approximately 1% of the genome of different species and each has hundreds of different mRNA targets [17]. miRNA biogenesis occurs in the nucleus, where a pri-miRNA hairpin is transcribed by RNA polymerase II and is subsequently cleaved by Drosha, a member of the RNA polymerase III family, into a 70–100 bp pre-miRNA that translocates in the cytoplasm, wherein it is cleaved by Dicer in 20–22 bp miRNA/miRNA* duplexes. Thereafter, the miRNA duplex is unwound and the mature miRNA strand binds to an Argonaute protein into a RNP complex, commonly known as RISC, that drives the mature miRNA strand to the 3′-UTR mRNA target sequence. Depending on the degree of complementarity between the miRNA and its target mRNA, miRNA binding to 3′-UTR represses translation or induces deadenylation and mRNA decay [13, 18, 19].

By regulating the expression of target genes, miRNAs control diverse cell functions such as proliferation, differentiation, and apoptosis [20]. Recent research has highlighted the role of certain miRNAs as tumor suppressors which inhibit oncogene expression, while several miRNAs are oncogenic modulators that inhibit the expression of tumor suppressor genes [13]. In the last decade, available information about miRNA expression in MM has significantly grown, disclosing several miRNAs controlling critical genes in MM pathobiology and revealing that miRNA expression pattern in MM is frequently associated with specific genetic abnormalities [14–16].

Firstly, Pichiorri et al. analyzed miRNA expression profile in a panel of 49 MM cell lines, 16 BM CD138$^+$ PCs isolated from MM, and 6 from MGUS patients, finding a common miRNA signature likely associated with the multistep transformation process of MM. Of note, they found miR-21, members of miR-106b-25 cluster, miR-181a, and miR-181b upregulated in MGUS patients; moreover, by comparing MGUS and MM samples with normal PCs, authors found some miRNAs, including miR-32 and miR-17-92 cluster, upregulated only in MM cells [21]. Research performed by our group indeed confirmed abnormal expression of miRNAs in MM samples, with miR-29b, miR-125b, miR-199a-5p, and miR-34a found expressed at low levels in MM cells and/or acting as tumor suppressor miRNAs [22–27], while miR-21, miR-125a-5p, miR-221, and miR-222 upregulated in MM cells and behaving as oncomiRNAs [28–32].

Similarly to protein-coding genes, the expression of miRNAs in MM cells is regulated by genetic and/or epigenetic mechanisms [33]; in addition, the BMM *per se* may alter the miRNA repertoire of MM cells, influencing their behaviour. On the other side, emerging evidence has shown that modulation of miRNA levels in MM cells might affect the phenotype of neighboring cells within the BMM.

The present review will focus on experimental findings underlying the relevant role of miRNAs as fine regulators of the cross-talk between MM cells and the BMM, with the perspective of novel miRNA-based therapeutic interventions targeting MM cells within their supporting *milieu*.

2. Cellular Components of the BMM

MM is the prototype of malignancies characterized by complex interactions between tumor cells and the host microenvironment. Survival and proliferation of malignant PCs rely on cell-to-cell contact with BMSCs [9], generally occurring through adhesion molecules expressed on BMSCs such as ICAM-1, VCAM-1, and β1- and β2-integrins and resulting in the activation of several signal transduction pathways promoting MM survival and drug resistance [34].

MM-BMSCs also express and produce many angiogenic factors as VEGF, basic-fibroblast growth factor (b-FGF), angiopoietin-1 (Ang-1), transforming growth-factor-β (TGF-β), platelet-derived growth factor-β (PDGF), and IL-1 [35]. Among others, NF-κB signalling is activated in BMSCs by MM-BMSCs interaction, which fosters IL-6 secretion by BMSCs and stimulates VEGF secretion by MM cells [36]. MM cell adhesion to BMSCs also promotes NF-κB-dependent production of BAFF, a member of the TNF protein superfamily, crucial for the maintenance and homeostasis of normal B-cell development, which confers a survival advantage on MM cells [37, 38] and promotes RANK-Lindependent osteoclastogenesis [39]. Moreover, the TGF-β family member activin-A, secreted by BMSCs and OCs after interaction with MM cells [40], modulates bone remodelling by acting as both OC promoter and inhibitor of OB differentiation. In MM, high activin-A levels in both BM and peripheral blood are associated with advanced bone disease (BD) [40]. The interaction between MM cells and BMSCs is also regulated by Notch, which activates growth promoting pathways and stimulates cytokines production both in MM and in BMSCs [41, 42]. MM-BMSCs and MM cells both produce exosomes that can be transferred between the two cell types and positively modulate tumor growth *in vitro* and *in vivo* [42, 43]. Exosomes, which may also carry miRNAs, will be discussed in a dedicated paragraph.

OBs and OCs are the two cellular components playing a pivotal role in the metabolism of bone tissues. The anabolic activities of OBs and the catabolic actions of OCs result in continuous self-renewal of bone, maintaining an adequate

bone mass and calcium homeostasis in vertebrates [44]. OBs derive from multipotent mesenchymal stem cells (MSCs), produce ECM, and are responsible for its mineralization, thus directly forming intramembranous bones; furthermore, OBs affect OC differentiation from hematopoietic cells [45, 46]. Suppression of OB activity accounts for both the MM osteolytic process and progression of MM. The Wnt signalling pathway inhibitor DKK1 suppresses OB activity in MM by binding to LR5/6 membrane coreceptors. Blockade of DKK1 by anti-DKK1 antibody (BHQ880) increases OB differentiation *in vitro* and trabecular bone formation *in vivo* [47]. Proteasome inhibitors (bortezomib) also display bone anabolic activity *in vivo* [48]. BD is the most frequent complication in MM resulting in osteolytic lesions affecting new bone formation; in MM-BD, the perfect balance between bone-resorbing OCs and bone-forming OB activity is completely abrogated in favour of OCs, thus resulting in skeletal disorders. Inside the BM niche, MM cells lie in close proximity to the sites of active bone resorption and are able to produce themselves or induce other cells to produce "osteoclast-activating factors." MM cells produce several factors, including RANK-L, MIP-1α, IL-3 and IL-6, which promote OC activation. RANK is a transmembrane receptor on OC cells which is activated by its ligand (RANK-L) expressed on MM cells; of note, adhesion of MM cells to BMSCs increases the surface expression of RANK-L on MM cell membrane. Binding of RANK-L to its receptor on OC-precursor cells increase their differentiation towards mature OCs by activating NF-κB and jun-N-terminal kinase pathway [49, 50]. Moreover, mounting evidence indicates that exosomes secreted by MM cells positively modulate OC function and differentiation [43], playing a key role in bone remodelling processes. Indeed, *in vitro* studies have demonstrated the prodifferentiative effects induced by MM-derived exosomes on both human primary OCs and murine pre-OCs. Specifically, MM-derived exosomes increased the expression of osteoclastic markers and their lytic activity; on the contrary, exosomes derived by healthy peripheral blood mononuclear cells did not elicit any effect [43].

Bisphosphonates that exert potent proapoptotic effects on OCs are the current standard treatment for MM-BD. However, severe side effects may occur in the mid-long-term treatment, limiting their real clinical usefulness [9]. Denosumab, a recently developed anti RANK-L monoclonal antibody, has unfortunately shown contradictory results in MM [51]; Bruton tyrosine kinase- (BTK-) inhibitors, such as ibrutinib, have conversely shown promising anti-OC activity in preclinical models of MM-BD [52].

Tumor angiogenesis has been linked to the pathogenesis and progression of hematological malignancies, including MM [53]. It is widely acknowledged that MM growth in the BM increases vascularity by altering the fine interplay, regulated by cytokines and growth factors, among pericytes, endothelial cells (ECs), dendritic cells (DCs), inflammatory cells, and hematopoietic stem cells [54]. Importantly, a progressive increase in microvascular density is observed during the transition from MGUS to SMM and from SMM to clinically active MM [55, 56], paralleled by the increase in the peripheral blood or in the BM of the serum levels of the major proangiogenic cytokines (VEGF, bFGF, HGF, and Syndecan-1) [57]. MM-derived ECs may secrete IL-6, bFGF, and HGF, which in turn promote MM growth and dissemination; on the other hand, MM cells secrete VEGF which stimulates IL-6 production by ECs [57–60]. A graphic overview of the interaction between MM cells and the most representative cellular components of the BMM is provided in Figure 1.

The progression of MM is also associated with an immunosuppressive microenvironment that promotes tumor growth and escape from physiological immune surveillance systems, where effector cells, mainly Natural Killer (NK) cells and cytotoxic T lymphocytes (CTLs), enable potent antitumor responses. Several immunosuppressive cell types have been identified in the context of the MM-BMM, such as myeloid derived suppressor cells (MDSCs) and regulatory T cells (Tregs). For detailed information on the MM-related immunological microenvironment, we recommend to the readers more specialized reviews [10, 11].

3. miRNA-Based Regulation of MM Cells by the BMM

The signals from the BM niche provide a viable environment for MM cell growth and survival. Such microenvironment-derived supporting role on MM cells mainly occurs through a close interaction between MM cells and BM components, which may exert their regulatory effects on cancer cells through miRNAs [21, 61–63].

It is now clear that the BMM is hypoxic and that low oxygen concentrations support MM cell angiogenesis, invasion, and disease progression [64]. Emerging data indicate that hypoxia regulates miRNA expression in cancer cells [65, 66]. In MM, we recently demonstrated that the hypoxic BMM strongly decreases the expression of miR-199a-5p [24, 50]. Of note, miR-199a-5p directly targets the transcription factor hypoxia-inducible factor-1α (HIF-1α), which is strongly overexpressed in MM cells [67–69]. Enforced expression of miR-199a-5p synthetic mimics in hypoxic MM cells reduced HIF-1α expression and impaired both MM and EC migration, increasing adhesion of cancer cells to the hypoxic BMSCs. The latest evidence was particularly interesting, since a previous report indicated that hypoxia reduces adhesion of MM cells to the BM stroma, thus promoting dissemination [70]. Importantly, miR-199a-5p synthetic oligonucleotides delivered in a mouse model of human MM reduced tumor growth and prolonged survival of treated animals [24], thus demonstrating the anti-MM potential of miR-199a-5p replacement strategies in overcoming the hypoxic microenvironment *in vivo*.

In the BMM, MM cells have an inhibitory effect on osteoprotegerin (OPG) secretion by BMSCs and OBs, thus inducing an imbalance in RANK-L/OPG ratio and leading to osteolytic lesions development. The TNF receptor ligand superfamily member OPG acts as a decoy receptor of RANK-L, thus antagonizing RANK-L binding to RANK and consequently preserving the integrity of bone mass [71, 72].

Recently, Pitari and colleagues reported the involvement of oncogenic miR-21 in MM-BD, validating OPG as direct target. The authors found that miR-21 was overexpressed in

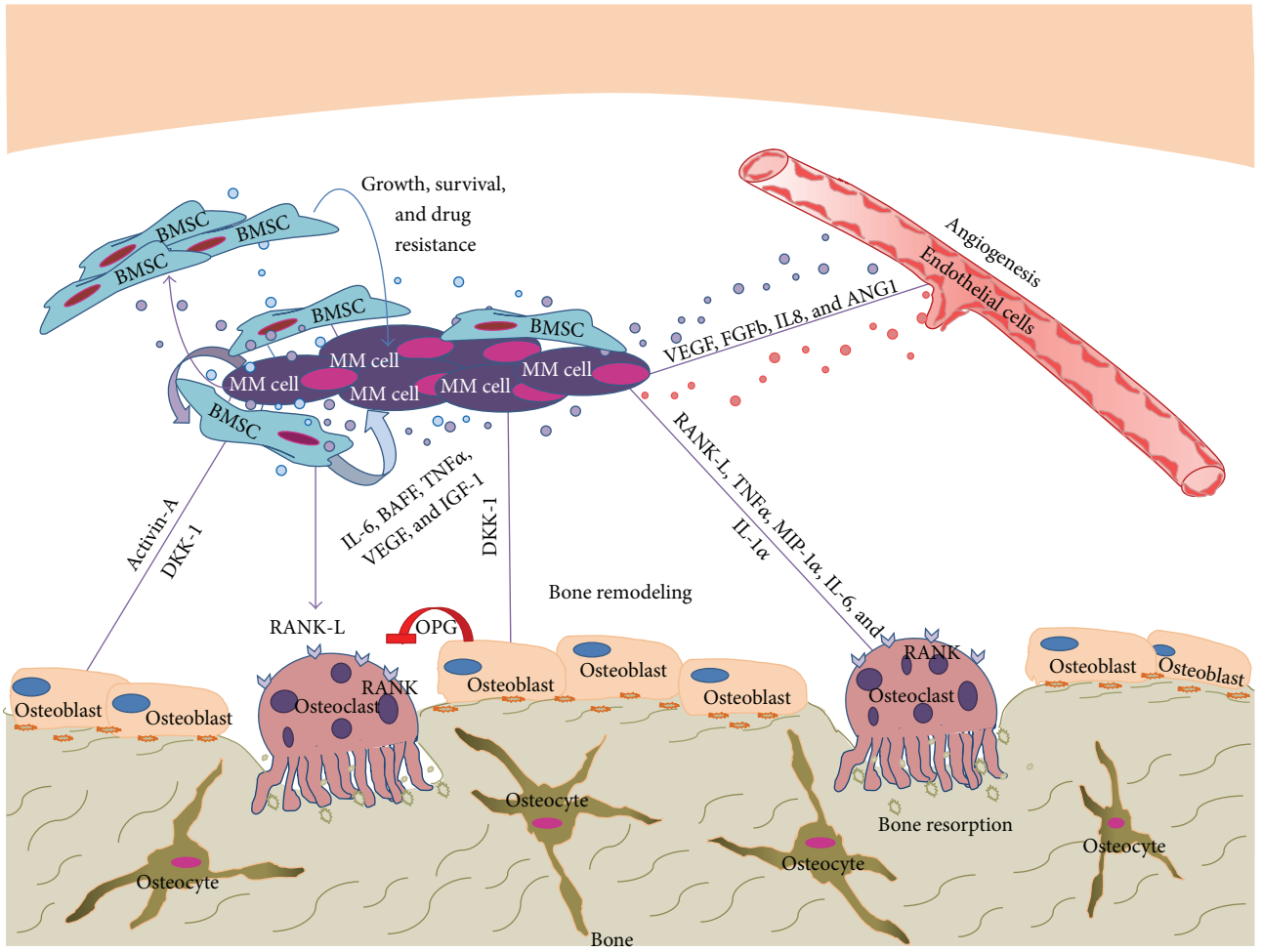

FIGURE 1: Cross-talk between MM cells and the BM microenvironment. MM cells support BM angiogenesis and disrupt normal bone remodelling process. Moreover, BMSCs sustain MM survival and regulate osteogenesis and angiogenesis by direct contact between BM cellular components and MM cells or by releasing molecules.

MM patient-derived BMSCs; furthermore, adhesion to MM increased miR-21 expression in stromal cells, while OPG secretion was impaired. On the contrary, constitutive miR-21 inhibition in BMSCs restored OPG secretion, reduced RANK-L production, and rescued RANK-L/OPG ratio in cocultures of MM patient-derived BMSCs. Importantly, authors found that inhibition of miR-21 negatively reduced bone resorption [29]. As discussed above, BMSCs are induced by MM cells to produce RANK-L, contributing to MM-BD [73]; however, several studies showed that also human MM cells express RANK-L [74]. Yuan and colleagues demonstrated that RANK-L promoter demethylation in MM cells was under the control of BMSCs. In detail, authors showed that coculture of MM cells with MM-BMSCs induced down-regulation of the DNA-methyltransferase DNMT1 along with RANK-L promoter demethylation in MM cells. The authors hypothesized that, among the soluble factors secreted by BMSCs, TNFα could be responsible for the phenomena described above [75]. Indeed, treatment with TNFα increased miR-140-3p and miR-126 expression in MM cells, which are under the control of TNFα, and led to repression of DNMT1 transcription and RANK-L expression; conversely, the anti-TNFα antibody partially abrogated RANK-L expression [76].

Studies on B cell-activating factor (BAFF), a member of the tumor necrosis factor family, demonstrated that the expression levels of this cytokine are significantly high in serum of MM patients. BAFF secreted by BMSCs positively controls MM survival, also sustaining adhesion of cancer cells to stromal cells. In turn, cell adhesion-activated NF-κB pathway in stromal cells further preserves myeloma cells against conventional drug treatment [37, 77]. Bioinformatic analyses evidenced that miR-202 targets BAFF [21, 78]. Shen and colleagues found that overexpression of miR-202 in BMSCs reduced adhesion of MM cells to the stroma;

furthermore, they observed inhibition of MM cell growth and survival, as well as an enhanced sensitivity of MM cells to bortezomib treatment [79].

In another study, adhesion of MM cells to the BMSCs was also demonstrated to trigger bortezomib resistance via suppression of the tumor suppressor miR-15a/-16 in MM cells. MiR-15a/-16 are located as a cluster on chromosome 13q14, an area frequently deleted in MM and strongly correlated with reduced survival in MM patients [80]. MiR-15a and miR-16 expression usually is low in MM PCs and totally absent in those patients carrying deletion [21]. Further studies indicated that the BMM might be involved in downregulation of miR-15a/-16: in fact, IL-6 produced by BMSCs was responsible for miR-15/-16 downregulation by BMSCs, and exogenous IL-6 induced a time- and dose-dependent reduction of miR-15a/-16 in MM cells; in addition, miR-15a inhibition rescued VEGF expression and contributed to disease progression [81, 82]. IL-6 also triggers the transcription of miR-21 gene, which contains two STAT3 binding sites within its putative regulatory regions; of note, IL-6-dependent miR-21 expression was completely abrogated when STAT3 motifs were removed by miR-21 promoter, thus demonstrating that miR-21 gene transcription by IL-6 occurred in a STAT3-dependent fashion [83].

The effect of the BMM on miRNA expression in MM cells was also investigated by Wang and colleagues, who showed that upregulation of oncomiR-21 in MM cells was a consequence of the adhesion to BMSCs and correlated with NF-κB activation in MM cells. Treatment with the proteasome inhibitor bortezomib, a strong inhibitor of NF-κB signalling pathway, led to downregulation of miR-21 even in MM/BMSCs cocultures [84]. Importantly, the authors evaluated the therapeutic efficacy of a combination between miR-21 synthetic inhibitors and dexamethasone, bortezomib, or doxorubicin, demonstrating that inhibition of miR-21 expression resensitizes MM cells to dexamethasone and bortezomib [84].

Mesenchymal stem cells (MSCs), the progenitors of OBs, readily contribute to MM-BD by promoting OC formation and activity at various levels (increasing RANK-L to OPG expression, augmenting secretion of activin A and production of Wnt5a, etc.), thus further contributing to OB/OC uncoupling in MM osteolytic lesions [85].

Several reports indicate a senescence-like state in BM-MSCs, promoting tumorigenesis in neighboring premalignant cells [86, 87]. In detail, a senescence-like state in MSCs seems to be correlated with an altered secretory profile, impaired osteogenesis, and inhibition of T-cell proliferation [88, 89]. Interestingly, two imprinted clusters in the human genome [90], namely, DLK1-DIO3 and C19MC, expressing several miRNAs, have been linked to the senescence process [91]. Berenstein and colleagues studied the correlation between senescence and miRNA expression in MM BM-MSCs. The authors evidenced an increased senescence in MSCs after coculture with MM cells; then they analyzed miRNAs deregulated in MSCs and likely associated with inflammation-induced cellular senescence [90, 92, 93] and identified miR-485-5p, whose hypermethylated locus is in the DLK1-DIO3 cluster, as a potential candidate accounting for the senescence status in BMSCs. Interestingly, overexpression of miR-485-5p in MM cells blocked cell cycle and senescence of MSCs [91].

4. miRNA-Based Strategies to Overcome the MM-Supporting BMM

The increasing number of preclinical studies demonstrates the ability of miRNA-based strategies to counteract the protective role of the BMM on MM cells.

Roccaro and colleagues identified several miRNAs deregulated in MM cells and, among others, miR-15a and miR-16 resulted to be significantly decreased in MM compared to healthy PCs. Therefore, the functional role of miR-15a and miR-16 in MM cells was investigated by transfecting synthetic pre-miRNAs and evaluating their anti-MM effect in the context of the BMM [94]. Restoration of miR-15a and miR-16 reduced MM cell proliferation and growth both *in vitro* and *in vivo*, abrogating the expression of validated targets involved in signalling pathways regulating proliferation, such as AKT3. MiR-15a and miR-16 restoration negatively affected VEGF secretion in MM cells and inhibited MM cell-dependent EC growth and capillary formation *in vitro*. Of note, miR-15a and miR-16 overexpression reduced the *in vitro* migratory capacity of MM cells and impaired MM adhesion to the BMM reducing tumor progression in mice [94].

A group of miRNAs in MM cells was found to be under the control of Argonaute 2 (AGO2) protein, a core component of the RISC complex that indirectly regulates gene expression by RNA degradation or translational repression. AGO2 directly binds to miRNAs and mediate target mRNA degradation. Wu et al. described the role of AGO2 as enhancer of MM angiogenesis, through upregulation of proangiogenic miRNAs such as let-7 family members and miR-92a and downregulation of the antiangiogenic miR-145. All these miRNAs have several angiogenic targets. Let-7 family members regulate VEGF level and promote angiogenesis by reducing HIF-3α expression, the negative regulator of HIF pathway in vascular cells. VEGF is also target of downregulated miR-145, a miRNA binding the 3′-UTR of VEGF. AGO2-induced angiogenesis is also triggered through the upregulation of miR-92a, which targets the antiangiogenic protein angiopoietin-like protein 1 (ANGPT1) [95].

Constitutively active canonical Wnt/β-catenin pathway has been described in MM cells [96], mostly due to increased expression of BCL9, the transcription coactivator for β-catenin [97]. Zhao and colleagues firstly demonstrated that BCL9 is a direct target of tumor suppressor miR-30 family members; furthermore, they showed that downregulation of miR-30s results from MM-BMSCs interaction [98]. Ectopic expression of miR-30c decreased BCL9 expression and inhibited components of Wnt pathway, such as CD44 and Axin-2 [99, 100]. CD44 being a functional component of cell adhesion-mediated resistance [6], the authors explored the potential involvement of miR-30s in BMM-dependent drug resistance: restoration of miR-30s expression resensitized MM cells to dexamethasone treatment, even when MM cells were cocultured with BMSCs. Importantly, miR-30c blocked tumor growth and dissemination in murine xenograft models

of human MM; in particular, microcomputed tomographic analyses of bones revealed a reduction of osteolytic lesions, suggesting miR-30s as new antiresorptive therapeutic agents in MM-BD [98].

Another tumor suppressor miRNA downregulated in MM is miR-125b-5p, which has been shown to target interferon regulatory factor 4 (IRF4), a lymphocyte-specific transcription factor with an oncogenic role in MM. IRF4 has several targets such as c-Myc, which has a prominent role in the pathogenesis of MM, or B-lymphocyte-induced maturation protein-1 (BLIMP-1), through which IRF4 regulates MM survival. Adhesion to BMSCs or exogenous cytokines (IL-6, IGF-1, and HGF) did not affect the *in vitro* tumor suppressive activity of synthetic miR-125b-5p mimics that was also confirmed *in vivo* after delivery of lipid-emulsion formulated oligonucleotides in SCID mice bearing MM xenografts [23].

miR-29 family members generally act as tumor suppressor in hematologic malignancies [33]. Recent studies by our group described the role of miR-29b in MM. Constitutive expression of miR-29b decreased cell proliferation and induced apoptosis in MM cells, reducing the expression of MCL-1 and CDK6, usually overexpressed in MM and associated with cell growth promotion [22]. Among miR-29b targets, we identified Sp1, a transcription factor with oncogenic activity in MM and other malignancies [47, 101], as involved in a negative feedback loop with miR-29b itself [22]. Later on, additional studies confirmed the biological role of miR-29b in the context of the BMM; in detail, miR-29b overexpression impaired MM and HUVEC migration and increased adhesion to BMSCs, downmodulating the expression of factors involved in both angiogenesis and disease progression as IL-8, MMP2, and VEGF-A [102]. We also reported the *in vivo* antitumor activity of synthetic miR-29b mimics in the context of the BMM, by using the SCID-*synth-hu* model [103]; in this system, $CD138^+$ cells from advanced MM patients are injected in SCID mice implanted with a 3D polymeric scaffold mimicking the bone architecture, which is previously reconstituted with human BMSCs. Of note, intrascaffold delivery of lipid-emulsion formulated miR-29b mimics induced apoptosis of MM cells, confirming the tumor suppressor role of miR-29b within the BMM [25]. We also investigated whether miR-29b, previously proven as involved in bone remodelling and osteoblastic differentiation [104], could have effects on osteoclastogenesis in MM-related BD. Importantly, we observed a reduction of miR-29b levels along *in vitro* human osteoclast generation from $CD14^+$ human monocytes exposed to M-CSF and RANK-L. Overexpression of miR-29b significantly impaired human OCs differentiation and bone resorption activity, by reducing expression of canonical targets C-FOS, MMP2, and also the master transcription factor for OC generation NAFTc-1 [105].

Loss of function p53 mutations are a rare event in early stage MM while they may occur in patients with primary plasma cell leukemia (PPCL) or in MM patients who progress to a leukemic phase (secondary PCL). Therefore, reactivating p53 may provide a therapeutic strategy against MM. Several miRNAs have been identified to regulate p53 expression and activity and/or are induced by p53 [106]. Among p53-induced miRNAs, we found that miR-34a, ectopically expressed by various means in MM cells, induced growth inhibition and apoptosis. By *in vivo* studies, we evaluated the antitumor effect of miR-34a-transduced MM cells engrafted in SCID mice, observing dramatic tumor growth inhibition and prolongation of survival in treated animals. The potential role of miR-34a as new antimyeloma agent was assessed *in vivo* by the SCID-synth-hu model [107].

miRNA profiling of primary MM samples has provided relevant information on miRNA dysregulation in MM [108]. We have demonstrated high miR-125a-5p levels in a subset of MM patients carrying the t(4;14) translocation. In an attempt to evaluate additional mechanisms of miR-125a-5p regulation, we found that adherence to BMSCs upregulated miR-125a-5p levels in MM cells. At the molecular level, miR-125a-5p was found to target the 3′-UTR of p53. Upregulation of p53 by miR-125a-5p inhibitors was paralleled by the activation of a subset of p53-induced miRNAs, like miR-192 and miR-194, and was associated with the inhibition of cell growth, migration, and induction in apoptosis only of MM cell lines carrying a wild-type *p53* gene [30].

miR-21 is an established onco-miRNA in human cancer. Adhesion of MM cells to human BMSCs has been described to trigger upregulation of miR-21 in MM cells, thus strengthening the relevant role of the BMM in the induction of onco-miRNAs. Using synthetic miR-21 oligonucleotide inhibitors, we observed *in vitro* and *in vivo* activity in SCID/NOD mice bearing human MM xenografts. miR-21 inhibitors triggered upregulation of tumor suppressor genes such as PTEN, BTG2, and Rho-B and reduced MM cell proliferation, survival, and clonogenicity in PTEN/AKT-dependent manner [28].

As discussed before, MM-MSCs play a critical role in MM pathophysiology. In a study by Xu and colleagues, primary MSCs derived from MM patients were analyzed for miRNA expression and were found to exhibit a reduced osteogenic potential along with enhanced expression of miR-135b, differently from MSCs from normal donors. In detail, authors noticed that increased expression of miR-135b in MM-MSCs was correlated with a decrease of both alkaline phosphatase activity and SMAD5 expression, a direct miR-135b target gene. Notably, by coculturing normal donors MSCs with MM cells, miR-135b expression significantly increased, suggesting a functional relationship between cancer and MSCs within the BMM [109].

A 3D bone cancer model was used by Reagan et al. in order to investigate MM growth and progression; in detail, this model recapitulates interactions among MM cells, MSCs, and ECs in the BMM, thus providing a physiologically relevant platform to study osteogenesis, BM angiogenesis, and cell survival. Interestingly, miRNA profiling in MSCs, cocultured in such a model with MM cells, revealed a strong downregulation of specific miRNAs (miR-199a, miR-24, miR-15a, and miR-16). Overexpression of miR-199a-5p increased mineralized bone matrix, while osteogenic marker genes, such as *runx2*, *ALP*, *OPN,* and *Col1a1*, were induced by both miR-199a-5p and miR-199a-3p overexpression [110]. Moreover, by pathway enrichment analysis, the authors identified MAPK and Semaphorin signalling pathways

Table 1: miRNAs acting in the context of the BMM.

miRNA	Expression pattern	Function in MM-BMM	Target	Reference
miR-15a/-16	Downregulated in MM cells	Tumor suppressors in MM cells, reduce growth and migration of MM and ECs and secretion of VEGF in MM cells	AKT3	[21, 80, 81, 83]
miR-29b	Downregulated in MM cells	Reduces growth and induces apoptosis in MM cells; regulates osteoclast differentiation	MCL-1, CDK6, C-FOS, MMP2, and NAFTc-1	[22, 106]
miR-30c	Downregulated in MM cells	Tumor suppressor miRNA, inhibits growth and survival of MM cells	BCL9	[96]
miR-34a	Downregulated in MM cells	Induces growth inhibition and apoptosis in MM cells	BCL2, CDK6, and NOTCH1	[25]
miR-125b	Downregulated in MM cells	Tumor suppressor miRNA inhibits growth and survival of MM cells	IRF-4 BLIMP-1	[23]
miR-145	Downregulated in MM cells	Regulates angiogenesis	ANGPTL1	[125]
miR-199a	Downregulated MM cells after hypoxia Downregulated in BM-MSCs	Induces osteogenesis, reduces MM and ECs migration, and increases adhesion to BMSCs	HIF-1α MAPK Semaphorin	[23, 110]
Let-7 family	Downregulated in MM cells	Regulates VEGF level promoting angiogenesis	HIF-3α	[125]
miR-21	Upregulated in BMSCs after MM contact, upregulated in MM cells	Affects RANK-L/OPG ratio in MM-BMSCs cocultures; oncomiR in MM cells, increases growth, survival, and clonogenicity	OPG PTEN	[28, 29]
miR-92a	Upregulated in MM cells	Regulates VEGF level promoting angiogenesis	VEGF	[125]
miR-125a-5p	Upregulated in MM cells	Induces growth and migration and inhibits apoptosis of MM cells	P53	[30]
miR-135b	Upregulated in MM BMSCs	Inhibits osteogenesis	SMAD5	[107]

as miR-199a-5p downstream pathways, highlighting their possible involvement in osteogenesis [111, 112]. A list of the most representative miRNAs whose activity has been studied in the context of the MM BMM is reported in Table 1.

5. Extracellular miRNAs in the BMM

In recent years, it has become evident that stroma-tumor interaction is not simply composed of paracrine signalling of soluble factors and cell-matrix adhesion. In fact, lipid membrane-bound small vesicles are secreted from both cancer and stromal cells and deliver their RNA and protein cargos, whereby they alter gene expression in the recipient cells [113, 114]. Extracellular miRNAs may exist in two main forms, that is, microvesicles- (MVs-) free and MVs-entrapped [115]. The first fraction, merely bound to AGO2 proteins, is the most represented both in blood/serum and cell culture media (90–99%) and displays resistance to nucleases [114–116]. On the other hand, mounting evidence indicates that cells selectively package and actively secrete certain miRNAs into MVs. Three different types of extracellular MVs have been so far described, that is, (1) exosomes (with a diameter ranging from 30 to 100 nm), which originate within the multivesicular bodies (MVBs) and are released upon fusion of MVBs with the plasma membrane; (2) shedding vesicles (with a diameter ranging from 0.1 to 1 μm), which derive from outward sprouting and fission of the plasma membrane; (3) apoptotic bodies (with a diameter ranging from 0.5 to 2 μm), the membranous vesicles shed from cells during programmed cell death. Different from AGO2-bound miRNAs, recent studies showed that MVs-entrapped extracellular miRNAs are indeed transferred to recipient cells where they regulate gene expression by directly binding to target mRNAs [114–116]. Therefore, at least this fraction of extracellular miRNAs can be considered as an active player in cell-to-cell communication, triggering signals from both living (exosomes, shedding vesicles) and dying (apoptotic bodies) cells. Furthermore, recent reports suggest that extracellular miRNAs may work in noncanonical ways. Specifically, both MVs-free and MVs-entrapped miRNAs can bind extracellular or intracellular Toll-like receptor (TLRs) acting as paracrine agonists

and, consequently, triggering the proinflammatory signalling downstream of TLRs [117, 118]. Extracellular miRNAs are also present and detectable outside the tumor microenvironment, for example, within the peripheral blood, and there is increasing evidence that these circulating miRNAs could represent a convenient and useful diagnostic/prognostic tool in human cancer, including MM [13, 116, 119]. Indeed, the availability of such less-invasive approach compared to BM PCs purified from human biopsies has opened a new field of investigation in MM [120]. Kubiczkova et al. identified 5 circulating miRNAs (miR-774, miR-130a, miR-34a, let-7d, and let-7e) differently expressed in serum from patients with MGUS or MM compared with healthy donors (HDs). Importantly, the combination of miR-34a and let-7e was able to discriminate MM from HDs with high sensitivity and specificity [121]. Jones et al. identified miR-720 and miR-1308 as circulating miRNAs able to discriminate between HDs from MGUS or MM patients, whereas the combination of circulating miR-1246/miR-1208 allowed distinguishing MM from MGUS patients [122]. Huang et al. profiled plasma samples from 12 MM patients and 8 HDs and found 6 miRNAs (miR-148a, miR-181a, miR-20a, miR-221, miR-625, and miR-99b) specifically upregulated in the peripheral blood of MM patients; moreover, the expression of miR-148a and miR-20a correlated with patients' clinicopathological features and survival, thus suggesting a prognostic value for these two circulating miRNAs [13, 123]. With a different experimental approach, based on NanoString-nCounter microRNA assay and subsequent stem-loop-RT-PCR validation, Rocci et al. found 2 circulating miRNAs (miR-16 and miR-25) positively associated with better OS in MM patients [120]. However, all these studies were conducted on MVs-free circulating miRNAs, and the expression levels of miRNAs detected in the peripheral blood did not reflect intracellular levels. Two recent reports demonstrated the involvement of exosomal miRNAs in both MM-MSCSs tumor-promoting activity and MM cell-mediated angiogenic switch [42, 124]. In the study by Roccaro et al., authors showed that exosomes released from MM BM-MSCs were actively transferred to MM cells resulting in sustained tumor growth *in vitro* and *in vivo* [42]. The ability of MM BM-MSCs-derived exosomes to modulate *in vivo* MM cell growth and dissemination was investigated by means of subcutaneously implanted tissue-engineered bones (TEBs). In this work, TEBs were loaded with MM cells and either MM or HD BM-MSCs-derived exosomes, while TEBs exclusively loaded with MM cells were used as control. Strikingly, MM and HD BM-MSCs exerted an opposite effect on tumor growth, with the latter negatively affecting the homing and proliferation of MM cells into the BM. These outcomes were associated with a different content in miRNAs, cytokines, and oncogenic protein cargos between MM BM-MSCs and HD BM-MSCs. Notably, the authors attributed to the tumor suppressor miR-15a a relevant role in regulating MM cell growth, since its abundance was much higher in exosomes from HD BM-MSCs compared to exosomes from MM BM-MSCs than [42]. In the study by Umezu et al., new insights on the mechanisms underlying the angiogenic switch in MM BM microenvironment were provided [124]. The authors firstly developed a new cellular model of hypoxia-resistant MM (HR-MM) as working platform and then focused on the potential angiogenic role of HR-MM cell exosomes. They clearly demonstrated that (1) HR-MM cells secreted a bigger amount of exosomes, as compared to isogenic cells; (2) exosomes derived from HR-MM cells induced tube formation in both normoxic and hypoxic HUVECs; (3) miRNA content differed between exosomes released from HR-MM cells and isogenic nonhypoxia resistant cells; (4) enhanced tube formation by HR-MM cell exosomes in HUVECs was mediated by exosomal miR-135b, which strengthened HIF-1α transcriptional activity by directly targeting hypoxia-inducible factor-1α subunit inhibitor FIH-1 [124].

6. Conclusions

Significant advances in understanding the pathogenesis of MM have highlighted the relevance of the BMM in PCs survival and resistance to conventional and novel drugs.

An intricate network composed of a plethora of signalling molecules regulates the cross-talk between MM cells and the surrounding microenvironment, inducing tumor growth by autocrine and paracrine mechanisms. In this context, miRNAs have emerged as contributors to tumor progression by regulating communication between cancer cells and other cellular components of the microenvironment [114]. Notably, several investigations have provided evidence of miRNAs playing a role in BMSC-triggered drug resistance of MM cells [81, 82], although the exact underlying mechanisms remain to be determined. Intriguingly, the findings that cytokines or adhesion to BMSCs may regulate levels of DNA-methyltransferases in MM cells [33, 76] suggest novel BMSC-driven epigenetic mechanisms regulating miRNA expression, which indeed deserve in-depth investigation.

Moreover, BMSCs have been proven to release miRNAs in exosomes [42, 124], which could influence the phenotype of MM or other cells of the BM *milieu* via a paracrine mechanism.

Preclinical studies taking advantage of murine models recapitulating the human BMM [25, 103, 107] suggest that miRNA manipulation in MM cells might activate diverse tumor suppressive pathways which potently inhibit MM survival and overcome the protective BMM, thus representing new tools against MM [13] and MM-related diseases [98]. However, additional research is needed to better disclose the regulatory role of miRNAs in the BMM, thus allowing the design of more effective miRNA-based therapeutic strategies targeting MM cells in the context of their natural microenvironment.

Conflict of Interests

The authors declare no competing financial interests.

Authors' Contribution

Lavinia Raimondi and Angela De Luca contributed equally to this paper.

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Review Article

ZNF423 and ZNF521: EBF1 Antagonists of Potential Relevance in B-Lymphoid Malignancies

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The development of the B-lymphoid cell lineage is tightly controlled by the concerted action of a network of transcriptional and epigenetic regulators. EBF1, a central component of this network, is essential for B-lymphoid specification and commitment as well as for the maintenance of the B-cell identity. Genetic alterations causing loss of function of these B-lymphopoiesis regulators have been implicated in the pathogenesis of B-lymphoid malignancies, with particular regard to B-cell acute lymphoblastic leukaemias (B-ALLs), where their presence is frequently detected. The activity of the B-cell regulatory network may also be disrupted by the aberrant expression of inhibitory molecules. In particular, two multi-zinc finger transcription cofactors named ZNF423 and ZNF521 have been characterised as potent inhibitors of EBF1 and are emerging as potentially relevant contributors to the development of B-cell leukaemias. Here we will briefly review the current knowledge of these factors and discuss the importance of their functional cross talk with EBF1 in the development of B-cell malignancies.

1. Introduction

The specification and development of the diverse blood cell lineages from haematopoietic stem cells have been extensively investigated during the past few decades, leading to substantial advances in our understanding of the regulation of haematopoiesis. In particular, B-lymphopoiesis has been characterised in great detail thanks to the identification of a wealth of molecular and genetic markers that have allowed for the accurate definition of the individual stages of development of the mature B-cell phenotype [1–3]. The B-lymphoid commitment of multipotent haematopoietic progenitors, as well as their progressive lineage restriction, that is, the stepwise acquisition of B-lymphoid features and the parallel loss of alternative developmental potential, is tightly controlled by the concerted action of a complex network of transcriptional and/or epigenetic regulators [2, 4–17]. Among these, early B-cell factor 1 (EBF1) is regarded as a master determinant of the specification, development, and maintenance of the B-lymphoid lineage [18].

EBF1 (also termed Olf-1 or COE1, for Collier/Olf-1/EBF1) is the founding member of a family of four DNA-binding proteins implicated in the control of the cell fate choice in multiple tissues [19–24]. In vertebrates, the EBF1 protein is characterised by an N-terminal atypical zinc finger motif that is referred to as "zinc knuckle" [25], responsible for its DNA-binding activity [26] and required for the transcriptional activation of target genes [27], and by an atypical helix-loop-helix (HLH) domain, containing duplication of the second helix motif, which mediates dimerisation. Between these domains is an IPT (IG-plexin transcription factor) domain, whose function is uncertain. At the carboxyl-terminal end, EBF1 presents a putative transactivation domain that is largely dispensable for its transcriptional activity [27].

The expression of *EBF1* in the haematopoietic system is restricted to the B-lymphoid lineage and is detectable

from the earliest lymphoid progenitors to mature B-cells and is subjected to complex control. Transcription of the *EBF1* gene, controlled by two distinct promoters [28, 29], is initiated in the B-cell biased subset of common lymphoid progenitors by the transcription factors E2A, FOX01, and STAT5 (activated in turn by IL-7R signalling). In later stages of B-cell differentiation, the levels of *EBF1* expression are maintained and further enhanced, by a positive feedback loop that involves EBF1 itself and the product of its target gene, PAX5 [29, 30].

The sustained expression of *EBF1* is essential in all stages of B-lymphopoiesis [31–33]. *Ebf1* gene knockout results in complete lack of B-lymphoid development, accompanied by loss of B-cell-specific gene expression [9]. Conversely, its enforced expression in primitive haematopoietic stem and progenitor cells restricts their differentiation potential to the B-cell lineage [34]. These effects are accomplished both via the transcriptional activation, induced by EBF1 alone or in combination with other factors, of a number of genes crucial for B-cell development (including those encoding EBF1 itself, PAX5, and components of the pre-B-cell receptor such as IGLL1, VPREB, CD79A, and CD79B) and through the repression of genes whose products promote the development of other haematopoietic cell lineages [35]. The latter mechanism is essential not only for lineage restriction, but also for preserving B-lymphoid identity, as indicated by several lines of evidence: conditional knockout of *Ebf1* in committed B-cell progenitors results in their conversion to non-B-lineages [33]; haploinsufficiency of *Ebf1* alone, or of *Ebf1* and *Runx1*, is associated with lineage-promiscuous gene expression in pro- and pre-B-cells [36]; heterozygous deletion of *Ebf1* and *Pax5* induces T-lineage conversion of $CD19^+$ pro-B-cells [37]. In immature B-cells, EBF1 strongly inhibits the expression of *B-limp1*, a transcription factor known to repress the *Pax5* gene [38]. In addition to its role as a transcriptional activator or repressor, EBF1 possesses properties of an epigenetic regulator and has been shown to initiate chromatin remodelling at the promoter of target genes thereby modulating its accessibility to transcriptional effectors [39–42]. Using a combination of CHIP-seq analyses and of gain- and loss-of-function gene profiling studies, Treiber et al. [11] have shown that EBF1 can induce chromatin remodelling in a set of target loci that poise these genes for expression at later stages of differentiation.

In light of its central role in the network of transcriptional and epigenetic regulators that promote the generation and maintenance of the B-lymphoid phenotype, it is not surprising that perturbations of the expression and/or function of EBF1, especially combined with those of other components of this network, are frequently associated with B-cell malignancies [43–46]. In a murine experimental model, ablation of a single allele of either *Ebf1* or *Pax5*, in combination with a constitutively active version of STAT5b, resulted in the development of B-cell acute lymphoblastic leukaemia (B-ALL) with complete penetrance [47]. More recently, *Ebf1* haploinsufficiency resulting from the insertion of a lentiviral vector in its locus was reported to trigger the occurrence of B-ALL [48]. *Ebf1* haploinsufficiency has also been linked to increased susceptibility of pro-B-cells to DNA damage in response to UV light and, though not highly leukaemogenic *per se*, induced pro-B-ALL development with high frequency when accompanied by *Pax5* heterozygosity [49].

The availability of methods that allow genome-wide, high-resolution detection of genetic lesions has led to the discovery of numerous novel genetic alterations that target genes encoding regulators of B-lymphopoiesis in approximately 60% of B-ALLs [34, 50–55]. Among these, mutations resulting in diminished expression and/or impaired activity of EBF1 are not as common as those affecting *PAX5* [51]. Interestingly, however, the frequency of *EBF1* deletions was considerably higher in pediatric high-risk B-precursor ALLs [56] and in relapsed ALLs [57], where copy number alterations were detected in 25% of the cases.

One alternative mechanism through which the activity of EBF1 may be impaired is the inappropriate expression of antagonist factors. Among the known inhibitors of EBF1, two related multi-zinc finger transcription cofactors, zinc finger proteins 423 and 521, have been repeatedly implicated in the development of B-ALL and lymphomas.

2. Zinc Finger Protein 423

ZNF423 (also referred to as Olf-1/Ebf1-associated zinc finger protein, OAZ or EBFAZ, and ZFP423 in mouse) is a nuclear protein containing 30 Krüppel-like zinc finger (ZF) motifs, first identified for its ability to bind to OLF-1/EBF1 and to inhibit its transcriptional activation of olfactory-specific genes [58] and to coordinate the expression of immature and mature stage-specific genes in olfactory-receptor neurons where its enforced expression induces maturation arrest [59]. It was determined that the binding between the two factors is mediated by the interaction of the last three zinc fingers of ZNF423/OAZ with the HLH domain of OLF-1/EBF1 [58], and this prevents the generation of transcriptionally active EBF1 homodimers. ZNF423 was also shown to possess direct DNA-binding activity to inverted GCACCCn repeats, mediated by ZF motifs located in the amino-terminal region of the protein [60]. Subsequent studies showed that, in response to bone morphogenetic protein (BMP) 2, ZNF423 can form complexes with SMAD1 and SMAD4 via its zinc fingers 14–17 and activate the transcription of BMP target genes [61]. However, the ZNF423-SMAD1/4 complex can also induce transcription of the inhibitory factor, SMAD6, thereby triggering a regulatory loop that limits the intensity and/or duration of BMP signalling [62]. Overexpression of EBF1/OLF-1 was found to modulate the activity of the ZNF423-SMAD1/4 complex, possibly by interfering with its formation through its binding to ZNF423 [61]. Additional relevant interactions of ZFP423 include that with the NOTCH1 intracellular domain, resulting in the selective upregulation of *Hes5* expression, which is potentiated by BMPs and antagonised by EBF factors [63]. Binding of ZNF423 with retinoic acid receptors has also been shown to represent an essential molecular partnership [64]. Cho et al. [65] reported the presence of a functional enhancer element containing overlapping EBF1 and ZFP423-binding sites in intron 5 of the *Zfp423* gene, whose activity was enhanced by EBF1 but

strongly suppressed by ZNF423, suggesting the existence of an autoregulatory feedback mechanism.

A wealth of recent experimental evidence has highlighted a central role for ZFP423 in the control of differentiation of adipocyte progenitors [57, 66–68], through the transcriptional activation of *PPARγ* genes whose products are essential preadipogenic factors. In this process, the activity of ZFP423 is enhanced by BMP4, via SMAD1/4-mediated displacement of WISP2, a WNT-induced adipokine that sequesters ZFP423 in the cytoplasm [69]. The proadipogenic effect of EBF1 has been in part ascribed to the stimulation of *Zfp423* expression in mesenchymal progenitors [70].

Finally, ZNF423 has been implicated in CNS midline patterning, vermis formation, and cerebellar development [59, 71, 72], in DNA damage response and ciliogenesis (through its interactions with the poly-ADP ribosyl polymerase 1 [62, 73] and the centrosomal/cilia protein CEP290 [73]), and in the transcriptional regulation of *BRCA1* [74].

3. Zinc Finger Protein 521

ZNF521/ZFP521 is the paralogue of ZNF423/ZFP423, and like ZNF423, it contains 30 Krüppel-like zinc fingers, and at the N-terminal end it harbours a 12-amino acid motif (NBD). This motif is shared with a number of transcriptional corepressors and recruits the nucleosome remodelling and histone deacetylase (NuRD) complex [75–77]. In ZNF521, the NBD is encoded by a short exon, raising the possibility that alternative splicing may generate a variant protein unable to bind the NuRD, whereas the NBD-containing isoform of ZNF423 is generated by the activation of an alternative upstream promoter [78].

Zfp521 was originally identified as a common target gene for retroviral integration associated with the occurrence of B-cell lymphomas in AKXD mice and hence termed ecotropic viral integration site 3 (*Evi3*) [71]. The cDNA encoding human ZNF521 (initially designated early hematopoietic zinc finger protein, EHZF) was cloned for its abundant and selective expression in primitive haematopoietic progenitors [75]. Within the haematopoietic system, *ZNF521* expression is almost completely restricted to stem and early progenitor cells [75, 76, 78–81]. Like ZNF423, this factor has been shown to cooperate with SMAD1/4 in the transcriptional activation of BMP target genes [75] and to strongly inhibit the expression of B-cell-specific EBF1 target genes with a mechanism that is largely independent of the NuRD complex recruitment [75, 82]. Silencing of *ZNF521* in human and murine haematopoietic progenitors considerably enhances the production of B-cells *in vitro* [82]. This suggests that ZNF521 counteracts the activity of EBF1 and other transcription factors that promote differentiation of haematopoietic progenitors such as GATA1 [83] and may contribute to the homeostasis of the immature haematopoietic cell compartment. Recently, using a mathematical model based on relevant literature to define key molecular interactions in the transcriptional network that governs B-lymphopoiesis, Salerno et al. [84] have identified the balance between EBF1 and ZNF521 as one major factor in B-lymphoid specification. According to this model, a shift of this balance toward ZNF521 is predicted to result in dedifferentiation of B-cell progenitors.

In addition to the haematopoietic system, the interplay between ZNF521 and EBF1 appears to be relevant in the determination of cell fate in other systems, including the developing striatum [85] and mesenchymal progenitors. In the latter, ZFP521 inhibits the proadipogenic activity of EBF1 and represses the EBF1-induced expression of *Zfp423*, acting both on the intronic enhancer and at the level of the *Zfp423* promoter, thereby favouring osteoblastic commitment at the expense of adipogenesis [70, 86]. *Zfp521* is in turn repressed by EBF1 [70]. In osteoblasts, ZFP521 stimulates bone formation by antagonising both RUNX2 [87, 88] and EBF1 [89]; in addition, ZFP521-mediated inhibition of EBF1 was reported to modulate both the intrinsic and osteoblast-dependent osteoclastogenesis [89]. Human articular chondrocytes appear to require *ZNF521* for the maintenance of their identity, and *ZNF521* silencing results in a markedly dedifferentiated phenotype when these cells are cultured in alginate beads [90]. Whether EBF1 contributes to this phenomenon remains yet to be determined.

A property of ZNF521 potentially relevant to cancer was discovered by La Rocca et al. [91] who showed that enforced expression of *ZNF521* enhances HLA Class I expression on the tumour cell surface, with particular regard to multiple myeloma cells, thereby preventing their recognition by natural killer cells.

A growing body of evidence has also delineated a prominent role for ZNF521/ZFP521 as a regulator of neurogenesis. Kamiya et al. [92] showed that ZFP521 promotes the spontaneous transition of epiblasts to neuroectodermal progenitors, through the activation of early neural genes in a process that requires the interaction of ZFP521 with the coactivator P300. ZNF521 transcript is abundant in the brain [75], particularly in neural stem cells and cerebellar granule neuron precursors [76], which are considered the cells of origin of a substantial fraction of medulloblastomas, the most common malignant brain tumours in children. Consistently, ZNF521 has been shown to stimulate the growth, clonogenicity, and tumorigenicity of human and murine medulloblastoma stem-like cells [93]. Unlike *Zfp423*, *Zfp521* knockout does not appear to dramatically disrupt cerebellar development but results in behavioural abnormalities and in the reduction in the number of neuronal progenitors in the dentate gyrus and in cerebellum [94]. Finally, a recent report has documented the existence of an incoherent feed-forward loop in which the RUNX1-induced expression of *Zfp521* in a subset of RUNX1-dependent sensory neurons activates gene expression programmes that lead to the development of VGLUT3$^+$ low-threshold c-mechanoreceptors while repressing genes driving the choice of alternative cell fates [95].

4. ZNF423 and ZNF521 in B-Lymphoid Malignancies

As highlighted in the previous section, *Zfp521*/*Evi3* was initially discovered because its dysregulated expression, induced by retroviral insertion, was associated with the development

of pre-B- or B-cell lymphomas in AKXD mice [71, 96]. A subsequent study [97] detected constitutive expression of *Zfp423*/*Ebfaz* (normally not expressed in haematopoietic cells) as a consequence of another frequent viral integration in AKXD-27 B-cell lymphomas. The integration in *Ebfaz* and in *Evi3* was mutually exclusive, suggesting functional redundancy of these two candidate oncogenes. In light of the shared EBF1-inhibitory activity of ZFP423 and ZFP521, it is conceivable that dysregulated expression of these factors might contribute to the development of B-cell malignancies. More recently, Hiratsuka et al. [98] reported that overexpression of *Zfp521* in SL/Kh mice, due to retroviral insertion in its locus, caused the upregulation of pre-BCR-associated signalling molecules, including BANK1, BLNK, and BTK. In the presence of concomitant viral integration targeting other regulatory genes such as *c-Myc*, *Zfp521* overexpression may eventually give rise to pre-B-cell lymphomas in these mice. It must be taken into account that the genetic background of AKXD-27 and SL/Kh mice, both prone to lymphoma development, may be relevant in determining the phenotypes observed in these studies.

Hiratsuka et al. [98] also detected expression of ZNF521 protein in human B-cell lymphoblastic lymphomas. It is puzzling, however, that the localisation of ZNF521 in these cells appeared to be predominantly cytoplasmic, raising the issue of potential staining artifacts. It will be interesting, in future studies, to assess whether aberrant expression of *ZNF521* in human lymphoma cells can be confirmed by gene profiling, *in situ* hybridisation, or mass-spectrometry-based proteomic analyses.

Hentges et al. [99] observed that upregulated expression of *Evi3* in aged female AKXD-27 mice was associated with the occurrence of B-lymphoid neoplasias resembling pro-B-cell leukaemias. In addition to overexpressing *Zfp521*/*Evi3*, the malignant cells displayed marked upregulation of *Ebf1* and of its target genes. Based on these data, it was postulated that ZFP521 may antagonise, or synergise with, EBF1 in a cell-type-specific manner [99]. This hypothesis was not confirmed by our subsequent investigation conducted in B-cells, where ZNF521 effectively repressed the expression of EBF1 target genes [82], and remains to be validated. However, a link between aberrant expression of *Zfp521* or of *Zfp423* and development of B-cell precursor leukaemias is supported by diverse experimental *in vivo* models of leukaemogenesis based on mice engineered to generate mutation backgrounds that mimic those associated with B-ALLs (reviewed in [100]). In an attempt to identify factors that cooperated with BCR-ABL to induce the progression of chronic myeloid leukaemia, Miyazaki et al. [101] used transgenic *BCR-ABL P210* mice crossed with BXH2 mice, which transmit a replication-competent retrovirus. They found that constitutive expression of *Zfp423*, resulting from viral integration in its 5′ noncoding region, led to the development of a B-lineage blast crisis with early onset. This was further supported by the detection of high expression of ZNF423 in cells from CML patients with B-lymphoid blast crisis, but not those in chronic phase [101]. van der Weyden et al. [102] generated a B-ALL mouse model in which the expression of the *ETV6-RUNX1* fusion gene (derived from the t(12;21)(p13;q22) translocation, the most common chromosomal rearrangement in B-ALLs) was combined to *Pax5* haploinsufficiency. Transposon-mediated insertional mutagenesis was then performed to identify cooperating B-ALL driver genes and led to the identification of five transposon common insertion sites, including one in the *Zfp423* gene, which was associated with a significant increase in the occurrence of B-cell precursor ALLs in these mice [102]. In a similar approach, Yamasaki et al. [103] sought to identify cooperating drivers for the *E2A-HLF* fusion gene generated by the t(7;19) translocation, whose rare occurrence characterises ALLs with extremely poor prognosis, by retroviral-mediated insertional mutagenesis in an *E2A-HLF* knock-in mouse. One of the three common integration sites identified in this study and associated with B-ALL development lay in the *Zfp521* locus. To confirm these findings, the authors generated transgenic mice with enforced expression of *Zfp521* in lymphoid cells, crossed them with *E2A-HLF* knock-in animals, and detected B-ALLs in 50% of the offspring but not in the parental mice [103].

Thus, several lines of experimental evidence suggest that ZFP423 and ZFP521 may cooperate with oncogenic lesions and contribute to B-ALL development, presumably through the inhibition of EBF1 and the consequent disruption of the functional network that governs normal B-cell differentiation. This notion is also supported by the results of some studies of human B-ALLs. As mentioned above, Miyazaki et al. [101] detected abundant levels of *ZNF423* transcript in patients with CML blast crisis, but not in those in chronic phase; more recently, a gene profiling analysis of human B-ALLs detected aberrant expression of *ZNF423* in most of the cases studied and established a significant correlation between high expression levels and adverse outcome in ETV6-RUNX1-negative B-ALLs [78]. The analysis of publicly available datasets, conducted and visualised using Oncomine (Compendia Bioscience, Ann Arbor, MI), confirmed that abundant *ZNF423* expression is typically found in B- and, to a lesser extent, in T-ALLs. However, this does not appear to be the case for *ZNF521*, whose expression is relatively high in a significant fraction of AMLs and T-ALLs, but (apart from rare instances, such as dic(9;18)(p13;q11) translocation in which its gene is fused with that encoding PAX5 and the expression of the resulting chimeric gene is driven by the B-lymphoid *PAX5* promoter [51]) is distinctly low or undetectable in virtually all B-ALLs (Figure 1; [76, 78] Mesuraca, in preparation).

This is consistent with the data of Aibar et al. [105], who designed and used an R package named geNetClassifier to discover subsets of genes that unequivocally differentiate and classify different leukaemia subtypes (cALL/pre-B-ALL, AML, CLL, and CML). In this study, *ZNF423* was second top ranking in a cohort of 799 genes whose expression is characterised as ALL specific, whereas *ZNF521* ranked sixth among 213 AML-specific genes.

How can the apparent lack of *ZNF521* expression in ALLs be reconciled with its proposed role as a driver in these leukaemias? One possible clue is offered by a recent report by Aoki et al. [106]. These authors investigated the leukaemia-initiating cells (LICs), a rare subpopulation of leukaemic cells endowed with stem-like features, capable of initiating leukaemia if transplanted into immunocompromised

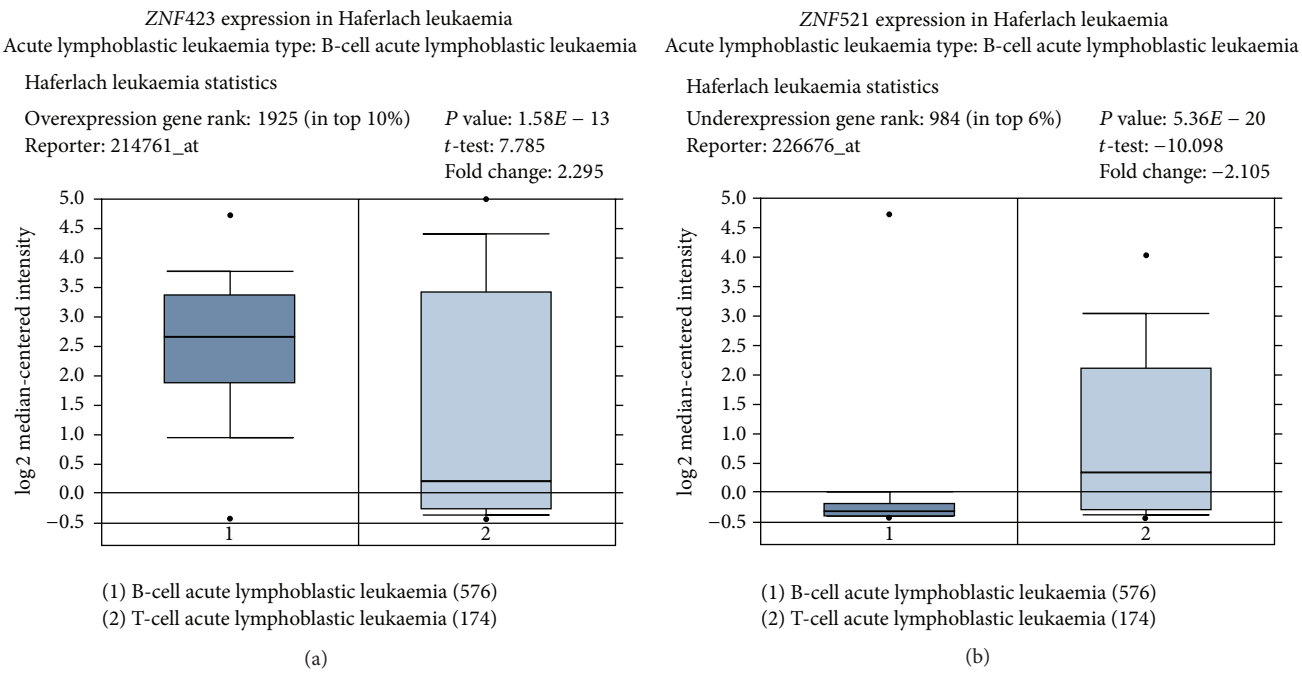

Figure 1: Expression of *ZNF423* and *ZNF521* in B- and T-ALLs. The Oncomine database was queried for the expression of *ZNF423* and *ZNF521* in DNA microarray studies of acute lymphoblastic leukaemias. The data shown are from [104] and document the overexpression of *ZNF423* and the underexpression of *ZNF521* in B-ALLs, whereas both genes display detectable expression in the T-ALLs studied.

animals, in B-ALLs bearing different rearrangements of the *MLL* gene. In particular, they determined that the LIC fraction of ALLs carrying the t(9;11) translocation, which generates the *MLL-AF9* fusion oncogene, was contained in the $CD34^{-}/CD19^{+}$ cell subset. A gene profiling analysis revealed that *ZNF521* was one of the genes whose expression was selectively enriched in these cells. Thus, aberrant expression of *ZNF521*, occurring in the LIC subset but not necessarily present in the bulk of leukaemic cells, may contribute to the development of some B-ALLs while remaining undetectable when the transcriptome of the whole leukaemic cell population is analysed.

ZNF521 is among the top 25 genes overexpressed in AMLs with *MLL* fusion genes [107], in particular those expressing *MLL-AF9* ([76]; Mesuraca in preparation), and is recognised as one of the prominent downstream targets of MLL-AF9 in AML cells [108]. Its expression may be activated by the AF9 moiety of the fusion protein via an epigenetic mechanism that involves the recruitment of 5-methylcytosine dioxygenase TET2, as it has been observed during the induction of neural differentiation of human ES cells [109]. MLL-AF9-transformed haematopoietic stem cells can give rise to myeloid or lymphoid leukaemias based on their intrinsic developmental potential and on signals provided by the microenvironment [110–112]. Intriguingly, overexpression of *ZNF521* was detected in $CD34^{+}$ cells transformed *in vitro* by MLL-AF9 and cultured in both myeloid and lymphoid conditions [112]. It could thus be hypothesized that if the $MLL\text{-}AF9^{+}$ LICs follow the B-ALL pathway, the expression of *ZNF521* is progressively attenuated by B-lymphoid regulatory factors that are known to repress its transcription including IKAROS [113], EBF1 [70], and possibly PAX5 [84]. Conversely, in $MLL\text{-}AF9^{+}$ AMLs, the sustained expression of *ZNF521* is ensured by the fusion oncoprotein in the presence of a permissive molecular context. Whether the presence of ZNF521 in ALL leukaemia-initiating cells is limited to those expressing MLL-AF9 or is a more general feature remains to be established.

A different scenario applies to ZNF423, whose expression is normally absent in the haematopoietic system. In their study, Harder et al. [78] determined that inappropriate expression of *ZNF423* was driven by the removal of epigenetic barriers, namely, demethylation of regulatory elements that normally prevent its expression in the haematopoietic system, combined with the transcriptional induction mediated by BMP2 whose expression is also upregulated in B-ALLs. Alternatively, aberrant expression of *ZNF423* in LICs may result from copy number gain, secondary to genomic instability caused by ROS-induced oxidative DNA damage, as observed by Bolton-Gillespie et al. [114] in a murine model of imatinib-refractory CML. However it is initiated, the sustained expression of *ZNF423* may then be maintained also by the positive transcriptional effect of EBF1 [65, 70].

5. Conclusions and Perspectives

Taken together, the evidence reviewed above indicates that aberrant expression of *ZNF423* and *ZNF521*, triggered by diverse mechanisms, may contribute to the pathogenesis of B-lymphoid malignancies by perturbing the activity of EBF1, a central component of the regulatory network that governs normal B-lymphopoiesis. Our knowledge of the biological

properties of these two factors is still incomplete and several questions remain, such as whether the repression of EBF1 target genes is the only mechanism responsible for their proleukaemogenic effect, the extent to which their expression contributes to the transformation of B-cell progenitors, and the role of epigenetic modifiers (e.g., the NuRD complex) that both proteins are able to recruit through their N-terminal domain, as well as other molecular partners of ZNF423 and ZNF521. Future studies addressing these issues will further our understanding of the biological and clinical relevance of ZNF423 and ZNF521 in the pathogenesis of B-ALLs and of their potential value as candidate molecular targets for therapeutic intervention.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Deep Response in Multiple Myeloma: A Critical Review

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Novel and more effective treatment strategies against multiple myeloma (MM) have significantly prolonged patients' survival and raised interest in the depth of response and its association with clinical outcome. Minimal residual disease (MRD) has emerged as one of the most relevant prognostic factors in MM and should be included in a new definition of complete response (CR). Although further standardization is still required, MRD monitoring should be applied in prospective clinical trials as a sensitive tool to compare and evaluate the efficacy of different treatment strategies, particularly in the consolidation and maintenance settings, and implement individualized therapy-monitoring approaches. Here, we review current definition of deep response in MM, advantages and limitations of current MRD assessment assays, clinical evidences for MRD monitoring as a prognostic tool for therapeutic decisions in MM, and challenges to develop uniform criteria for MRD monitoring.

1. Introduction

Multiple myeloma is a complex disease characterized by the presence of profound intratumoral heterogeneity that increases progressively from the stages of monoclonal gammopathy of undetermined significance (MGUS) and asymptomatic MM to symptomatic or clinical MM [1–3]. The introduction of novel therapies for the treatment of multiple myeloma (MM) patients has significantly improved clinical outcome [4]; however, majority of the patients relapse, making myeloma still an incurable disease [5, 6]. The challenge now is to identify the population of patients with aggressive disease and therefore poor prognosis [7, 8]. Although the ideal way to classify patients with different prognosis is at diagnosis, usually it is extremely difficult, and therefore response monitoring is becoming more relevant in MM.

Complete response (CR), defined by negative immunofixation (IFX) and less than 5% bone marrow plasma cells, has been accepted as a relevant surrogate marker of survival [9]. This definition of clinical response criteria and clinical end points has largely remained the same over the past 15 years [8, 10–12] and presents several relevant limitations [8, 13]. The challenge is to identify the patients that despite reaching CR status relapse very quickly (unsustained response) compared to other patients that only achieve partial response but have prolonged survival.

As CR rates have improved, more rigorous definitions of response have been developed. In the last consensus criteria of response in MM, three new concepts have been incorporated: stringent CR (sCR), immunophenotypic CR (iCR), and molecular CR (mCR) (Table 1). These deep response criteria are all based on different methodologies and provide discordant results [14–25] making the scenario very confusing. Importantly, published data show that establishing some levels of deep response in MM could translate in different prognosis impact: patients achieving grade CR^3 (0,1% deep complete response) had a projected progression-free survival of 35–45 months, while patients achieving CR^5 (0.001% deep complete response grade) had a projected progression-free survival of more than 80 months [26, 27] (Table 2). These levels of disease reduction have prognosis impact, independently of the techniques employed.

A growing body of evidence demonstrates that detection of subclinical levels of myeloma (i.e., minimal residual disease, MRD) provides powerful independent prognostic information [23], and categories defining deep response should be

Table 1: Definition of response according to the last classification of the IMF.

Categories of response according to IMF 2011	Level of detection
PR	MC < 50%
VGPR	MC < 90%
nCR	MC 0.1–0.5 g/dl (EF−/IF+)
CR	MC ≤ 0,5 g/dl (IF−)
	PC in BM < 5%
Stringent CR	sFLC ratio +BM ICH
Immunophenotypic CR	sCR+ nonaberrant PC in 1,000,000 cells
Molecular CR	CR+ nonclonal plasma cells with sensitivity > 10^{-5}

updated according to the levels of MRD. Chronic myeloid leukemia (CML) is the first disease in which this approach was applied to normalize the criteria for a deep response [28, 29]. Consequently, there is an increasing interest in the use of MRD detection to provide early end points in clinical trials and to inform myeloma patient management.

Therefore, a new definition of CR including different levels of MRD is needed in MM to compare different treatment strategies and develop a truly personalized approach to MM therapy. Likewise, this definition will be applied in all clinical settings and will be interchangeable between different centers.

2. Methodologies for Assessing Minimal Residual Disease in Myeloma

Improving CR rates have made the measurement and monitoring of MRD in MM a relevant task. However, implementation of MRD assessment into clinical practice is a major challenge, hampered by differences in the assays and analytical methods employed between different routine laboratories. Most patients who achieve MRD-negative status eventually relapse, indicating that the sensitivity and specificity of traditional techniques for MRD assessment can be improved. Recent data by Rawstron et al. [27] suggests that a lower cutoff provided by more sensitive assays (e.g., next generation sequencing (NGS) or high-sensitive multiparameter flow cytometry (MFC)) will likely improve outcome prediction further. This has already been confirmed by Martinez-Lopez et al. using NGS [26] who identified 3 groups of patients with different time to progression (TTP): patients with high (<10^{-3}), intermediate (10^{-3} to 10^{-5}), and low (>10^{-5}) MRD levels showed significantly different TTP (27, 48, and 80 months, resp.). Accordingly, 10^{-5} should currently be considered as the target cutoff level for definition of MRD negativity.

In this section, we will consider the various methodologies available at present for MRD detection, taking account of their relative advantages and limitations.

2.1. Serologic Methods, to Determine the Tumor Production. Measurements of monoclonal protein biomarkers, which can be in the form of intact immunoglobulin, immunoglobulin fragments, or free immunoglobulin light chains (FLC), in either the serum or the urine are all widely available and noninvasive methods used for diagnosis and monitoring of disease burden in MM. One of the earliest identified biomarkers is Bence Jones protein, described in 1848 [30].

During the past decade the measurement of serum kappa and lambda free light chains (sFLCs) has also become part of routine clinical testing, particularly for the diagnosis and follow-up of patients with nonsecretory and oligosecretory myeloma, light chain myeloma, and amyloidosis [31].

The International Myeloma Working Group (IMWG) in 2006 has introduced normalization of sFLCs and absence of clonal PCs in BM biopsies by immunohistochemistry and/or immunofluorescence as additional requirements to define more stringent CR criteria [10]. sFLC ratio has been shown at diagnosis to be an independent prognostic factor and predict more aggressive disease [32] with potential to improve risk stratification as well [33]. However, several other studies show contradictory results [31, 34, 35], even with regard to response [36, 37], and it remains controversial how to incorporate sFLCs measurement into MRD monitoring in MM. These studies found that normalization of sFLCs was not associated with increased survival in patients in conventional CR. In addition, it has been suggested that the sFLC might be replaced by the heavy-light format and become merely a surrogate for recovery of the immune system rather than MRD monitoring tool. Therefore, in our opinion, sFLCs should not be considered as a method for MRD assessment in myeloma.

The US Food and Drug Administration- (FDA-) approved heavy/light chain (HLC) assay (Hevylite) measures suppression of the uninvolved HLC pair (e.g., IgG-lambda, IgA-kappa, and IgA-lambda for a patient with IgG-kappa disease). The HLC ratio reflects the balance between monoclonal and polyclonal immunoglobulins of involved and uninvolved isotypes taking into account the polyclonal plasma cell suppression or expansion that occurs with the treatment. However, only few studies have shown the ability of the Hevylite assay to give additional prognostic information in MGUS and MM [38]. In general, the assay does not add any value to immunofixation or sFLC tests, although it could have some advantages in monitoring patients with M component migrating in β regions [39].

2.2. Bone Marrow (BM) Methodologies to Determine the Tumor Burden. Morphologic bone marrow examination is one of the most commonly used methods to measure tumor burden in MM. Two different studies demonstrate that microscopic assessment of the BM can have prognostic value [40, 41]. However, the sensitivity of morphology alone is limited by the number of cells evaluated as well as sampling variability. Moreover, BM biopsies are expensive and invasive, posing some risk to patients.

Multiparameter flow cytometry (MFC) and Ig allele-specific oligonucleotide-based quantitative polymerase chain reaction (ASO-PCR) have emerged as the most attractive, well-suited, and sensitive approaches to detect MRD in the BM of MM patients during and after therapy [35]. Molecular

Table 2: Proposed new definition for deep response in multiple myeloma.

New proposal for deep response	Level of detection	Project PFS
Deep CR grade 3 CR[3]	Nonclonal plasma cells below 10^{-3}, highly sensitive techniques FCM or sequencing should be employed	>35–45 months
Deep CR grade 5 CR[5]	Nonclonal plasma cells below 10^{-5}, highly sensitive techniques FCM or sequencing should be employed	>80 months

PR, partial response; MC, monoclonal component; PC, plasma cell; CR complete response; SFLC, serum free light chain; BM, bone marrow.

monitoring of disease by MFC and PCR has been commonly used in chronic myelogenous leukemia, acute lymphoblastic leukemia, and acute promyelocytic leukemia to help determine prognosis and guide therapy [42–44].

In myeloma, several reports have demonstrated the ability of both MFC and quantitative PCR to stratify patient cohorts with different prognoses [21, 25, 45]. However, both techniques have some disadvantages and neither has become a standard of care in MM. ASO-PCR in MM is associated with high technical complexity and low applicability [46]. MFC has a higher applicability, virtually covering all patients without requiring patient-specific diagnostic phenotypic profiles [21, 22, 25, 47]. In recent years, the sensitivity of MFC has increased (between 10^{-4} and 10^{-5}) because of simultaneous assessment of ≥8 markers in a single tube that can readily identify aberrant PC phenotypes at MRD levels if sufficient cell numbers (e.g., $\geq 5 \times 10^6$) are evaluated [47]. The requirement for extensive expertise in MFC analysis and the lack of a well-standardized flow-MRD method are important disadvantages of MFC immunophenotyping. Additionally, no tumor cells are detectable by MFC or PCR in a fraction of patients who ultimately relapse, indicating that further improvement and standardization efforts are required. Recent reports have demonstrated the utility of high-throughput sequencing- (HTS-) based MRD assessment in lymphoid malignancies [26, 48]. This quantitative method, termed the LymphoSIGHT platform, relies on consensus primers to universally amplify and sequence all rearranged immunoglobulin gene segments present in a myeloma clone. Preliminary studies have shown that NGS of Ig genes might be applicable for MRD detection in BM of MM patients [8]. The sequencing method demonstrated applicability higher than 90% and assay sensitivity $\leq 10^{-6}$, with the potential to be distributed across multiple laboratories, because it relies on automated data analysis and does not involve expert interpretation by an operator. Moreover, molecular techniques are not influenced by genetic heterogeneity and clonal tiding throughout patients' treatment. However, additional validations are needed to prove and confirm the utility of this technology for patient risk stratification.

2.3. Imaging Techniques. Unlike other hematologic disorders such as acute leukemia, the pattern of BM infiltration in MM is not uniform. Moreover, hemodiluted BM aspirates may lead to false-negative results. These aspects, together with extramedullary disease, represent a potential challenge and pitfall common to all techniques that use BM samples for MRD assessment, as nonrepresentative samples of disease infiltration are sometimes obtained. For this reason, MRD-negative results may correspond to a false-negative case. The use of alternative methods for disease assessment such as imaging techniques [24, 49], monitoring of clonogenic MM progenitors [49, 50], or MM circulating tumor cells [51] could provide complementary information to MRD and improve the estimation of the risk of progression.

Multiple myeloma presents a high frequency of extramedullary relapses, and sensitive imaging techniques have become relevant in assessing low levels of disease outside BM. Magnetic resonance imaging (MRI) is the most sensitive noninvasive imaging technique for detection of bone involvement in the spine and also provides relevant information on the extent and nature of soft tissue disease and the pattern of marrow infiltration (normal, focal, heterogeneous, or diffuse). However, due to treatment-induced necrosis and inflammation, focal lesions may remain hyperintense in both responding and nonresponding patients for several months after therapy, making MRI-based CR inconsistent. While MRI does not properly identify myeloma active lesions after treatment, imaging by fluorodeoxyglucose-positron emission tomography (PET) has been shown to have prognostic significance [24, 52] and would represent the most effective imaging tool to monitor MRD in MM. A specific advantage of PET imaging relies on its ability to detect extramedullary disease, which represents an adverse prognostic event. PET imaging is widely available; however it has some major issues: not all MM patients have PET-avid lesions and interpretation of data can be a challenge considering heterogeneity of visual criteria and poor interobserver reproducibility. Therefore, standardization of response definitions by PET as well as comparison with other sensitive BM-based MRD methods is needed to implement this imaging technique across different clinical studies.

3. Standardization and Harmonization

MRD monitoring variability between different clinical laboratories is a major challenge. Because of the prognostic value of MRD in MM, a key goal of the standardization effort is to eliminate or correct the relative differences between MRD negativity assessment and response rates across laboratories.

Optimal use of clinical guidelines for disease diagnosis and patient management requires first standardization and then harmonization, to maximize compatibility, interoperability, safety, repeatability, and quality as well as achieve uniformity of results [53, 54]. Results that are neither standardized nor harmonized may lead to erroneous clinical,

financial, regulatory, or technical decisions. While some initiatives on standardization have been performed in chronic myeloid leukemia on molecular MRD [29], there is a lack of standardization and harmonization for MRD assessment by flow cytometry [47]. Different MFC groups need to adopt standardized and validated antibody panels, sample processing, and cell-analysis methods such as those developed by the EuroFlow consortium for MFC, in order to become a universal and fully standardized option for MRD assessment. Standardization of flow cytometric and molecular MRD testing is vital to ensure better and uniform assessment of response and clinical prognostication.

We here propose a roadmap for standardization of MRD assessment in MM:

(1) Development of reference standard and referral material to define CR grades 3 and 5.

(2) Manufacturing internal calibrators.

(3) Evaluation of current degree of measurement equivalence.

MRD assessment in MM based on test results from a specific clinical laboratory measurement procedure (CLMP) without considering the possibility or likelihood of differences between various CLMPs should be flawed. When this happens, aggregation of data from different research clinical trials and development of appropriate clinical practice guidelines will be flawed by the lack of standardized or harmonized results.

Previous experiences of standardization and harmonization of molecular techniques have been difficult and not very well accepted for many laboratories; furthermore the implementation of standardization and harmonization has been hard and long. However, when application in real clinical practice has started the results have been positive. In MM, standardization and harmonization of MRD assessment techniques should be considered, especially if these techniques will be considered biomarkers of response in clinical trials and in the regular clinical practice.

4. MRD Assessment in Myeloma: Clinical Applications

Intra- and interpatient heterogeneity in multiple myeloma underscore the need for personalized treatment approaches. In an era of increased treatment options, there are substantial data showing the association of depth of response and clinical outcome. Achievement of CR is considered one of the strongest prognostic biomarkers in MM, both in the transplant and nontransplant settings, although the sCR criteria have failed to unequivocally demonstrate superior prognostic value compared with CR [37].

4.1. MRD Adapted Treatments. MRD assessment is a relevant concept in myeloma and several studies using different MRD techniques have shown its value for evaluation of the efficacy of specific treatment stages and, therefore, potential treatment decisions. Overall, persistence of MRD is always an adverse prognostic feature, even among CR patients, but, so far, no clinical trial has randomized MM patients according to their MRD status and, thereby, investigated the role of MRD for individualized therapy. Achievement of MRD negativity may ultimately serve as a primary end point in clinical trials for MM and should be included in the CR criteria. However, patients achieving MRD negativity eventually relapse, and at this point we still do not know if these patients should receive the full-programmed treatment besides reaching the MRD negativity status or asymptomatic relapse. Importantly, considering the patchy pattern of BM infiltration observed in MM that leads to a degree of ambiguity regarding MRD-negative results, it would be safer to make clinical decisions based on MRD positivity rather than on MRD negativity. Several other questions on how to incorporate MRD evaluation in the treatment strategy for MM patients remain to be answered. Can we decide which patients should receive consolidation therapy based on MRD measurement? Can MRD monitoring be used to determine the need for or duration of maintenance therapy? How does maintenance therapies modify MRD levels?

4.2. Timing of MRD Evaluation. An important aspect to be considered is the time of MRD monitoring during the course of the treatment. Most studies have been carried out after transplantation in younger patients and after induction in elderly patients, but it is still unclear when the best time is to measure and integrate MRD measurement into therapeutic decision-making.

4.3. MRD Kinetics. Changing of MRD levels over time (MRD kinetics) could be relevant for a better evaluation of MM patients and needs further evaluation. For example, both the Spanish and the United Kingdom study groups have shown that MRD kinetics before and after HDT/ASCT allow the identification of chemosensitive (MRD-negative cases at 2 time points), intermediate, and chemoresistant patients (MRD-positive patients at 2 time points). A small clinical study of the Italian group showed three patterns of kinetics: high tumor burden, low tumor burden, and active disease, which could predict the relapse [55]. These clinical studies suggest that MRD kinetics are more informative than single time point assessments and may be useful to address specific clinical questions (e.g., early versus delayed HDT/ASCT for CR patients after induction). Therefore, additional studies should be performed in this regard to avoid overtreatment and undertreatment, particularly during consolidation and maintenance.

4.4. MRD Detection Methods. Finally, sensitive methods of MRD detection (1 in 10^5 cells) may contribute to the design of patient-specific treatment approaches. Extensive research is still warranted to determine how to best integrate medullary and extramedullary MRD monitoring, and a process of standardization and harmonization of these methodologies is required. Harmonized MRD approaches not only will provide backwards compatibility with established assays but will also offer sufficiently high enough sensitivity as treatment strategies evolve to remain relevant for the next decade.

In summary, (i) MRD assessment is ready for clinical application as biomarker to evaluate the response to different therapies in MM; (ii) MRD could be used to measure the efficacy of different treatments in clinical trials; (iii) MFC and NGS are both equally valid for MRD assessment in MM; (iv) standardization and harmonization are the next steps for MRD assessment in MM.

5. Conclusions

Improvement in MM patient outcomes can be achieved through adaptive clinical trials involving risk models based on multiple biomarkers, but several questions are still unanswered. Different clinical trials integrating these approaches to confirm the clinical benefits of MRD monitoring are currently ongoing in myeloma and will hopefully provide the rationale for the use of MRD assessment in the evolving MM clinical paradigm. Moreover, the new generation of biomarkers, including epigenetics, novel imaging, clone burden, GEP signature, and next generation sequencing, coupled with established prognostic biomarkers holds promise for improved stratification of patients with myeloma into specific therapies and clinical trials.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Mechanisms of Drug Resistance in Relapse and Refractory Multiple Myeloma

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Multiple myeloma (MM) is a hematological malignancy that remains incurable because most patients eventually relapse or become refractory to current treatments. Although the treatments have improved, the major problem in MM is resistance to therapy. Clonal evolution of MM cells and bone marrow microenvironment changes contribute to drug resistance. Some mechanisms affect both MM cells and microenvironment, including the up- and downregulation of microRNAs and programmed death factor 1 (PD-1)/PD-L1 interaction. Here, we review the pathogenesis of MM cells and bone marrow microenvironment and highlight possible drug resistance mechanisms. We also review a potential molecular targeting treatment and immunotherapy for patients with refractory or relapse MM.

1. Introduction

Multiple myeloma (MM) is a clonal B-cell malignancy that is characterized by the proliferation of a plasma cell clone that produces a monoclonal immunoglobulin. MM leads to end-organ damage diseases such as anemia, hypocalcemia, renal insufficiency, or osteolytic bone lesions [1]. The incidence of MM is around 15,000 per year in the US and Europe, and the median survival is about 4-5 years [2]. In addition to the International and Durie-Salmon staging systems [1], biological markers, including cytogenetic abnormalities such as presence of hypodiploidy, t(4;14), t(14;16), del(17p), and del(13), serum β2-microglobulin levels greater than 2.5 mg/L, an elevated plasma cell labeling index, and detection of circulating plasma cells, are predictors of poor prognosis in newly diagnosed MM patients [1, 3–10]. Over the past decade, new therapeutic strategies for MM have been developed on the basis of a deeper understanding of the biology of myeloma cells and their interaction with the bone marrow (BM) microenvironment. These therapies include novel proteasome inhibitor agents such as bortezomib [11, 12] and immunomodulatory drugs such as thalidomide [13, 14] and lenalidomide [15, 16]. Implementation of these therapies has led to increased longevity in MM patients, with median survival of over 5 years [17]. However, many patients still relapse or become refractory to treatment [18]; therefore, MM still is an incurable disease, and understanding the disease mechanism is important, specifically for the development of effective treatments.

2. Myeloma Tumor Cells and the BM Microenvironment

Plasma cells are derived from hematopoietic cells via Ig VDJ rearrangement, somatic mutation, and Ig class switching [19].

Myeloma cells are postgerminal, long-lived plasma cells with mutated homogeneous clonal sequences [19, 20]. MM cells express CD38 and CD138 antigens on the cell surface but lack CD45 and surface Ig expression [19]. Chromosomal alterations have been detected by conventional karyotyping, interphase fluorescence in situ hybridization (FISH) [21, 22], and spectral karyotyping analysis [23] in 30%–50% of MM patients. The results of these analyses have suggested two different pathways of pathogenesis: (1) nonhyperdiploid tumors with a very high incidence of IgH translocations involving five well-defined recurrent chromosomal translocation areas (11q13 [cyclin D1], 6p21 [cyclin D3], 4p16 [fibroblast growth factor receptor 3, FGFR3], multiple myeloma SET domain [MMSET], 16q23 [c-maf], and 20q11 [mafB]) [24] and relatively high incidence of chromosome 13/13q14 loss and (2) hyperdiploid tumors associated with multiple trisomies involving chromosomes 3, 5, 7, 9, 11, 15, 19, and 21, but low incidence of both chromosome 13/13q14 loss and IgH translocation [25]. These chromosomal alterations lead to dysregulation of cyclin D and selective expansion during interaction with BM stromal cells (BMSCs), which produce interleukin-6 (IL-6) and other cytokines [25].

The BM microenvironment is important for MM pathogenesis. The very-late antigen-4 (VLA-4) on MM cells binds to fibronectin in the serum, and the lymphocyte function associated antigen-1 (LFA-1) on MM cells binds to intercellular adhesion molecule-1 (ICAM1) on BMSCs [26], causing MM cells to home in to the BM. Other cytokines such as tumor-necrosis factor-α (TNF-α) in the BM can modulate the adhesion of MM cells in the BM by inducing nuclear factor- (NF-) κB. NF-κB-dependent upregulation of cell surface adhesion molecules such as ICAM1 and vascular cell-adhesion molecule-1 (VCAM1), on both MM cells and BMSCs, increases the binding capacity of tumor cells and BMSCs and induces the transcription and secretion of cytokines such as IL-6 and VEGF in BMSCs [27]. Cytokines in the BM microenvironment, such as IL-6, insulin-like growth factor-1 (IGF-1), vascular endothelial growth factor (VEGF), and TNF-α, mediate the growth of MM cells. However, IL-6, IGF-1, and IL-21 are associated with tumor cell survival and resistance to apoptosis [28–36]. This association is mediated through the Janus kinase (JAK)/signal transducer and activator of transcription 3 (STAT3) and phosphatidylinositol 3-kinase (PI3K)/AKT pathways. The proliferation of MM cells is triggered by cytokines such as IL-6, IGF-1, VEGF, TNF-α, stromal cell derived factor-1α (SDF-1α), and IL-21 and is mediated through the RAF/mitogen-activated protein kinase kinase (MEK)/p42/p44/mitogen-activated protein kinase (MAPK) signaling cascade [27, 30, 32, 36–39]. VEGF and SDF-1α play important roles in cell migration, and the migration of MM cells is mediated through a protein kinase C- (PKC-) dependent, p42/p44/MAPK-dependent pathway [37, 40, 41].

Immune compromise is a major complication in MM patients. Programmed death receptor-1 (PD-1, CD279) is a receptor of the Ig superfamily that negatively regulates T cell antigen receptor signaling by interacting with specific ligands (PD-L1). PD-1 is suggested to play a role in the maintenance of self-tolerance. PD-1 is induced on activated T cells and is expressed on exhausted T cells [42]. Engagement of PD-1 by its ligands, PD-L1 (B7-H1, CD274) or PD-L2 (B7DC, CD273), results in the activation of phosphatases that deactivate signals emanating from the T-cell receptor [43]. Moreover, PD-1 engagement upregulates the expression of basic leucine ATF-like transcription factor (BATF), which in turn impairs T-cell proliferation and cytokine secretion [44]. PD-L1 plays a crucial role in the evasion of the host immune system by tumor cells [45]. PD-L1 is more ubiquitous than PD-L2, and MM cells express elevated levels of PD-L1 [46]. T cells from myeloma-bearing mice and MM patients express higher levels of PD-1. These PD-1-positive T cells were found to be exhausted and produced IL-10 [47, 48]. Stimulation by interferon-γ (IFN-γ) and Toll-like receptor (TLR) ligands upregulated PD-L1 expression in MM cells from MM patients via the MyD88/TRAF6, MEK, and STAT1 pathway [46].

MicroRNAs (miRNAs) play crucial roles in cancer progression [49], and many miRNAs are deregulated in multiple myeloma. Al Masri et al. reported that the expression levels of *miR-125b*, *miR-133a*, *miR-1*, and *miR-124a* vary in multiple myeloma [50]. Among the 464 miRNAs analyzed, 95 were shown to be expressed at higher levels in patients with MM than in healthy donors [51]; this dysregulation of miRNA expression included upregulation of *miR-let-7a*, *miR-16*, *miR-17-5p*, *miR-19b*, *miR-21*, *miR-531*, *miR-335*, *miR-342-3p*, *miR-25*, *miR-32*, *miR-20a*, and *miR-93*; increased expression of the miRNA cluster containing *miR-106a*, *miR-106b*, *miR-181a*, *miR-19b*, *miR-181b*, *miR-92a*, and *miR-17-92* [52–54]; and downregulation of *miR-372*, *miR-143*, and *miR-155* [52]. In patients with monoclonal gammopathy of undetermined significance (MGUS), 41 miRNAs were shown to be upregulated, with *miR-181*, *miR-21*, *miR-106a*, *miR-25*, and *miR-93* showing the greatest upregulation, whereas seven miRNAs were shown to be downregulated, compared with the levels in healthy plasma cells [55]. These abnormally regulated miRNAs target genes regulating the cell cycle, apoptosis, survival, and cell growth; for example, the *miR-17-92* cluster regulates Bcl-2 [56], *miR-29b* regulates MCL1 [57], *miR-21* regulates STAT3 in an IL-6-dependent manner [53], and *miR-125b* regulates BLIMP1 and IRF4 [58].

3. Current Biological Based Therapies for MM

Improved understanding of the pathogenesis and importance of the BM microenvironment in MM has led to the development of two therapeutic categories for MM treatment: proteasome inhibitors and immunomodulatory drugs. These therapies have significantly improved treatment response and survival in MM patients.

3.1. Proteasome Inhibitor. Bortezomib is a proteasome inhibitor that inhibits the activity of the 26S proteasome [59]. Bortezomib blocks the degradation of IκBα, an inhibitory protein that is constitutively bound to cytosolic NF-κB, thereby inhibiting the nuclear translocation and activation of NF-κB. Bortezomib induces apoptosis by activating caspase-8 and caspase-9 in drug-resistant MM cell lines and primary cancer cells derived from MM patients. Moreover,

bortezomib downregulates the expression of adhesion molecules on MM cells and BMSCs and their related binding. Bortezomib also inhibits IL-6 and/or BMSC/MM cell adherence-induced p42/p44 MAPK phosphorylation and proliferation of MM cells [60, 61].

Bortezomib has received full FDA approval for the treatment of relapse, refractory, and newly diagnosed MM patients based on the results of phase III trials [11, 12]. Treatment regimens including bortezomib have become the standard treatment for multiple myeloma patients, particularly for hematopoietic stem cell transplantation-eligible patients, because of the improved response rate and survival compared to chemotherapy and steroid treatment alone [11, 62–75].

3.2. Thalidomide and IMiDs. Thalidomide and the more potent second-generation thalidomide analogues, IMiDs, target myeloma cells in the BM microenvironment. They inhibit TNF-α production [75, 76] and angiogenesis by blocking the angiogenic growth factors, basic fibroblast growth factor (bFGF), and VEGF [77]. Specifically, these agents trigger caspase-8-mediated apoptosis and enhance both caspase-8-mediated MM cell apoptosis, triggered by FAS or TRAIL, and caspase-9-mediated MM cell killing, triggered by dexamethasone [78–80]. They also block the induction of cytokines such as IGF-1 and IL-6 and VEGF secretion triggered by MM cell adherence to BMSCs. In addition, they inhibit angiogenesis and augment natural killer cell activity against autologous MM cells [79–82]. Several clinical trials have demonstrated the benefits of using regimens involving thalidomide or IMiDs (lenalidomide) for MM treatment, particularly in combination with proteasome inhibitors [15, 16, 63–66, 69–71, 74, 83–96]. This combined therapy has become the standard regimen for MM treatment. Pomalidomide therapy has afforded prolonged progression-free survival in patients who relapsed or became refractory to lenalidomide treatment [97].

The choice of therapy for patients is influenced by a variety of factors, including age, comorbidities, and eligibility for stem cell transplantation. Treatment strategies for MM patients include two-drug regimens such as bortezomib-dexamethasone [62], lenalidomide-dexamethasone [15, 16, 86, 94], or thalidomide-dexamethasone [63, 84, 92, 93] and three-drug regimens such as bortezomib-thalidomide-dexamethasone [63–66], bortezomib-melphalan-prednisone [68, 69], or lenalidomide-bortezomib-dexamethasone (RVD) [98]. However, RVD has shown the most promising effect.

4. Mechanisms of Drug Resistance

During conventional chemotherapy such as treatment with vincristine and doxorubicin, accumulation of drugs induces the expression of multidrug resistance (MDR) genes and p-glycoprotein in tumor cells [99–101]. The BM microenvironment can confer drug resistance through two major mechanisms (Figure 1(a)) [102]: (1) tumor cell adhesion, which involves MM cell binding to fibronectin, which in turn induces KIP1 and G1 growth arrest and confers cell-adhesion mediated drug resistance [103, 104] and (2) cytokine-mediated antiapoptotic sequelae, which involve the induction of JAK/STAT and PI3K/AKT signaling by cytokines in the BM microenvironment, which in turn mediates resistance to conventional and novel therapies. IL-6 induces resistance to dexamethasone by activating JAK/STAT signaling and upregulating the antiapoptotic proteins, BCL-XL [105, 106] and myeloid cell leukemia sequence-1 (MCL1) [107, 108]. IL-6 also activates SRC-homology tyrosine phosphatase 2 (SHP2), which blocks dexamethasone-induced activation of RAFTK and apoptosis [109]. Both IL-6 and IGF-1 inhibit drug-induced apoptosis of MM cells through PI3K/AKT signaling and NF-κB activation, which in turn induces the intracellular expression of downstream inhibitor of apoptosis proteins (IAPs), FLICE-inhibitory protein (FLIP), survival, cellular inhibitor of apoptosis-2 (cIAP2), A1/BFL1, and X-linked inhibitor of apoptosis protein (XIAP) [32, 35, 110, 111]. Neither bortezomib nor thalidomide/IMiDs can block JAK/STAT or PI3K/AKT signaling [102].

MicroRNAs play a key role in multidrug resistance in cancers by modulating drug transporter-related proteins, cell cycle-related proteins, drug targets, autophagy, the tumor microenvironment, cell survival signaling, and apoptosis pathways [112, 113]. Roccaro et al. reported that the expression of *miR-15a* and *miR-16* decreased, while the expression of *miR-221*, *miR-222*, *miR-382*, *miR-181a*, and *miR-181b* increased in patients with relapse/refractory MM compared with the levels in healthy volunteers [114]. Moreover, *miR-15a* and *miR-16* regulate the cell cycle by inhibiting the expression of cyclin D1, cyclin D2, and CDC25A and the phosphorylation of Rb, resulting in G_1 arrest. *miR-15a* and *miR-16* can also reduce the expression of Bcl-2. Cells transfected with pre-*miRNA-15a* and pre-*miRNA-16-1* exhibit the following effects: (1) increased phosphorylation of the inhibitory protein IκB in the cytoplasm, indicating involvement of these miRNAs in both the canonical and noncanonical NF-κB pathways; (2) significantly decreased VEGF secretion, suggesting an antiangiogenic role for these miRNAs; (3) and inhibition of migration in response to SDF-1. Significant inhibition of the adhesion of MM to primary BM stromal cells upon application of these miRNAs has been confirmed in mouse models. In addition, the *miR-15a*/*miR-16-1* cluster upregulates several genes, including *NEDD9*, *Snai2*, *MALAT1*, and *VEGF*, and leads to the inhibition of tumor progression by enhancing tumor cell survival, metastasis, and the angiogenic properties of MM cells [115]. Neri et al. reported the dysregulation of several miRNAs related to bortezomib resistance, including the overexpression of *miR-155*, *miR-342-3p*, *miR-181a*, *miR-181b*, *miR-128*, and *miR-20b* and the downregulation of *miR-let-7b*, *miR-let-7i*, *miR-let-7d*, *miR-let-7c*, *miR-222*, *miR-221*, *miR-23a*, *miR-27a*, and *miR-29a* [116]. The predicted targets genes include genes involved in cell cycle regulation, cell growth, apoptosis, and the ubiquitin-conjugation pathways [115]. *miR-21* targets Rho-B, PTEN, and BTG2 and controls STAT-3/IL-6-dependent pathways as well as AKT and NF-κB signaling via myeloma cell adhesion to BMSCs [117–119]. *miR-21* inhibitor exhibits synergistic effects with dexamethasone, doxorubicin, and bortezomib [117], indicating that *miR-21* may be involved in mediating drug resistance. Another

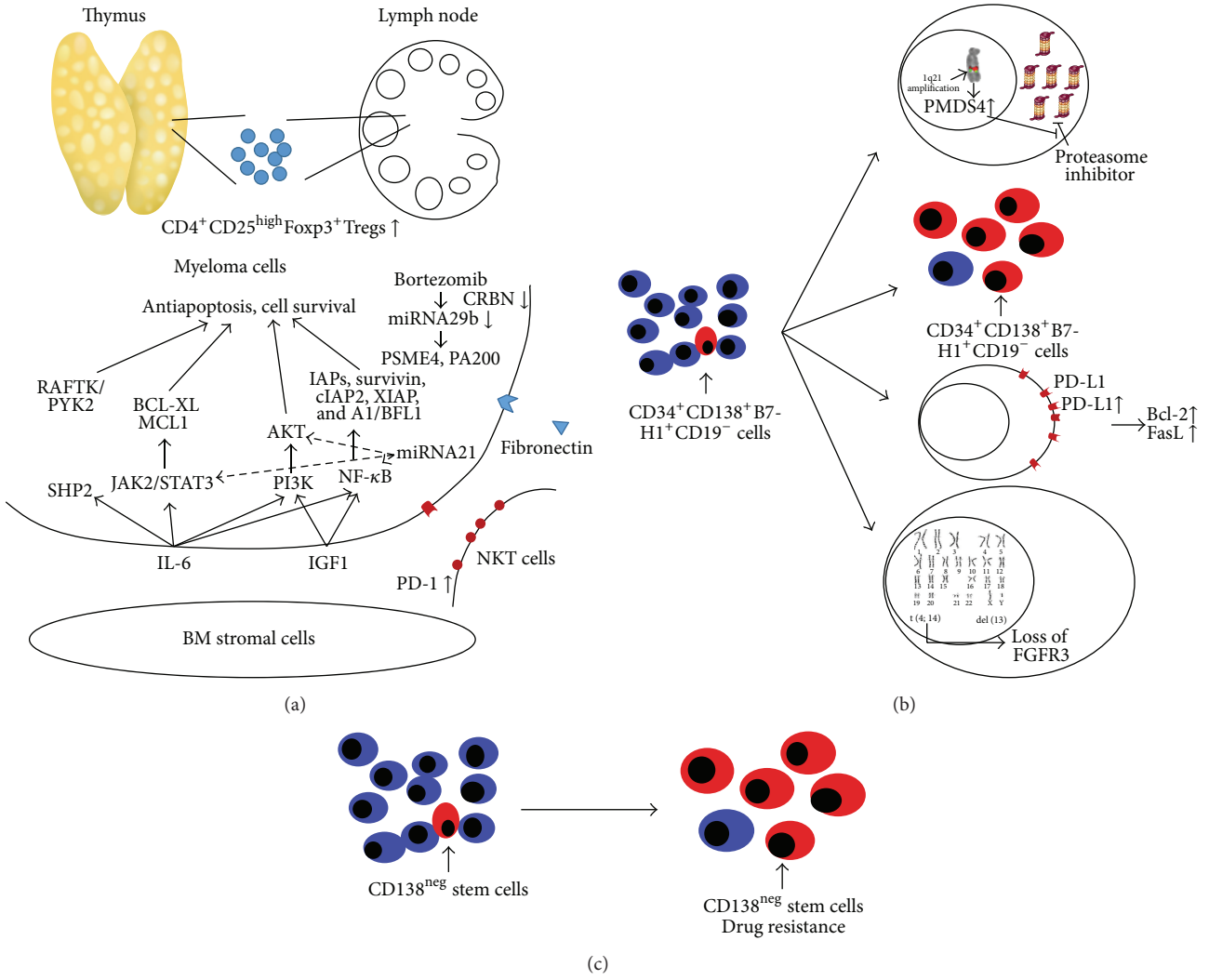

Figure 1: The mechanism of drug resistance of refractory and relapse multiple myeloma. (a) Microenvironment, (b) clonal evolution of myeloma cells, and (c) cancer stem cell.

miRNA, *miR-29b*, has been shown to target *PSME4*, which encodes the proteasome activator PA200; this miRNA is significantly downregulated in bortezomib-resistant cells and in cells resistant to second-generation proteasome inhibitors, for example, carfilzomib and ixazomib [120]. Bortezomib promotes the accumulation of polyubiquitinated proteins and induces aggresome and autophagosome formation to promote protein clearance, tumor survival, and relative drug resistance. Activating transcription factor 4 (ATF4), an endoplasmic reticulum-resident transmembrane protein, and microtubule-associated protein 1 light chain 3B (LC3B), one of the key factors in autophagosome formation, play a critical role in activating autophagy and protecting breast cancer cells from bortezomib-induced cell death, representing another potential mechanism of resistance to bortezomib [121].

Clonal evolution of MM cells is another possible mechanism of drug resistance (Figure 1(b)). Hyperexpression of the proteasome-related gene, *PSMD4*, is highly sensitive to chromosome 1q21 amplification and is reported to be associated with bortezomib resistance [122]. MM with gain of chromosome 1q has demonstrated poor prognosis [123], and patients with relapse or refractory MM who received treatment with lenalidomide and dexamethasone in the presence of del(13) and t(4;14) chromosomal abnormalities exhibited lower response rates and shorter median progression-free survival (PFS) [4]. Chromosome t(4;14) is likely to evolve over time, first to a chimeric and ultimately to an unbalanced translocation, with the associated loss of FGFR3 expression, which indicates disease progression [124]. B7-H1 (PD-L1) expression is upregulated on the surface of cells from MM patients. Compared to B7-H1$^-$ human myeloma cell lines (HMCLs), B7-H1$^+$ HMCLs were found to be more proliferative and less susceptible to dexamethasone and melphalan treatment and were accompanied by higher Bcl-2 and FasL expression [125, 126]. The expression levels of PD-L1 were found to be upregulated after myeloma patients relapsed or became refractory to therapy [126]. Kuranda

et al. also reported that a small subpopulation of cycling $CD34^+CD138^+B7\text{-}H1^+CD19^-$ plasma cells were found in MM patients, and these cells often expressed Ki67, a marker for proliferation, and limited the clinical benefits of autologous $CD34^+$ cell transplantation [127]. However, a population of suppressive $CD4^+CD25^{high}Foxp3^+$ regulatory T cells (Tregs) accumulated in the thymus and lymphoid peripheral organs during disease progression [128].

Another mechanism of drug resistance involves epigenetic inactivation of genes such as *RASD1*. Methylation of *RASD1*, which encodes a Ras family protein that is induced by dexamethasone and suppresses cell growth, was found to be associated with its inactivation, which correlated with resistance to dexamethasone [129].

The concept of cancer stem cells was introduced in the late 1990s. Traditionally, cancer cells that survive chemotherapy and acquire drug resistance are thought to give rise to a population of drug-resistant cancer cells through modulation of mechanisms such as drug inactivation, changes in the expression of cellular targets, suppression of drug accumulation, and inhibition of drug activation [130–132]. The Notch, Wnt, and Hedgehog pathways play a role in regulating normal stem cells and the pathogenesis of a wide variety of human cancers, including MM [133–137]. Aberrant activation of Hedgehog signaling has been identified in MM. Pathway activation by ligands results in the expansion of immature myeloma cells, whereas the inhibition of signaling with a ligand-neutralizing monoclonal antibody or antagonists of the positive mediator of the pathway signaling induces plasma cell differentiation [137, 138]. Matsui et al. identified a group of $CD138^{neg}$ MM cells that possess high drug efflux capacity and intracellular drug detoxification activity. Alternatively, MM cells expressing the memory B-cell markers CD20 and CD27 from the peripheral blood could give rise to clonogenic MM growth *in vitro* and in SCID/NOD mice [139]. These data support the hypothesis that MM cells exhibit stem cell characteristics.

Cereblon (CRBN) is the primary target of thalidomide teratogenicity [140]. Thalidomide binds to CRBN, alters the function of the E3 ubiquitin ligase complex, and induces downstream effects, including cell cycle arrest caused by the upregulation of the cyclin-dependent kinase inhibitor $p21^{WAF\text{-}15}$ and the downregulation of interferon regulatory factor 4 (IRF4), which targets critical genes, including *MYC*, *CDK6*, and *CASP* [141–143]. CRBN is also required for the anti-MM action of the thalidomide derivatives lenalidomide and pomalidomide; decreasing the expression of *CRBN* results in resistance to IMiDs, as evidenced by both *in vitro* and clinical studies [144–148]. However, the majority of patients with low CRBN levels do not harbor genomic mutations [149].

5. Potential New Therapies for Refractory and Relapse MM Patients

5.1. Second-Generation Inhibitors of the Ubiquitin-Proteasome Cascade [150]. Recently, potent inhibitors with chymotryptic activity have been developed. These include carfilzomib, ONX 0912, and MLN 9708 [151, 152], which can overcome bortezomib resistance, as demonstrated in preclinical and early clinical trials. Carfilzomib was approved by the FDA in July 2012 to treat relapse and refractory MM patients who had received prior treatment with bortezomib and thalidomide/lenalidomide [153, 154]. The safety and efficacy of carfilzomib were demonstrated in the PX-171-003-A1 trial, a prospective phase II trial in patients with relapse or refractory MM who had received at least two prior therapies, including a proteasome inhibitor and an immunomodulatory agent [155]. A randomized phase III clinical trial comparing carfilzomib-lenalidomide-dexamethasone and lenalidomide-dexamethasone treatment regimens in patients with relapse MM [156] and another randomized phase III clinical trial comparing carfilzomib-dexamethasone and bortezomib-dexamethasone in patients with relapse MM [157] are ongoing. ONX 0912 [151] and MLN 9708 [158] are novel orally bioavailable proteasome inhibitors that trigger apoptosis by activating caspase-3, caspase-8, and caspase-9. Ongoing phase I and II clinical trials for these inhibitors have shown promising results [159–161].

P5091 is another second-generation proteasome inhibitor that targets the deubiquitinating enzyme USP7 and induces apoptosis in MM cells resistant to conventional and bortezomib therapies [162]. NPI-0052 is a broader proteasome inhibitor that targets chymotryptic, tryptic, and caspase-like activities to overcome bortezomib resistance in preclinical studies [163]. PR-924, an inhibitor of the LMP-7 immunoproteasome subunit, also blocks MM cell growth *in vitro* and *in vivo* [164].

5.2. Immunomodulatory Agents [150]. Pomalidomide is a distinct oral IMiD immunomodulatory agent with direct antimyeloma, stromal-support inhibitory, and immunomodulatory effects. Pomalidomide can synergize *in vitro* with proteasome inhibitors such as bortezomib [79]. Phase 1 clinical studies of pomalidomide in combination with low-dose dexamethasone have demonstrated the effectiveness of this therapy in MM patients who were resistant to other agents, including thalidomide, lenalidomide, and bortezomib [165, 166]. The pivotal multicenter, open-label, randomized phase III trial, MM-003, compared pomalidomide and low-dose dexamethasone with high-dose dexamethasone in 455 patients with refractory or relapse MM after failure of bortezomib and lenalidomide treatment. Pomalidomide and low-dose dexamethasone induced better progression-free survival and favorable overall survival without cross-resistance of prior treatment of lenalidomide and/or thalidomide [167].

5.3. PD-1/PD-L1 in Multiple Myeloma [150]. PD-L1 expression is increased in MM cells, and PD-1 is expressed on a relatively large number of T cells in myeloma-bearing mice, but only in sites of tumor accumulation [48]. Binding of PD-L1 to PD-1 expressed on the surface of activated T cells delivers an inhibitory signal, thereby reducing cytokine production and proliferation [168]. Preclinical data have confirmed the important role of the PD-1 pathway in immune evasion by MM cells [46, 48, 168]. In phase I clinical trials, objective responses were observed in patients with

melanoma, renal cell carcinoma, and non-small cell lung cancer, who underwent immunotherapy with an anti-PD-1 monoclonal antibody [169–172]. In addition, an anti-PD-L1 monoclonal antibody exhibited antitumor activity in patients with melanoma, renal cell carcinoma, non-small cell lung cancer, and ovarian cancer [172, 173]. Pidilizumab (CT-011), an anti-PD1 antibody, enhances NK-cell activity against autologous, primary MM cells. In addition, lenalidomide downregulates PD-L1 in MM cells and may augment CT-011-mediated enhancement of NK-cell activity against MM [47]. However, another anti-PD1 antibody, nivolumab (BMS-936558), did not show objective responses in MM [174]. This may be attributed to the fact that the mechanism of action of T-cell activity against MM cells does not involve PD-1/PD-L1 interaction. Clonal cytotoxic $CD8^+$ T cells are the only definitive T cells that have a protective role and impact on survival in MM [175]. Cytotoxic T-cell clones ($CD57^+CD28^-$TCR$V\beta$ restricted) were found to be present in 51% of 264 patients with MM. These protective T cells exhibit telomere-independent senescence, rather than the exhausted or anergic phenotype [176]. Suen et al. demonstrated that PD-1 expression is downregulated in clonal BM cytotoxic T cells, compared with the levels in nonclonal T cells, in MM patients [177]. Thus, the role of PD-1 or PD-L1 blockade needs to be investigated in detail, and clinical trials need to be performed to evaluate its therapeutic potential.

5.4. Antibody-Related Therapies. Several antigens that exhibit strong expression in MM cells, including CD38, CD138, CD56, CD74, CD40, insulin-like growth factor-1 receptor (IGF-1R), signaling lymphocyte activating-molecule F7 (SLAMF7), and immunoglobulin superfamily member FcRL5, may be candidates for antibody-related immunotherapy [178]. Numerous naked antibodies have been tested in preclinical myeloma models, and antibodies against six antigens, that is, CD38, CD74, CD40, SLAMF7, IL-6, and IGF-1R, have been examined in clinical trials. Daratumumab [179] and SAR650984 [180] are anti-CD38 monoclonal antibodies that have shown satisfactory response rates in patients with relapse/refractory MM and $CD38^+$ hematological malignancies (including 27 patients with MM) in separate phase I clinical trials. A phase II study of daratumumab plus proteasome inhibitor in patients with IMiD refractory myeloma and a phase I/II study of the combination of lenalidomide and dexamethasone are currently underway [181, 182]. SAR650984 is currently being tested in a phase I dose-escalation study and a phase Ib combination study with lenalidomide and dexamethasone [183, 184]. Milatuzumab [185], an anti-CD74 monoclonal antibody, resulted in only 26% of patients achieving stable disease (SD), with a 0% overall response rate (ORR) in patients with refractory/relapse MM. Dacetuzumab [186] and lucatumumab [187] are anti-CD40 monoclonal antibodies that yielded ORRs of 0% (20% of patients achieving SD) and 4% (43% of patients achieving SD), respectively. However, there are no trials currently underway in patients with MM. Elotuzumab, an anti-SLAMF7 (CS1) monoclonal antibody, yielded no objective responses in a phase I clinical trial [188]. However, the combination of elotuzumab, lenalidomide, and dexamethasone yielded an ORR of 84% in patients with refractory/relapse MM in a phase II clinical trial [189]. In a recent phase III study, 321 patients with relapse/refractory MM received elotuzumab plus lenalidomide and dexamethasone, and 325 patients with relapse/refractory MM received the control treatment of lenalidomide and dexamethasone. After a median follow-up of 24.5 months, the rates of progression-free survival (PFS) at 1 and 2 years were 68% and 41%, respectively, in the elotuzumab group as compared with 57% and 27%, respectively, in the control group. Median PFSs were 19.4 and 14.9 months in the elotuzumab and control groups, respectively, and the ORRs were 79% and 66% in the elotuzumab and control groups, respectively [190]. The anti-IGF-1R antibody figitumumab (CP-751871) and AVE 1642 showed disappointing results in phase I studies [191, 192]. However, treatment with the IGF-1R inhibitor OSI-906 or transfection with IGF-1R-targeting small hairpin RNA had synergistic effects on bortezomib sensitivity in cell lines and patient samples [193]. Siltuximab, another monoclonal antibody targeting IL-6, had minimal effects in a phase I study [194, 195] and exhibited no benefits in a phase II clinical trial in patients with refractory/relapse MM [196].

Another type of antibody-related therapy is antibody-drug-conjugated therapy. The anti-CD138 antibody-drug conjugate (ADC), indatuximab ravtansine (BT062), had an ORR of 11%, with 41% achieving SD, in 27 patients with relapse/refractory MM in a phase I study [197]. Combined with lenalidomide and dexamethasone, this ADC resulted in an ORR of 78% in nine patients [198]. The anti-CD56 ADC lorvotuzumab, mertansine, yielded an ORR of 17%, with 28% achieving SD, in selected patients with MM exhibiting CD56 expression in a phase I study [199]. A few additional ADCs are currently being examined in preclinical studies, including ADCs targeting CD74, Fc receptor-like 5 (FcRL5), and B-cell maturation antigen (BCMA). Milatuzumab, an anti-CD74 antibody conjugated to doxorubicin, shows *in vitro* and *in vivo* activity against MC/CAR cells and MC/CAR xenografts in SCID mice [200]. The anti-FcRL5 maytansine analog (DM4) and monomethyl auristatin E (MMAE) have activities similar to those of bortezomib (biweekly treatment) in the inhibition of tumor growth in subcutaneous xenografts of OPM2-FcRL5 and EJM-FcRL5 cells in SCID mice and have been shown to be well tolerated in monkeys in a preclinical study [201]. An anti-BCMA antibody conjugated to monomethyl auristatin F (MMAF) has been reported to show rapid internalization, efficient trafficking to lysosomes, and high antigen recycling rates by 6 h after administration [202]. The anti-BCMA ADC GSK2857916 also resulted in elimination of xenografts arising from myeloma cells [203].

Chimeric antigen receptor- (CAR-) modified T-cell therapy is a new type of immunotherapy. Adoptive transfer of T cells engineered to express chimeric antigen receptors (CARs) can specifically recognize tumor-associated antigens, combining the advantages of non-major histocompatibility complex- (MHC-) restricted recognition with efficient T-cell activation and expansion [204–207]. CARs combine the antigen recognition domain of the antibody with the intracellular domain of the T-cell receptor-ζ (TCR-ζ) chain

or FcγRI protein into a single chimeric protein that is capable of triggering T-cell activation in a manner very similar to that of the endogenous TCR [208, 209]. CS-1 is a cell surface glycoprotein of the signaling lymphocyte activation molecule (SLAM) receptor family that is highly and selectively expressed on normal plasma cells and MM cells, with lower expression on NK cells and little or no expression on normal tissues. CS1-CAR NK cells exhibit enhanced MM cytolysis and IFN-γ production and exhibit tumor suppressive effects on MM cell lines, primary MM tumor cells, and MM xenograft mouse models [210, 211]. CD138 is highly expressed on MM cells and is involved in the development and/or proliferation of these cells [212]. Guo et al. reported that four out of five patients with chemotherapy-refractory MM treated with CART-138 therapy achieved SD longer than 3 months [213]. In a preclinical study, anti-BCMA-CAR-transduced T cells exhibited BCMA-specific functions, including cytokine production, proliferation, cytotoxicity, and *in vivo* tumor eradication. Importantly, anti-BCMA-CAR-transduced T cells recognize and kill primary MM cells [214]. A clinical trial examining CART-19 combined with autologous stem cell transplantation (ASCT) in patients with early refractory/relapse MM is currently underway [215].

5.5. Histone Deacetylase Inhibitors [150]. Deacetylases are a group of enzymes that affect various intracellular proteins, including histones, transcription factors, and molecular chaperones, which modulate gene expression, cellular differentiation, and survival [102]. Deacetylase inhibitors (DACi), including panobinostat and vorinostat, have been evaluated for the treatment of MM. The addition of proteasome inhibitors to DACi treatment regimens enhances the sensitivity of MM cells to DACi to induce mitochondrial dysfunction, caspase-9, caspase-8, and caspase-3 activation, and poly (ADP-ribose) polymerase degradation, which is associated with NF-κB inactivation, c-Jun NH_2-terminal kinase activation, p53 induction, caspase dependent cleavage of $p21^{CIP1}$, $p27^{KIP1}$, and Bcl-2, and cyclin D1 downregulation [216]. The mechanism of this synergistic apoptotic effect on MM cells is multifactorial and includes disruption of protein degradation and inhibition of the interaction of MM cells with the tumor microenvironment [217]. Rocilinostat (ACY-1215) is HDAC6 inhibitor that targets aggressomal protein degradation systems. A synergistic antitumor effect of ACY-1215 and proteasome inhibitors was observed in MM. In addition, a potential benefit was observed in MM-related bone diseases with the combination of these two drugs [218, 219].

5.6. Other Agents. Other drugs, including cell signaling targeted therapies (PI3K/AKT/mTOR, p38 MAPK, Hsp90, Wnt, Notch, Hedgehog, and cell cycle) and strategies targeting the tumor microenvironment (hypoxia, angiogenesis, integrins, CD44, CXCR4, and selectins) are candidates for the treatment of refractory and relapse MM [220]. PI3K/AKT is upregulated during refractory and relapse MM. Bortezomib and IMiDs (thalidomide and lenalidomide) do not impact PI3K/AKT signaling [102]. The PI3K/AKT pathway regulates apoptosis, cell cycle, and tumor proliferation [221]. AKT indirectly activates mTOR, a complicated checkpoint of cellular growth influenced by growth factor signaling, adenosine monophosphate levels, and nutrient and O_2 availability [222]. Perifosine (KRX-0401) is an oral bioactive alkylphospholipid that is thought to target cell membranes and modulate multiple signaling pathways, including the inhibition of AKT and promotion of apoptosis in MM cells [223]. A phase I study with perifosine in combination with lenalidomide and dexamethasone [224] and a phase I/II study with perifosine in combination with bortezomib with or without dexamethasone in refractory and relapse MM [225] demonstrated high treatment tolerance and beneficial effects on survival. Rapamycin and some analogues (temsirolimus or CCI-779 and everolimus or RAD001) are inhibitors of mTOR and have shown preclinical potential as MM therapies. Phase I/II clinical trials using temsirolimus and everolimus in heavily pretreated MM patients showed high tolerance and acceptable response rates [226, 227]. NVP-BEZ235 is a dual pan inhibitor of the PI3K/AKT/mTOR pathways at the levels of PI3K and mTOR, which inhibits growth and proliferation in MM. Moreover, synergism studies have revealed synergistic and additive effects of NVP-BEZ235 in combination with melphalan, doxorubicin, and bortezomib [228]. P38 is constitutively activated in human myeloma and has been implicated in osteoclast and osteoblast activity and bone destruction [229]. The effect of a p38 alpha-selective MAPK inhibitor, SCIO-469 (indole-5-carboxamide, ATP-competitive inhibitor), or its structural analogue, SD-282 (indole-5-carboxamide, ATP-competitive inhibitor), reduced human myeloma cell growth *in vivo* at early and advanced phases of the disease; the same study also provided evidence of the potential for cotherapy with dexamethasone in mouse models of MM [230]. However, LY2228820, a p38 MAPK inhibitor, significantly enhanced toxicity in MM patients [231]. Therefore, more studies on this pathway are required for the development of safe and effective compounds.

Tanespimycin, an Hsp90 inhibitor, reduces tumor cell survival *in vitro* by affecting the IL-6 receptor and elements of the PI3K/AKT and MAPK signaling pathways, through abrogation of the protective effect of BMSCs. Tanespimycin is known to inhibit angiogenesis [232]. A phase I/II study with tanespimycin and bortezomib in relapse/refractory MM patients showed acceptable toxicity and durable response rates [233].

Cancer stem cells use many of the same signaling pathways that are found in normal stem cells, such as Wnt, Notch, and Hedgehog (Hh). Agents targeting these pathways would complement current treatment approaches [234–237]. Other agents targeting the cell cycle, such as seliciclib [238] and LCQ195 [239], cyclin D kinase (CDK) inhibitors, and MLN8237 [240], an aurora-A kinase inhibitor, have demonstrated therapeutic benefits in MM in a preclinical setting.

TNF-related apoptosis-inducing ligand or Apo ligand (TRAIL/Apo2L) is a member of a superfamily of cell death-inducing ligands which also includes TNF-α and Fas ligand (FasL or CD95L) [241]. In a preclinical study, TRAIL/Apo2L selectively induced apoptosis in human MM cells, including cells that were sensitive or resistant to dexamethasone

and doxorubicin [78], and reversed the bortezomib-induced upregulation of β-catenin, MCL1, and FLIP, thereby enhancing the cytotoxicity of combination therapy [242]. This treatment may represent a promising candidate for targeted therapy.

In MM, the impacts of tumor microenvironmental factors such as hypoxia, angiogenesis, and interactions between MM and BMSCs have become an important consideration for understanding disease progression and resistance to therapy and have been incorporated into novel drug screening approaches. VEGFR antagonists inhibit angiogenesis in the MM microenvironment [243]. However, the clinical data for VEGFR antagonists, including pazopanib [244], vandetanib [245], and SU5416 [246], have demonstrated disappointing results.

SDF-1 is produced by BM-derived stromal cells, and its receptor CXCR4 is expressed on the surface of normal and MM cells. The SDF-1/CXCR4 axis is a key regulator of MM cell homing, adhesion, and motility [247]. The CXCR4 antagonist AMD3100 was shown to block MM cell interactions with the BM microenvironment and consequent signaling responses, leading to enhanced sensitivity to therapy [248]. In a phase I trial of plerixafor and bortezomib as a chemosensitization strategy in relapse or relapse/refractory MM patients, preliminary results showed that the combination is well tolerated and demonstrates an acceptable response rate [249].

Mitochondria are important organelles involved in apoptosis under conditions of oxidative stress. Chauhan et al. reported that combining PK-11195, an antagonist of the mitochondrial peripheral benzodiazepine receptors (PBRs), with bortezomib triggers synergistic anti-MM activity, even in MM cells resistant to doxorubicin, melphalan, thalidomide, dexamethasone, and bortezomib. The mechanism through which apoptosis is induced includes loss of mitochondrial membrane potential, superoxide generation, release of the mitochondrial proteins cytochrome-c and Smac, activation of caspase-8/caspase-9/caspase-3, and activation of c-Jun NH_2-terminal kinase (JNK) [250].

6. Conclusion

Based on a thorough understanding of the mechanism and importance of the MM microenvironment, proteasome inhibitors, such as bortezomib, have been developed in combination with IMiDs and steroids to provide dramatic improvement in treatment response and survival in MM patients. However, MM is still an incurable disease. The possible mechanisms of drug resistance include *MDR* gene polymorphism and p-glycoprotein overexpression in MM cells, microenvironmental changes (cell adhesion, activation of cytokine-related antiapoptosis pathways such as the JAK/STAT and PI3K/AKT pathways), clonal evolution such as hyperexpression of the proteasome-related gene, *PSMD4*, related to chromosome 1q21 amplification, t(4;14) unbalanced translocation, and selected $CD34^+CD138^+B7\text{-}H1^+CD19^-$ plasma cell accumulation after treatment. The up- and downregulation of various miRNAs modulate MM cell survival, cell cycle, and microenvironment, thereby contributing to drug resistance, including against bortezomib. PD-1 is enriched on T cells in MM patients, and PD-L1 expression on MM cells is enhanced. PD-1/PD-L1 interactions have been shown to mediate tumor escape from immune control in a number of animal models. Moreover, PD-1/PD-L1 interactions are related to immune dysfunction in MM patients. PD-L1 in MM cells and PD-1 in T cells surrounding tumors contribute to drug resistance mechanisms. Potential therapies, including second-generation proteasome inhibitors, new immunomodulatory agents, DACi, and kinase inhibitors such as the mTOR inhibitor, as well as drugs targeting cytokine-related pathways, anti-PD-1/anti-PD-L1 monoclonal antibodies, and monoclonal antibodies (naked or conjugated with drugs), and CAR-T therapy, are under preclinical and clinical investigation to provide better treatment responses in MM patients. Study of the pathophysiology of MM and the mechanisms of drug resistance will enable the development of novel therapeutic strategies to cure this disease. Further clinical trials of the novel agents described here are also necessary, especially for refractory/relapse MM patients.

Conflict of Interests

The authors declare that there no financial or nonfinancial competing interests.

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Research Article

The Applicability of the International Staging System in Chinese Patients with Multiple Myeloma Receiving Bortezomib or Thalidomide-Based Regimens as Induction Therapy: A Multicenter Analysis

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The International Staging System (ISS) is the most important prognostic system for multiple myeloma (MM). It was identified in the era of conventional agents. The outcome of MM has significantly changed by novel agents. Thus the applicability of ISS system in the era of novel agents in Chinese patients needs to be demonstrated. We retrospectively analyzed the clinical outcomes and prognostic significance of ISS system in 1016 patients with newly diagnosed multiple myeloma in Chinese patients between 2008 and 2012, who received bortezomib- or thalidomide-based regimens as first-line therapy. The median overall survival (OS) of patients for ISS stages I/II/III was not reached/55.4 months/41.7 months ($p < 0.001$), and the median progression-free survival (PFS) was 30/29.5/25 months ($p = 0.072$), respectively. Statistically significant difference in survival was confirmed among three ISS stages in thalidomide-based group, but not between ISS stages I and II in bortezomib-based group. These findings suggest that ISS system can predict the survival in the era of novel agents in Chinese MM patients, and bortezomib may have the potential to partially overcome adverse effect of risk factors on survival, especially in higher stage of ISS system.

1. Introduction

Multiple myeloma (MM) is the second most common hematological malignancy, accounting for 10% of all neoplastic hematologic disorders. It is characterized by significant heterogeneities in clinical manifestations and prognosis. The median overall survival (OS) for MM is about 4-5 years, but the OS is highly variable in different MM patients; some patients with aggressive disease courses may die in a few months after diagnosis, while other patients with indolent courses may live more than ten years [1]. Consequently, it is important to find a simple and robust stratification system to predict prognosis and help to optimize treatment strategy early in the course of myeloma [2].

Exploring a useful prognostic system has been a topic of interest in the myeloma filed since the past forty years, and considerable progresses have been made now. The Durie-Salmon system was established in 2005 and worldwide used since then. This system can divide patients into three stages by tumor burden [3]. However, this system is complicated and not objective. In 2005 the International Myeloma Working Group (IMWG) developed a new stage system called the

Table 1: The definition of the ISS system.

ISS stage	Definition	Median OS
Stage I	Sβ_2M less than 3.5 mg/L plus serum albumin ≥3.5 g/dL	64 months
Stage II	Neither stage I nor stage III	44 months
Stage III	Sβ_2M ≥5.5 mg/L	29 months

ISS: International Staging System, OS: overall survival.
Sβ_2M: serum beta 2 microglobulin.

International Staging System (ISS), which was relying on the combinations of two easily available and objective prognostic variables (serum beta 2 microglobulin (Sβ_2M) and serum albumin): ISS stage I, Sβ_2M less than 3.5 mg/L plus serum albumin ≥3.5 g/dL; ISS stage II, neither stage I nor stage III; and ISS stage III, Sβ_2M ≥ 5.5 mg/L [4] (Table 1). The ISS system is the most important and commonly used stage system today. However, difficulties have been encountered now. When ISS system was defined, it contained data from patient with MM between 1981 and 2002. All of these patients were treated by conventional agents, not exposed to novel agents. However current treatment strategies for MM have been completely changed during the last decade by the introduction of novel agents. Novel agents such as bortezomib and thalidomide have become the most important component in MM therapy and dramatically improved the response rate, progression-free survival (PFS), and even OS of MM patients [5]. Thus the prognostic value of ISS system in the era of novel agents is still in debate.

Although recent studies had been focused on the applicability of ISS in the era of novel agents, until recently there was no conclusion. One study indicated that the ISS system was still robust after introduction of thalidomide in Greece [6]. A study from Dimopoulos et al. then demonstrated the applicability of ISS in MM patients with renal dysfunction [7]. Another study from Yang et al. indicates that ISS was not suitable for patients who underwent hematopoietic stem cell transplantation (HSCT) after the introduction of thalidomide [8]. Novel agents are well tolerated and have been recommended as the first-line choice at induction chemotherapy in National Comprehensive Cancer Network (NCCN), European Society for Medical Oncology (ESMO) guideline [9, 10]. The majority of patients received at least one kind of novel agents, such as bortezomib, thalidomide, or lenalidomide at induction therapy now. However, data is still less accurate for the prognostic significance of ISS in those who were acutely treated with novel agents at induction therapy in Chinese patients.

Therefore, we investigated a consecutive cohort of patients with MM who were treated with bortezomib- or thalidomide-based regimens as induction treatment in three Chinese centers, to validate the prognostic significance of the ISS in the era of novel agents in Chinese patients.

2. Methods

2.1. Patients and Treatment. A total of 1016 newly diagnosis symptomatic MM patients were enrolled between August 2006 and December 2012, from three Chinese myeloma centers (Department of Hematology at Changzheng Hospital, Peking University People's Hospital and Chaoyang Hospital). All of patients were diagnosed according to IMWG criteria [11]. The approvals were obtained from the Scientific Committee of three hospitals for the use of patients' medical records and publication of these data.

Patients who received at least two courses of one novel agent based therapy in the first-line treatment were included in this study. Thalidomide and bortezomib were introduced in the treatment of MM patients in China in 1999 and 2005, respectively, while lenalidomide was not available until 2013, before this analysis was conducted. The patients were divided into two groups by the type of the first-line regimens, bortezomib-based group and thalidomide-based group.

Patients' characteristics at diagnosis including gender, age, immunophenotype, ISS stage, peripheral neuropathy (PN), hemoglobin, platelets, bone marrow (BM) plasma cell percentage, serum calcium, serum albumin, Sβ_2M, serum lactate dehydrogenase (LDH), and serum creatinine were collected. Table 2 reported details regarding patients' clinical and hematological features.

2.2. Follow-Up. The last follow-up was conducted in March 2013. The primary end point for this study was OS, while secondary end points were PFS and response rate. OS was defined as the time between the diagnosis and death of any cause or until the last follow-up. PFS was defined as the time between the diagnosis and progression or until the last follow-up. Response rate to induction therapy was defined according to IMWG criteria. Patients were considered responsive when they achieved at least partial response (PR) in the first-line treatment.

2.3. Statistical Analysis. Statistical analyses were performed using SPSS version 18.0. Survival curves were plotted by using the Kaplan-Meier method. OS between the stages were tested using the log-rank test, with $p < 0.05$ taken as level of significance.

3. Results

3.1. Patient Characteristics. As showed in Table 2, 1016 patients with MM were enrolled in this study, 60.5% were male, the median age was 59 years, and the major subtypes were IgG (44.1%), IgA (22.2%), and light chain (23.1%). At diagnosis, 61.5% of patients had anemia (defined as hemoglobin <10 g/dL), 19.2% had renal dysfunction (defined as serum creatinine (Cr) ≥ 2 mg/mL), and 36.3% of patients' bone marrow plasma cell in filtration was more than 40%.

We divided 1016 patients into two groups by the types of novel agents in the first-line treatment, 709 patients in bortezomib-based group (defined as at least received 2 cycles of bortezomib-based treatment in first-line treatment) and 307 patients in thalidomide-based group (defined as at least received 2 cycles of thalidomide-based treatment in first-line treatment). The regimens of first-line therapy in each group were also listed in Table 2. The number of patients exposed to both bortezomib and thalidomide in our database is too

Table 2: Patient characteristics at diagnosis according to the type of novel agents in the first-line therapy.

Parameters	Total (%)	Bortezomib-based group (%)	Thalidomide-based group (%)	p value
Patient (n)	1016	709	307	
Male	59.7%	60.1%	59.0%	0.394
Age >60 years	44.2%	40.3%	53.1%	0.0001
Hemoglobin <10 g/dL	61.5%	60.2%	64.8%	0.253
Creatinine ≥2 mg/mL	19.2%	20.3%	16.4%	0.183
BM plasma cell percentage ≥40%	36.3%	38.8%	30.2%	0.012
Platelet counts $<130 * 10^9$/L	30.9%	31.7%	29.2%	0.454
PN	12.3%	10.7%	15.4%	0.063
LDH ≥245 U/L	14.6%	14.9%	13.8%	0.689
Albumin (<35 g/L)	47.4%	43.6%	56.1%	0.0001
β_2-MG (≥3.5 mg/L)	55.3%	56.1%	53.4%	0.028
DS stage				0.483
I	2.5%	2.2%	3.6%	
II	9.5%	9.3%	10.1%	
III	88.0%	88.5%	86.3%	
ISS stage				0.343
I	22.7%	24.0%	26.4%	
II	33.6%	33.3%	34.2%	
III	43.7%	42.7%	45.9%	
Myeloma type				0.050
IgG	44.1%	42.6%	47.6%	
IgA	22.2%	23.6%	19.2%	
IgD	7.4%	8.5%	4.9%	
κ light chain	12.0%	12.1%	11.7%	
λ light chain	11.1%	11.0%	11.4%	
others	3.2%	2.2%	5.2%	
Regimens of the first-line therapy		PAD/VD/BCD/V-DECP	TAD/TD/MPT/CTP/T-DECP	
≥PR to the first-line therapy	80%	84.1%	68.8%	0.0001

BM: bone marrow, PN: peripheral neuropathy, LDH: lactate dehydrogenase, DS stage: Durie-Salmon stage, ISS: International Staging System, OS: overall survival, PAD: Bortezomib (Velcade), Adriamycin, and Dexamethasone, VD: Bortezomib (Velcade) and Dexamethasone, BCD: Bortezomib (Velcade), Cyclophosphamide, and Dexamethasone, V-DECP: Bortezomib Cisplatin, Etoposide, Cyclophosphamide, and Dexamethasone; TD: Thalidomide and Dexamethasone, TAD: Thalidomide, Adriamycin, and Dexamethasone, MPT: Melphalan, Prednisone, and Thalidomide, T-DECP: Thalidomide, Cisplatin, Etoposide, Cyclophosphamide, and Dexamethasone; CTP: Cyclophosphamide, Thalidomide, and Dexamethasone, and PR: partial response.

small (41 patients). Thus the data of these patients were not included in this study. Compared to patients in thalidomide-based group, patients in bortezomib-based group had more elder patients ($p = 0.0001$), higher BM plasma cell percentage ($p = 0.012$), and better response rate ($p < 0.0001$).

3.2. Patient Outcome in the Entire Cohort. The median estimated follow-up for the cohort was 24.3 months with 72.1% alive at last follow-up. The median OS was 55.8 months and PFS was 28.0 months for the entire patients. In this study, it showed a significant better survival in patients who at least achieve PR in the first-line therapy than those who did not, median OS was 63.4 versus 53.7 months, and 5-year survival was 50.9% versus 38.3% ($p < 0.0001$) (Figure 1).

3.3. Validate Prognostic Value of ISS System in the Entire Cohort. The prognostic value of ISS system was evaluated in the total population of 1,016 patients. Patients were divided into stages I/II/III according to ISS system, and corresponding proportion in each stage was 22.7%/33.6%/43.7%, respectively. The median OS for ISS stages I/II/III was not reached/55.4 months/41.7 months (Figure 2), and the median PFS was 30/29.5/25 months ($p = 0.072$), respectively. From these data, we can conclude that ISS system can predict prognosis for OS, but for PFS in MM patients in the entire cohort.

3.4. Validate Prognostic Value of ISS System in Bortezomib-Based or Thalidomide-Based Group. In order to discern the impact of novel agents to the ISS system, subgroup analyses for OS were also performed in patients who received bortezomib-based treatment and thalidomide-based treatment in the first-line therapy.

In bortezomib-based group, 170 patients were in ISS-I, 236 in ISS-II, and 303 in ISS-III, with median OS being not reached/57.5 months/42.0 months and 3-year survival was 77.7%/75.8%/53.4%, respectively (Figure 3). Statistical difference was verified between ISS-I and ISS-III ($p < 0.0001$), ISS-II and ISS-III ($p < 0.0001$), and ISS-I and

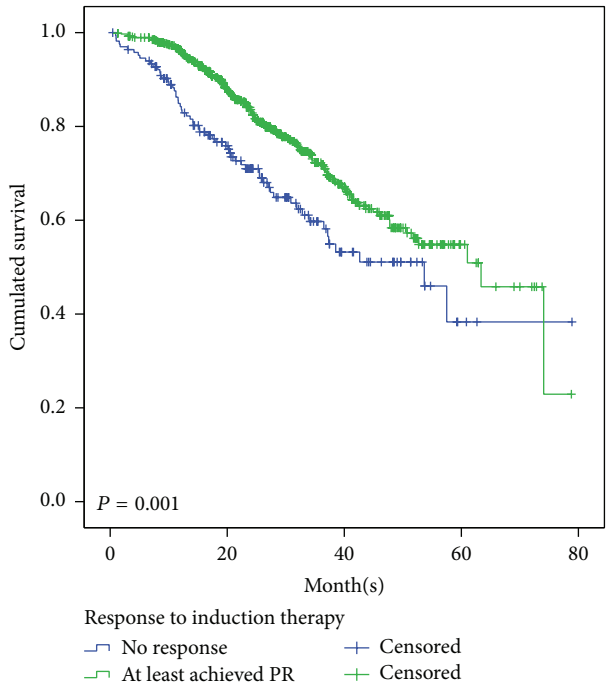

Figure 1: Overall survival (OS) for 1016 patients according to the response to the first-line therapy who at least achieved PR and who were below PR.

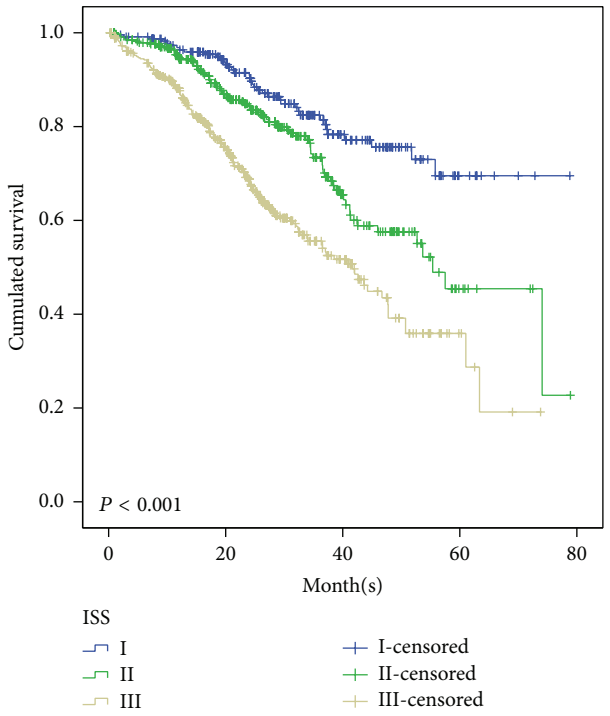

Figure 2: Overall survival for 1016 patients with newly diagnosed symptomatic multiple myeloma according to the ISS stages I, II, and III, who were treated with novel agents as the first-line strategy.

ISS-II ($p = 0.038$). In order to validate ISS system in transplant and nontransplant patients, we further stratified bortezomib-based patients into two subgroups according to whether they underwent transplantation after induction therapy. In bortezomib-based group, 177 patients underwent transplantation and 532 patients did not, the median OS was 51.8 months versus 57.5 months, respectively. The 3-year survival was 76.1% versus 69.2% versus 56.2% in nontransplantation group and 89.7% versus 86.7% versus 42.3% in transplantation group, respectively. The similar result was showed in transplantation and nontransplantation group, and no statistical difference was showed between ISS-I and ISS-II in bortezomib-based transplantation ($p = 0.413$) and nontransplantation groups ($p = 0.056$).

In thalidomide-based group, 61 patients were classified in ISS-I, 105 in ISS-II, and 141 in ISS-III, with median OS being not reached, 55.4 months, and 41.7 months, respectively (Figure 4). Statistically significant difference in survival was confirmed between three stages, ISS-I and ISS-II ($p = 0.024$), ISS-I and ISS-III ($p < 0.0001$), and ISS-II and ISS-III ($p = 0.047$), respectively. Among these patients, 282 patients underwent transplantation and 25 patients did not. Because the number in transplantation group is quite small, we do not carry out analysis in transplant group. In nontransplant group, 54 patients were in ISS-I, 95 in ISS-II, and 133 in ISS-III. There was statistical difference between ISS-I and ISS-II ($p = 0.009$) and ISS-I and ISS-III ($p < 0.0001$), but no statistical difference between ISS-II and ISS-III ($p = 0.089$). Although ISS prognostic significance disappeared between ISS-II and ISS-III in thalidomide-based nontransplant group, this may be due to the small number in ISS stages II and III; more studies are needed in the future.

4. Discussion

Multiple myeloma (MM) is characterized by heterogeneity in the clinical course and risk stratification is vital for prediction of prognosis. ISS is the most important prognostic system for MM in the past ten years. This system predicts survival of newly diagnosed MM patients by using two routine and inexpensive pieces of laboratory data and separated patients into three stages with a distinct prognosis [4]. Although ISS system was wildly used in Chinese myeloma patients in the past decade, the original analysis of ISS system from Greipp et al. did not include Chinese patients' data. Besides these, the survival of MM has dramatically changed by the introduction of novel agents, and nowadays the majority of patients received novel agent based treatment in the first-line therapy. Thus, the initial question that motivates our study was to determine whether ISS is suitable in the era of novel agents and in Chinese MM patients. This study aimed to provide outcome data for patients actually exposed to novel agents at first-line treatment.

In this analysis, we enrolled consecutive patients; thus the results may be more appropriate to the general group of myeloma patients, for patients enrolled in clinical trials were selected by some screening conditions. In this study, all of patients exposed to novel agents in first-line therapy. Survival in patients who achieved at least PR at induction

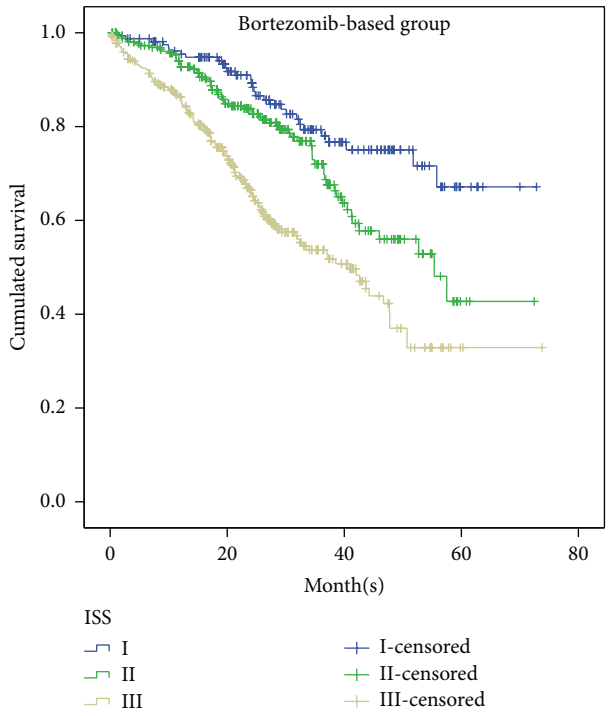

Figure 3: Overall survival for 709 patients with newly diagnosed symptomatic multiple myeloma according to the ISS stages I, II, and III, who were treated with bortezomib-based treatment as first-line therapy.

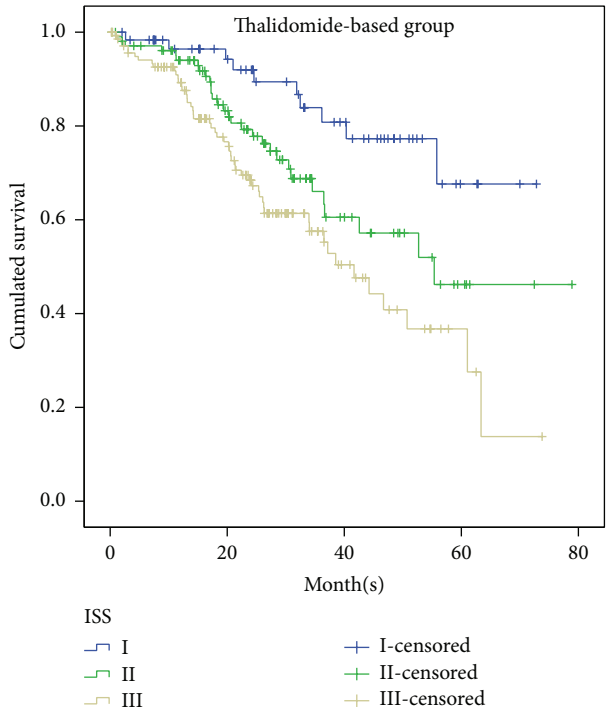

Figure 4: Overall survival for 307 patients with newly diagnosed symptomatic multiple myeloma according to the ISS stages I, II, and III, who were treated with thalidomide-based treatment as first-line therapy.

therapy is much better than those who did not responsd, due to better outcome with more aggressive therapy. These results confirmed the Dimopoulos et al.'s observations [12].

The ISS system still has prognostic significance value when applied to the total 1016 patients, with median OS for ISS stages I/II/III being not reached/55.4 months/41.7 months ($p < 0.001$). When ISS was proposed the median OS of MM patients in ISS stages I/II/III was 64, 44, and 29 months, respectively. Thus this study indicates that the survival of patients in each ISS stage is significantly improved in the era of novel agents. This significance partly disappeared when ISS implied to patients who received bortezomib-based regimens in the first-line treatment. We demonstrated the ISS system still has prognostic value in the era of novel agents in Chinese patients with MM, while in subgroup analysis it is not fully applicable and limited prognostic value in patients receiving bortezomib-based treatment in first-line therapy.

There is few data on the applicability of ISS in bortezomib-based treatment in the first-line therapy in literature. From previous studies we can indicate that MM patients can achieve deeper response by the use of novel agents, improved PFS and OS [13]. A meta-analysis performed by Zou et al. showed the addition of bortezomib to first-line therapy did significantly prolong OS compared with conventional therapy alone [14]. Some studies have showed that bortezomib-based regimens can improve outcome of patients with t(4;14), deletion of chromosome 13, and deletion of 17p, respectively [15–17]. This study also figured out patients with either t(4;14) or del(17p) presented in a higher ISS stage. We can find out that bortezomib has shown survival benefit in myeloma and overcome specific cytogenetic risk features in MM patients. This may partly explain in our study no statistical difference in OS between ISS stages I and II.

The ISS system was used as an independent prognostic system in the past, but it was unable to reflect the cytogenetic abnormalities of MM. Some new prognostic factors were increasingly found, such as fluorescent in situ hybridization (FISH), karyotype, and serum-free light chain [18–21]. These new prognostic factors can overcome this deficiency and provide cytogenetic or molecular genetics-based risk classification for MM patients. Many efforts have been made, such as proposing a new stage system by combination of ISS with FISH [22]. A recent study from IMWG combined ISS, CA, and LDH data to define Revised International Staging System (R-ISS) by following three risk categories: R-ISS I including ISS stage I, no high-risk CA [del(17p) and/or t(4;14) and/or t(14;16)], and normal LDH level; R-ISS III including ISS stage III and high-risk CA or high LDH level; and R-ISS II including all the other combinations. The data of R-ISS were enrolled on 11 clinical trials from 2005 to 2013. All patients received new drugs based chemotherapy as up-front treatment. The 5-year OS rate in R-ISS I, II, and III was 82%, 62%, and 40%, respectively. In our data, few patients had the data of chromosomal abnormalities (CA) detected by interphase fluorescent in situ hybridization after CD138 plasma cell purification. Compared with these IMWG studies, the majority of patients were in intermediate-risk group; in our study, 43.7% of patients were in high-risk group (ISS stage III). This distribution may explain worse survival in

our study in three stage groups. The R-ISS system can predict prognosis on OS in patients who did receive proteasome inhibitor based treatment, while in our study the ISS system cannot clearly distribute the OS of MM patients in ISS stages I and II. One interpretation might be that, compared with R-ISS system, ISS system may wrongly allocate a certain group of patients with poor prognosis in lower ISS stage [23].

There are many restrictive conditions for these new variables, for example, no consensus in standard classifications, not being easily available, and being too expensive. Their applications were limited by these passive factors. Thus, although novel prognostic factors such as FISH, karyotype, and serum-free light chain are important in MM risk stratification, the prognostic value of traditional serum markers still deserves attention. It can be an important component of new staging system in the future. Reevaluating the prognostic value of ISS system now is beneficial for the future research for a new staging system.

Because the initial retrospective study design from which these data are obtained was focused on clinical features and outcome in Chinese patients, the data analysis was presented with a number of challenges including (1) inconsistencies in patients feature among two groups, (2) the inability to study the effect of novel agents by using the same regimens in each group, and (3) needing a very long follow-up time and a very large patients' series to prove OS benefit in the era of new agents. Unfortunately, we could not evaluate the impact of adverse genetic markers in our cohort of patients because FISH studies were performed in a minority of patients.

5. Conclusion

In conclusion, our data is the first multicenter retrospective study in Chinese myeloma patients that validates ISS value in a large number of unselected patients. The results demonstrated that International Staging System still has prognostic value in the era of novel agents in Chinese patients with MM. However, that ISS system is not fully applicable in patients receiving bortezomib-based therapy at first-line treatment. These findings suggest that ISS system is predictive for OS of Chinese MM patients in the era of novel agents, but value is limited in PFS and in patients who were exposed to bortezomib in the first-line therapy. Bortezomib may have the potential to partially overcome adverse effect of risk factors on survival, especially in higher stage of ISS system. Further study is needed to develop more suitable staging system applied to MM patients in the era of novel agents which can reflect not only tumor burden and patient's condition, but also genetic risk classification.

Conflict of Interests

The authors declare that they have no competing interests.

Authors' Contribution

Jing Lu, Jin Lu, and Aijun Liu are equal contributors and are co-first authors.

Acknowledgments

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Review Article

Mechanism of Action of Bortezomib and the New Proteasome Inhibitors on Myeloma Cells and the Bone Microenvironment: Impact on Myeloma-Induced Alterations of Bone Remodeling

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Multiple myeloma (MM) is characterized by a high capacity to induce alterations in the bone remodeling process. The increase in osteoclastogenesis and the suppression of osteoblast formation are both involved in the pathophysiology of the bone lesions in MM. The proteasome inhibitor (PI) bortezomib is the first drug designed and approved for the treatment of MM patients by targeting the proteasome. However, recently novel PIs have been developed to overcome bortezomib resistance. Interestingly, several preclinical data indicate that the proteasome complex is involved in both osteoclast and osteoblast formation. It is also evident that bortezomib either inhibits osteoclast differentiation induced by the receptor activator of nuclear factor kappa B (NF-κB) ligand (RANKL) or stimulates the osteoblast differentiation. Similarly, the new PIs including carfilzomib and ixazomib can inhibit bone resorption and stimulate the osteoblast differentiation. In a clinical setting, PIs restore the abnormal bone remodeling by normalizing the levels of bone turnover markers. In addition, a bone anabolic effect was described in responding MM patients treated with PIs, as demonstrated by the increase in the osteoblast number. This review summarizes the preclinical and clinical evidence on the effects of bortezomib and other new PIs on myeloma bone disease.

1. Introduction

Bone disease, the hallmark of multiple myeloma (MM), is characterized by the presence of pure lytic lesions instead of solid tumors [1, 2]. Radiological bone lesions are found to be present in about 70–80% of newly diagnosed MM patients. It has been reported that 67% of MM patients display lytic lesions and 20% osteoporosis or pathologic fractures [3]. Up to 84% of the patients were found to develop skeletal lesion during the disease [3]. Skeletal-related events (SREs) consist of pathological or vertebral fractures, hypercalcemia, severe bone pain, and need for surgery/radiotherapy that affect the MM patients by decreasing the quality of life [4]. Although conventional radiography is the standard diagnostic procedure for the detection of skeletal involvement defining the presence of lytic lesions, its utility is limited as lytic lesions can be detected only after 30% trabecular bone loss [5]. The whole-body low-dose computed tomography (CT) is a reproducible technique for defining bone disease in MM patients with higher sensitivity compared to the conventional X-ray [6]. Magnetic resonance imaging (MRI) can show increased marrow cellularity due to myeloma cell infiltration, which is extremely useful in identifying the focal lesions in the absence of evident osteolysis [7]. Positron emission tomography combined with CT (PET/CT) using an 18-F labeled deoxyglucose (FDG) is being used to identify the focal growth of the myeloma cells in the skeleton [8, 9].

Osteolytic lesions are due to a profound alteration of the unbalanced and uncoupled bone remodeling process along with an increase in the osteoclast formation and activity together with the absence of osteoblastic response [2, 10]. Nitrogenous bisphosphonates are the mainstay therapy

approved for myeloma bone disease that induces osteoclast apoptosis by inhibition of mevalonate pathway, preventing SREs and reducing bone pain [11]. However, anabolic agents are not available for the treatment of myeloma bone disease. Therefore, this review aims to explore the mechanisms of action of the proteasome inhibitors (PIs), including bortezomib and other next generation PIs, with particular interest in their effects on osteoclast activity and anabolic effects on osteoblasts. The potential effect of PIs on patients with bone disease in a clinical setting will also be summarized and discussed in the paper.

2. Pathophysiology of Myeloma-Induced Alterations of Bone Remodeling

The interaction between myeloma cells and the bone marrow (BM) microenvironment, through vascular cell adhesion molecule-1 (VCAM-1) and $\alpha 4\beta 1$ integrin, stimulates the production of several proosteoclastogenic factors, including the receptor activator of nuclear factor kappa B (NF-κB) ligand (RANKL) [12]. The alteration of the RANK/RANKL pathway is the main mechanism involved in the bone destruction in MM [13, 14]. RANK is a transmembrane signaling receptor located on the surface of osteoclast precursors, whereas RANKL is expressed on BM stromal cells (BMSCs) and osteoblasts and secreted by activated lymphocytes [13, 14]. Through the NF-κB and JunN terminal kinase pathways, the RANK/RANKL signal enhances the osteoclast survival by increasing the bone resorption [13, 14]. Myeloma cells can disrupt the interplay between RANKL and its soluble decoy receptor osteoprotegerin (OPG) by increasing the RANKL and decreasing the OPG expressions and promoting the formation and activation of osteoclasts [15]. Moreover, several studies have demonstrated that the levels of soluble RANKL and OPG correlated with advanced bone disease having a prognostic impact [13]. The role of RANKL/OPG pathway in bone destruction has also been confirmed in murine MM models. These models have demonstrated that RANKL, either blocked by a soluble form of RANK receptor or OPG, has inhibited the bone destruction [13, 16]. The interaction between BMSCs and myeloma cells also stimulated the activation of NF-κB and p38 mitogen-activated protein kinase (MAPK) pathways. Specifically, the inhibition of p38 decreased the adhesion of myeloma cells to BMSCs, reduced the myeloma cell proliferation, and shortened the tumor burden in the murine MM model [17, 18]. Chemokine (C-C motif) ligand 3 (CCL3), being an RANKL independent inducer of osteoclast formation, can enhance both RANKL and interleukin- (IL-) 6 stimulated osteoclast formation [19]. The level of CCL3, produced directly by the human myeloma cells, correlates with the osteolytic bone lesions in MM patients [20, 21]. Moreover, either an antisense sequence anti-CCL3 or a neutralizing antibody against CCL3 reduces the bone destruction in mouse MM models [21]. IL-3 and IL-7 are also involved in osteoclastic bone resorption in MM [1, 22]. Moreover, Activin A, a member of TGF-β family, has been identified as a factor involved in IL-3 induced osteoclast activation in MM patients [23, 24].

Along with increased bone resorption, myeloma bone disease is characterized by suppressed osteoblast activity. MM patients show lower levels of bone formation markers, such as alkaline phosphatase (ALP) and osteocalcin (OC), and increased bone resorption markers [25]. Osteoblast suppression occurs mainly due to the blockage of the osteoblast differentiation from progenitors into the BM. The osteogenic differentiation of stromal cells requires the activity of the runt-related transcription factor 2 (Runx2/Cbfa1) [26]. The role of Runx2 in MM-induced osteoblast inhibition has been demonstrated in coculture systems performed between myeloma cells and osteoprogenitor cells [27]. Myeloma cells can inhibit osteoblast differentiation by reducing the number of both the early and late osteoblast precursors and decreasing the expression of ALP, OC, and type I collagen [27]. MM-induced Runx2 inhibition in the osteoprogenitor cells is mediated by the cell-to-cell contact between myeloma and osteoprogenitor cells [27]. Moreover, it has been reported that the MM patients had increased levels of transcriptional repressor Gfi1 compared with controls and that Gfi1 was a novel transcriptional repressor of Runx2 [28, 29]. IL-7 is involved in the Runx2 inhibition in osteoblast progenitors and in the consequent suppression of the osteoblast formation [27, 30]. Tumor necrosis factor- (TNF-) α is an inflammatory cytokine increased in MM and BM microenvironment that block osteogenic differentiation by suppressing the Runx2 and osterix expressions [31, 32]. Consistently, both anti-IL-7 and anti-TNF-α antibodies blocked the Gfi1 upregulation in BMSCs [28]. IL-3 has a dual role in myeloma bone disease; apart from stimulating the bone resorption, IL-3 can also inhibit the differentiation of preosteoblast at concentrations similar to those seen in BM plasma from MM patients [22, 33]. The inhibitors of the canonical wingless-type (Wnt) signaling, such as soluble frizzled-related proteins, sFRP-2, sFRP-3, and Dickkopf-1 (Dkk-1) [34–38], are involved in the pathogenesis of myeloma bone disease. The canonical Wnt signaling, through binding of Wnt proteins to the frizzled receptor and low-density lipoprotein receptor-related protein (LRP-5/6) coreceptor, leads to the translocation of β-catenin to the nucleus. Here, it interacts with members of the T-cell factor (TCF)/lymphoid enhancer factor (LEF) family in order to activate the osteoblast transcription factors and osteoblast formation [39]. Previous literature data indicate that the deregulation of canonical Wnt signaling in myeloma cells causing overexpression of Dkk-1 or frizzled-related protein gene FRZB is associated with a high incidence of bone lesions in MM patients [36, 37]. Moreover, higher Dkk-1 levels in BM correlate with the presence of focal bone lesions in MM patients [37].

Besides negative regulation of osteoblast differentiation, myeloma cells may affect osteoblast proliferation and induce osteoblast apoptosis in coculture systems by sensitizing cell death mediated by TRAIL [40–42]. In the last few years, studies have focused on the role of osteocytes, the terminally differentiated cells derived from osteoblasts, to partially regulate bone remodeling through cell death [43, 44]. Recently, studies have reported an increase of osteocyte death in MM patients bone disease in relation to the presence of bone lesions and the number of osteoclasts [45]. These data,

which were confirmed by ultrastructural *in vitro* analysis on coculture system, showed that myeloma cells can induce cell death in human preosteocytes, [45] which also regulate the osteoclast activities. In particular, living osteocytes produce soluble factors that inhibit osteoclast formation, whereas the apoptotic or autophagic osteocytes lose this inhibitory effect and promote bone resorption [46]. Indeed, apoptotic bodies produced from the osteocyte-like cells support osteoclastogenesis [46].

3. The Proteasome Complex and Its Inhibition

The proteasome, a multicatalytic enzyme complex located in the cytoplasm and cell nucleus, is involved in the adenosine triphosphate- (ATP-) dependent intracellular proteolysis by ensuring the rapid degradation of the target proteins with a chain of ubiquitin [47]. The ubiquitin-proteasome pathway (UPP) is the principal pathway by which the cellular proteins, such as the proteins involved in cell cycle, transcription, DNA repair, and apoptosis, are degraded [47, 48]. The control of the timed protein degradation is essential for controlling the intracellular protein levels and the cellular function [47–50]. The 26S proteasome is formed by 20S proteolytic core region and 19S regulatory particle [47–50]. The 20S core region is made up of 28 subunits arranged in four stacked heptameric rings to form a chamber where the proteolysis can occur [51]. The two outer and inner rings are composed of 7α and 7β different subunits, respectively, arranged one above the other as α-β-β-α [51]. Degradation of a protein involves coupling of a polyubiquitin chain through the action of three enzymes in an ATP-dependent manner [49, 51, 52]. This polyubiquitin chain acts as a flag to target the protein for degradation. When the ubiquitin molecules are removed, the protein is transferred into the inner catalytic chamber of the 20S proteasome where three different catalytic activities cleave the ubiquitinated protein into small peptides [52, 53]. The catalytic activities, linked to two central β-rings, are classified into three categories: chymotrypsin-like (CT-L), trypsin-like (T-L), and caspase-like (C-L) activities [51, 53]. Since UPP is involved in essential biological processes, the malfunction in this pathway is associated with a variety of diseases leading to the development of PIs. The malignant cells are more sensible to the inhibition of proteasome compared to the normal cells due to their high proliferation and protein synthesis rate. In particular, the clonal myeloma plasma cells secret high amount of immunoglobulin (Ig) which are generally transported out of endoplasmic reticulum through the unfolded protein response (UPR) pathway, for proteasomal degradation [54–56]. However, if the stress is prolonged and severe as caused by PIs, the UPR pathway leads to cell cycle arrest and apoptosis [54, 57, 58]. Thus, the proteasome inhibition occurring in MM patients is sufficient to kill the malignant plasma cells but not the normal cells [59, 60]. One of the first mechanisms attributed to PIs was the inhibition of the transcription factor NF-κB activity. It is well known that NF-κB plays an important role in promoting growth, survival, and chemoresistance of myeloma cells in BM through the regulation of IL-6 and insulin-like growth factor 1 (IGF-1) expression [61, 62]. Moreover, it regulates various tumor-related processes such as induction of angiogenesis and suppression of apoptosis [61, 63]. Inhibition of proteasome activity prevents degradation of the NF-κB inhibitor I-κB, which blocks the binding of NF-κB to the promoters of the target genes such as antiapoptotic genes and IL-6 [63, 64].

4. Proteasome Inhibition and Bone Microenvironment Cells

Proteasome inhibition is involved in bone remodeling. As described above, the binding of RANKL to RANK on the surface of osteoclast precursors activates NF-κB that promotes the osteoclast maturation and bone resorption [13, 14]. Thus, the proteasome-dependent inhibition of NF-κB leads to a reduction in the RANKL-mediated osteoclast differentiation. Moreover, it has been demonstrated that the PIs, MG-132 and MG-262, inhibit both osteoclast formation and resorption capacity, and this correlates with the extent of NF-κB binding capacity [65, 66].

On the other hand, the proteasome pathway also regulates the bone formation. It has been shown in an MM mouse model that treatment with PIs resulted in an increase in the bone mineral density and a concomitant reduction in the osteoclast numbers [67–69]. The compounds that inhibit proteasome activity, such as lactacystin and epoxomicin, stimulate bone formation in a dose-dependent manner affecting the increased expression of bone morphogenetic protein- (BMP-) 2 by osteoblasts [69]. This impact suggests that PIs and the proteasome pathway may have a role in bone remodeling.

Bortezomib, also known as PS-341, is the first class of PIs approved for treatment of MM [70–72]. Chemically, it is a dipeptidyl boronic acid that binds reversibly to CT-L subunit of the proteasome [73, 74] (Figure 1). It has also been reported to bind to C-L and T-L subunits with lower affinity [73, 74]. Although bortezomib is a reversible inhibitor, the boronate-proteasome complex has a low degree of dissociation and remains stable for several hours [74].

An increasing number of studies focused on the role of bortezomib in MM-related bone disease. It has been demonstrated that bortezomib affects RANKL-induced osteoclast differentiation in a dose-dependent manner in both the early and late stages through the modulation of p38, activator protein-1 (AP-1), and NF-κB pathways [65, 66]. The SCID-rab mice bearing myeloma, additionally, showed a reduction in the osteoclast number after the bortezomib treatment [75].

Bortezomib not only inhibits the osteoclast function but also affects the osteoblast differentiation. In preclinical models, it has been reported that bortezomib can induce osteoblast phenotype in human mesenchymal stromal cells (MSC) without affecting the number of osteoblast progenitors and viability of mature osteoblasts [76]. The *in vitro* effect was associated with an increase in both the Runx2 activity and expression of osteoblast markers such as type I collagen, without affecting the canonical Wnt signaling [76]. These *in vitro* observations also confirmed the bone biopsies of MM patients treated with bortezomib showing

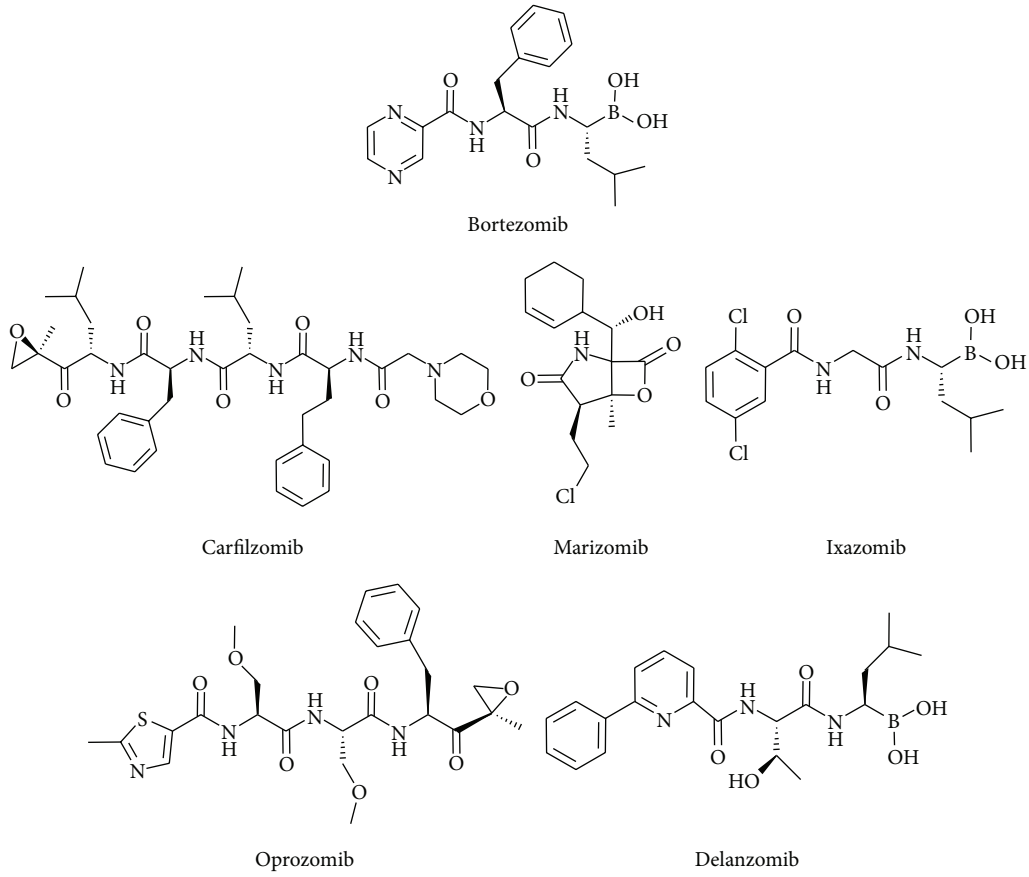

Figure 1: Bortezomib and the new PIs. Chemical structure of bortezomib and the new PIs. PIs: proteasome inhibitors.

that the responding patients had more osteoblastic and Runx2-positive cells compared to the control groups [76]. The bone anabolic effect of bortezomib relies on the activation of β-catenin/TCF signaling. This effect shows that bortezomib promotes matrix mineralization by osteoprogenitor cells through the stabilization of β-catenin and induction of TCF transcriptional activity [77]. It has been demonstrated that bortezomib can enhance the differentiation of murine MSCs towards osteoblasts, rather than the more differentiated osteoblast progenitors [78]. Moreover, in both the mouse models implanted with MSCs and osteoporosis, the treatment with low doses of bortezomib resulted in an increase in the bone formation. No effect on the osteoclast activation and differentiation generated from murine BM mononuclear cells was observed [78]. It was also demonstrated that bortezomib stabilizes Runx2 activity consistently with the previous studies concluding that PIs should prevent Runx2 degradation [69]. Further, bortezomib and other PIs also stimulate the bone formation in mouse calvarial organ culture by increasing the BMP-2 production. This is positively correlated with their ability to inhibit the proteasome activity [69, 79]. One of the possible mechanisms in which the PIs stimulate BMP-2 expression involved the protein Gli-3. Gli-3 is degraded in a proteasome-dependent manner and its truncated form is a potential inhibitor of BMP-2 transcription. Its overexpression in osteoblast precursors has been reported to inhibit the effects of PIs on BMP-2 expression. The PIs are also able to prevent the proteolytic processing of Gli-3, the generation of its truncated form, and the suppression of BMP-2 gene transcription [69]. On the other hand, in another study, bortezomib was found to increase the expression of ALP and OC in mesenchymal cell line with an effect similar to BMP-2, but without affecting the BMP-2 target gene expression [80]. Bortezomib inhibits Dkk-1 gene expression and protein level in both mice treated with *calvariae* and BMSCs, which also suggested its ability to modulate the canonical Wnt signaling [79]. Using the severe combined immunodeficiency- (SCID-) rab mouse as a model, it has been reported that the mice responding to bortezomib showed a significant increase in both BMD and osteoblast and decrease in osteoclast numbers [67]. The increased BMD is not seen in responsive melphalan-treated mice, suggesting that the effect of bortezomib on bone is not only due to the tumor burden reduction. A histomorphometric analysis revealed that the myelomatous bones from bortezomib-treated hosts showed increased trabecular thickness and trabecular numbers associated with a higher number of osteoblasts and a lower number of osteoclasts in comparison to the control groups [67]. Osteoblasts and MSCs express the vitamin D receptor (VDR), and the effects of vitamin D on osteogenic differentiation have been demonstrated both *in vitro* and in mouse models [81, 82]. Recently, it has been

showed that the simultaneous treatment with bortezomib and vitamin D strongly stimulated the VDR signaling and increased the vitamin D-dependent expression of osteoblastic differentiation markers, such as OC and osteopontin, by both the human MSCs and osteoblasts. Bortezomib also blunts the downregulation of OC and osteopontin, induced by coculture with myeloma cells [83]. Moreover, the stimulatory effect of bortezomib on VDR signaling may be due to the decreased proteasomal degradation of the VDR [83].

Recently, *in vitro* data indicated that the bortezomib or MG262 treatment for 12–24 hours would significantly blunt the osteocyte cell death induced by the myeloma cells. In addition, treatment with PIs reduced the high doses of dexamethasone-induced death of MLO-Y4. Parathyroid hormone (PTH) short-term treatment also potentiated the *in vitro* effects of bortezomib and MG262 on the dexamethasone-induced death of osteocytes [84]. The data also indicated that the anabolic effects of bortezomib and PIs may have been mediated by their impact on the osteocytes rather than on osteoblasts.

Thus, several mechanisms underlying the effects of PIs and bortezomib on bone remodeling demonstrate that these drugs inhibit osteoclast formation and activity with a significant anabolic effect (Figure 2).

5. Second Generation of PIs and Their Possible Effects on Bone Remodeling

Recently, novel PIs have been developed to overcome bortezomib resistance. The second generation of PIs, such as carfilzomib, marizomib, ixazomib, oprozomib, and delanzomib, differed in the chemical structure, biological properties, and mechanisms of action [85] (Figure 1).

Carfilzomib (PR-171) is a tetrapeptide epoxyketone analog of epoxomicin, an epoxyketone family member of natural PIs [86, 87]. It binds irreversibly to CT-L catalytic subunits of proteasome so that the reestablishment of proteasome function is possible only by the synthesis of new single subunits [88, 89]. In high doses, it also inhibits the T-L and C-L activities [87]. In contrast to bortezomib, which binds with different serine proteases contributing to some of the neurotoxicity, carfilzomib binds irreversibly with proteasome only and not with other proteases [86–89]. Preclinical studies have demonstrated that the greater selectivity of carfilzomib for the CT-L, compared to bortezomib, revealed little off-target activity and dose flexibility in the xenograft models [87–89].

Recently, it has been demonstrated that carfilzomib stimulates, *in vitro*, MSCs differentiation into bone-forming osteoblasts by increasing the matrix mineralization and calcium deposition [68, 90, 91]. Osteoblasts derived from MM-MSC patients, treated with clinically relevant doses of carfilzomib, showed an increase in the ALP activities [68]. Carfilzomib inhibits osteoclast differentiation and function at cytotoxic concentrations to myeloma cells without affecting the precursor viability. This effect seems to be due to the disruption of RANKL-induced NF-κB signaling and the reduced $\alpha V\beta 3$ integrin expression involved in bone resorption activities of osteoclasts [68]. During the osteoblast differentiation, carfilzomib reduced RANKL expression by inhibiting their ability to stimulate osteoclastogenesis. The *in vitro* evidences were confirmed by the *in vivo* studies on both the non-tumor bearing mice and 5TMG1 model, which suggested that the potential efficacy of the treatment in other pathological disorders is characterized by bone disease [68]. The molecular mechanisms by which carfilzomib promotes MSC differentiation are still under investigation. It has been reported that β-catenin/TCF pathway is involved in regulating the MSCs and osteoblasts differentiation [39]. Carfilzomib also induces the Wnt-independent nuclear accumulation of active β-catenin as well as the activation of the transcription factor TCF in both osteoblastic-like cell and stromal cell lines in the MM-MSC patients [90]. In the last years, several authors have shown that Notch1 pathway regulates the osteogenic differentiation by suppressing the Runx2 activity in BM mesenchymal progenitors [91–93]. Moreover, the induction of osteogenic differentiation suppresses the Notch1 activity. Recently, it has been demonstrated that the carfilzomib-induced stimulation of osteogenesis is associated with Notch1 signaling inhibition [91]. The role of carfilzomib in PTH signaling is to inhibit the PTH-induced *RANKL* mRNA expression by blocking the histone deacetylase 4 (HDAC4) proteasomal degradation in osteoblasts [94]. However, carfilzomib fails to affect the PTH-dependent inhibition of OPG. Using coculture system between osteoblastic cell line and osteoclast precursors cells, it has been shown that high concentrations of carfilzomib can inhibit PTH-induced osteoclast formation and activity. This inhibition decreases the NF-κB activation without affecting the cell viability [94].

Marizomib (NPI-0052) is the first natural PI included in the MM clinical research [95, 96]. It is an orally bioactive β-lactone derived from obligate marine bacteria actinomycetes, *Salinispora tropica*, and is structurally different from bortezomib and carfilzomib [96]. Marizomib inhibits all the enzymatic activities of proteasome binding with high affinity to the CT-L and T-L catalytic sites and lower affinity to the C-L site [96]. Similarly to bortezomib, marizomib also inhibits the canonical NF-κB pathway and secretion of IL-6, TNF-α, and IL-1β but at lower concentrations than bortezomib [97, 98]. Bortezomib requires caspase-8 and caspase-9, whereas marizomib induces the apoptotic effect mainly through caspase-8 signaling that allows it to overcome the resistance of myeloma cells conferred by Bcl-2 mutations [97, 98]. The overexpression of Bcl-2 is demonstrated to protect the myeloma cells by bortezomib and to some extent by marizomib too, due to its caspase-9 activation [98]. The marizomib potentiated apoptosis is induced by TNF-α, bortezomib, and thalidomide with a concomitant downregulation of cell proliferation and survival proteins (such as cyclin D1, c-Myc, Bcl-2, Bcl-xl, and survivin). The protein involved in migration and angiogenesis, such as matrix metalloproteinase (MMP-9) and vascular endothelial growth factor (VEGF), also induces the apoptosis [99]. Marizomib did not affect the viability of BMSCs, rather blocked the production of IL-6 that is triggered by myeloma cells and BMSC interaction. It also induced apoptosis in myeloma cells in the presence of IL-6 and IGF-1 [98, 99]. The potent

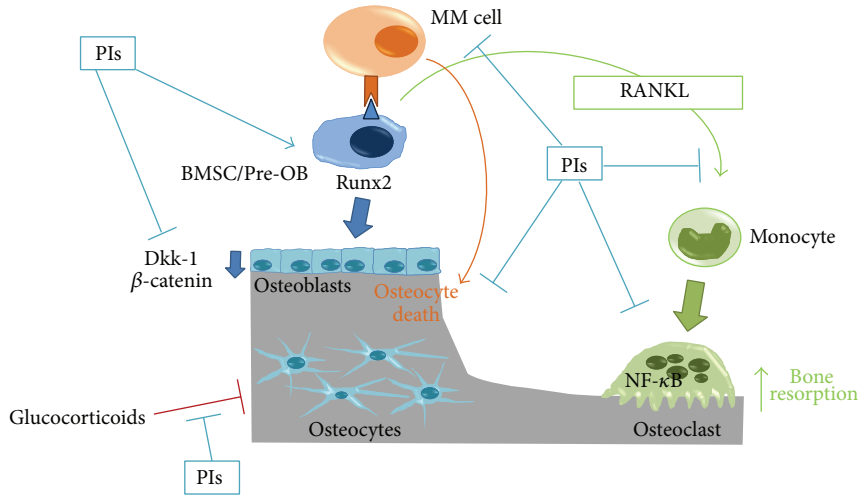

Figure 2: Effects of PIs on osteoblast and osteoclast remodeling in MM. PIs directly act on myeloma cells and on MM-induced alterations of bone remodeling. PIs block osteoclast formation from monocyte and the effects of RANKL on osteoclastogenesis. A direct effect of PIs on mature osteoclast has been shown. PIs stimulate osteogenic differentiation of BMSCs and osteoblast progenitors increasing osteoblast number and function. A stimulatory effect of PIs on the osteogenic transcription factor Runx2 has been demonstrated. PIs reduce Dkk-1 production and consequently affect β-catenin. PIs stimulate osteocyte viability and blunt the effect of glucocorticoid on osteocytes. PIs: proteasome inhibitors; RANKL: receptor activator of nuclear factor kappa B (NF-κB) ligand; BMSC: bone marrow stromal cell; Pre-OB: osteoblast progenitor.

antitumor activity of marizomib has been confirmed *in vivo* studies on a human plasmacytoma xenograft mouse model [98].

A few studies have focused on the marizomib's effect on osteoclastogenesis and osteoblastogenesis. Nevertheless, it was well demonstrated that carfilzomib inhibits RANKL-induced osteoclastogenesis without affecting the viability of osteoclast-like cells [99].

Ixazomib (MLN9708), an analog of boronic acid, is orally administered with greater potential activity against myeloma cells than bortezomib [96, 100]. In comparison to bortezomib, ixazomib hydrolyzes immediately in the aqueous solution or plasma to its biologically active form MLN2238, displaying shorter half-life and wider distribution in blood [100–102]. It inhibits not only the CT-L subunit in higher concentrations, but also the C-L and T-L subunits of the proteasome. Ixazomib showed a shorter proteasome dissociation half-life than bortezomib by improving its pharmacokinetic and pharmacodynamic profile [100–103]. The antitumor activity of ixazomib depends on the activation of caspase-8, caspase-9, and caspase-3 and the upregulation of p53 and p21. Treatment with ixazomib upregulates the transcription factors that respond to the endoplasmic reticulum stress. It also inhibits both the canonical and noncanonical NF-κB signaling in myeloma cells by reducing the BMSCs-induced proliferation of myeloma cells [96].

Ixazomib inhibits *in vitro* osteoclastogenesis and osteoclast resorption. These effects involved the F-actin ring damage, blockade of the NF-κB activation induced by the RANKL, and downregulation of $\alpha V\beta 3$ integrin. Ixazomib also promotes *in vitro* osteoblastogenesis and osteoblast activity, at least in part, by the activation of β-catenin/TCF signaling. It also encourages the upregulation of the inositol-requiring enzyme 1 (IRE1) component of the unfolded protein response. Finally, ixazomib demonstrates significantly reducing the bone disease in MM mouse model [100].

Oprozomib (ONX0912) is a novel orally administered epoxyketone that is derived from carfilzomib [104]. It binds irreversibly to CT-L subunit of proteasome, resulting in longer duration of inhibition compared to bortezomib [104]. In an *in vitro* study, it has been demonstrated that oprozomib inhibits growth and migration of myeloma cell lines and induces apoptosis through the activation of caspase-8, caspase-9 and caspase-3 and poly(ADP) ribose polymerase (PARP) [104].

Compared to carfilzomib, oprozomib inhibits osteoclast differentiation and functions without affecting the osteoclast precursor viability. The treatment of osteoclast precursors with oprozomib inhibited RANKL-induced NF-κB activation caused by the damaged proteasomal degradation of I-κB. Similarly, oprozomib promotes osteogenic differentiation *in vitro* increasing the ALP activity and other osteogenic markers that modulate the transforming growth factor β (TGF β), MAPK, and UPR pathway [68]. Both the antitumor and anabolic effects of oprozomib were confirmed in an *in vivo* mouse model [68].

Delanzomib (CEP-18770) is a boronic acid-based PI that inhibits, reversibly, CT-L and C-L activities [105]. Delanzomib has been demonstrated to decrease the NF-κB activity and induce apoptosis in myeloma cells with a good cytotoxic profile towards normal cells [105, 106]. In the preclinical mouse model of MM, it has been demonstrated that delanzomib can efficiently induce an improved response against bortezomib-resistant cells [106]. During the culture, delanzomib showed antiangiogenic and antiosteoclastogenic

activities [105]. In the preclinical studies, delanzomib showed an enhanced anti-MM activity of bortezomib and melphalan, and it also reduced the tumor growth in combination with dexamethasone and lenalidomide [107, 108].

6. Effects of PIs Treatment on Bone Disease in MM Patients

The original observation by Zangari et al. [109] on ALP increase in a 63-year-old woman affected by relapse MM responding to bortezomib encouraged more large-scale analysis in three data sets from clinical trials. This confirmed a correlation between ALP increase and its response to bortezomib therapy [109]. Retrospective analysis of ALP variation in SUMMIT and APEX trials displayed a statistically significant difference in the median levels of ALP in responders to bortezomib versus nonresponders maximum in the eighth and sixth week, respectively [110–112]. In the APEX trial, considering only the responding patients of both the groups, median ALP variation was higher in bortezomib group in comparison to the dexamethasone group. This observation suggests that both the direct and indirect effects on the bone disease occurred during the bortezomib treatment [112]. Similarly, a recent retrospective analysis of 67 relapse or refractory MM patients who were treated with carfilzomib demonstrated that elevation in ALP levels correlated with the response to the treatment [113].

Several studies after the APEX trial analysis confirmed the positive effects of PIs on bone formation and resorption markers [114–121].

Biochemical markers of the bone remodeling represent an important tool to check the alterations in the bone turnover that occurs in MM patients with extensive bone disease. They are particularly useful in evaluating the response to the antiresorptive or anti-MM therapy with a significant impact on the bone turnover. Bone resorption markers are known to include collagen N-terminal cross-linking telopeptide of type I collagen (NTX), C-terminal cross-linking telopeptide of type I collagen (CTX), and I-terminal cross-linking telopeptide of type I collagen (ICTP) that represent bone-specific products of osteoclast-mediated degradation of triple-helix collagen. Tartrate-resistant acid phosphatase isoform-5b (TRACP-5b) is an osteoclast-specific serum enzyme that reflects the total osteoclastic number and activity. Bone formation markers include procollagen type I N-propeptide (PINP) and procollagen type I C-propeptide (PINC) derived from degradation of procollagen during the deposition of bone matrix. Bone-specific ALP (bALP) and OC are well-known indicators of osteoblast bone formation and activity [122].

Terpos et al. [119] showed an increase in bALP and OC in the relapse MM patients treated with twice-weekly bortezomib for four cycles. The change in bALP was marked in responders versus nonresponders and correlated significantly with the type of response. Dkk-1 levels at baseline were increased both in the study population compared to the control groups and in the MM patients with high of bone disease compared to all other groups [119]. After four cycles of bortezomib, Dkk-1 serum levels decreased significantly compared to the baseline, irrespective of the response to the treatment. Markers of bone resorption TRACP-5b and CTX and osteoclast regulator soluble RANKL (sRANKL) were significantly reduced after the treatment [119]. In another study, serum CTX and urinary NTX were evaluated before and after three days of each bortezomib administration performed on three MM patients [123]. Bortezomib induced a significant reduction percentage after two days compared to that in the baseline with a trend of increment after three days [123]. Lund et al. [117] assessed that the variations in bone turnover markers included bALP, PINP, Dkk-1, and NTX-I in the bisphosphonate-naïve and untreated MM patients. All patients received four cycles of twice-weekly bortezomib, initially as monotherapy and then combined with dexamethasone from the second to the fourth cycle. In the responders, bone formation markers bALP and PINP increased to the maximum value on day 42 [117]. A temporary decrease of PINP was also observed every time dexamethasone was added. Dkk-1 and NTX levels decreased to 25% and 50% in the responding patients, respectively. No changes in the bone remodeling markers were detected in nonresponders, except for a little decrease in NTX [117].

A post hoc analysis of phase III VISTA was conducted to assess the clinical skeletal events and the serum modifications in ALP and Dkk-1 during the treatment. The untreated MM patients, not eligible for transplantation, were randomized to bortezomib-melphalan-prednisone (VMP) or melphalan-prednisone (MP) alone. Bisphosphonate therapy was allowed during the treatment and follow-up period. The increase in maximum median ALP from baseline to any time point was higher by response in VMP group versus MP group, both in patients achieving CR and PR [118]. It was also noted that a statistically significant Dkk-1 reduction in serum from baseline to the day 4 of the first cycle showed opposite results to the increase in the MP subgroup. Six out of 11 patients in the VMP arm were assessed by skeletal imaging (X-ray or CT) both before and after baseline, and they showed signs of bone sclerosis suggesting an initial process of bone healing, but none was observed in MP arm [118].

In a multicenter prospective study, the primary endpoint was the bone markers variation recorded before and after four cycles of twice-weekly bortezomib in association with other agents in relapse MM patients [120]. A reduction in the Dkk-1 levels was recorded after bortezomib treatment, and the levels of OC and bALP were also found to decrease both in the responders and in nonresponders. Remarkably, the same bone markers variation was not significant in the patient group without steroid combination, which confirms the detrimental role of steroids on bone neoformation to overcome the bortezomib positive effect on osteoblast function.

Recently, a prospective study was conducted to compare the bone markers changes in 99 relapse MM patients treated with drugs combinations of lenalidomide-dexamethasone (LD) or bortezomib-lenalidomide-dexamethasone (VRD) [121]. In the VRD arm, a marked increase in bALP and OC and a reduction in sRANKL/OPG, Dkk-1, and CTX were observed after the third and sixth cycle, irrespective of

the response to the treatment. RD arm patients showed an increase of Dkk-1 after six months of therapy and a significant reduction of CTX levels in responders as compared to the nonresponding patients without any other significant alterations on bone biomarkers. Additionally, two refractory patients in RD subgroup developed SREs but none in VRD. This study supports the positive bortezomib role in enhancing the bone formation and preventing bone resorption while the lenalidomide alone retains a minor effect on the bone resorption.

In addition to the studies on the markers of bone turnover, a histomorphometric study was conducted by Giuliani et al. [76] on the BM biopsies of 21 MM patients before and after the sixth to eighth cycles of twice-weekly bortezomib. This study, for the first time, displayed a significant increase in the number of osteoblastic cells/mm^2 of bone tissue in MM patients responding to the bortezomib treatment but not in the nonresponders. Immunohistochemical staining observed a significant increase in the number of Runx2-positive osteoblastic cells in the responding MM patients compared to the nonresponders [76]. This study clearly consolidates the notion of the anabolic effect of bortezomib treatment in the MM patients.

The positive anabolic effect of bortezomib on bone healing and new matrix deposition has also been investigated by bone imaging techniques [118, 124–128]. The BMD was evaluated by dual-energy X-ray absorptiometry (DEXA) after the completion of eight cycles of twice-weekly bortezomib-dexamethasone therapy and bisphosphonates used in the 27 relapse MM patients. A total of 66% of the patients had lytic lesions in less than three areas, and 51% had osteoporosis at baseline DEXA. A significant increase in BMD was detected in the axial skeleton (L2–L4) and not in the appendicular skeleton (femoral bones). The BMD improvement correlated with the reduction of urinary NTX and increase in the serums bALP and OC [124]. Zangari et al. [125] assessed BMD changes by DEXA in 13 smoldering MM patients treated with weekly low-dose bortezomib (0,7 mg/m^2) for nine cycles. They showed an improvement in the T-score of hip and lumbar spine at the end of the treatment in sixth and third cycle, respectively. In a case report, the effect of bortezomib as a single agent or in combination with other drugs on myeloma bone lesions was assessed using technetium-99m-(99mTc-) methyl-diphosphonate (MDP) bone scans in two MM patients.

Tc-99m MDP bone scans after the treatment revealed multiple densities with an increase in the uptake of the radiotracer on bone surfaces that is consistent throughout the new bone deposition [126]. Bone structure and remodeling alterations were also assessed by bone markers, micro-CT, bone histomorphometry, and tetracycline labeling in 16 relapse MM patients treated with twice-weekly bortezomib as a single agent. Serums bALP and OC increased considerably in the responding patients after the first cycle. In addition to bALP and OC, the increase in PTH levels was observed in the responders on day 11. Micro-CT measurements on biopsy specimens obtained on the baseline and at the end of the study showed an increase in the bone volume/total volume (BV/TV) and trabecular thickness (TbTh) after 12 doses of bortezomib and tetracycline incorporation in 63% of the analyzed biopsy samples [127]. A recent study evaluated the frequency, extent, and the patterns of BM sclerosis detected by whole-body reduced-dose CT in 79 MM patients. CT examinations were performed at baseline, during therapy, at the end, and 12 months after the termination of bortezomib treatment. Sclerosis was found to develop in 14 patients, either focal or diffuse. The mean time for the detection of skeletal sclerosis was eight months. In six patients, the mean size reduction of lytic lesions was >40%. Two patients, who were evaluated after one year from bortezomib discontinuation due to the absence of subsequent specific therapy, showed a size decrement of 17% and 100%, respectively. A considerable sclerotic modification in cancellous bone was seen in patients having no evaluable lytic bone lesions at baseline evaluation [128]. These clinical evidences further confirmed that bortezomib treatment may induce the bone healing in MM patients.

7. Conclusions

Osteolysis is the hallmark of MM. Bortezomib and the new PIs, which are currently being investigated in clinical trials, can affect bone remodeling. Osteoclastic formation and activity are inhibited by PIs, mainly through the blockade of RANKL signaling pathway in the osteoclast progenitors. However, the more significant impact of the bone remodeling by this class of drugs is the capacity to stimulate either the osteogenic differentiation of MSC or the osteoblastic function, leading to the consequent bone formation with a considerable anabolic effect. Osteocytes are also possible targets of PIs with a stimulatory effect on their viability. The preclinical evidence, thus, is confirmed in MM patients treated with bortezomib and more recently with carfilzomib. An improvement of the bone remodeling markers was observed in the patients treated with PIs. The histomorphometric data in MM patients treated with bortezomib prominently indicated that PIs can stimulate the bone formation process and induce the bone regeneration process. Bone healing, as well as an increase in the BMD, has also been reported in some of the patients treated with bortezomib. Overall, the literature data support the use of these drugs to restore bone integrity in MM patients.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Fabrizio Accardi and Denise Toscani equally contributed to the paper.

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Review Article

Mechanisms and Clinical Applications of Genome Instability in Multiple Myeloma

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Ongoing genomic instability represents a hallmark of multiple myeloma (MM) cells, which manifests largely as whole chromosome- or translocation-based aneuploidy. Importantly, although it supports tumorigenesis, progression and, response to treatment in MM patients, it remains one of the least understood components of malignant transformation in terms of molecular basis. Therefore these aspects make the comprehension of genomic instability a pioneering strategy for novel therapeutic and clinical speculations to use in the management of MM patients. Here we will review mechanisms mediating genomic instability in MM cells with an emphasis placed on pathogenic mutations affecting DNA recombination, replication and repair, telomere function and mitotic regulation of spindle attachment, centrosome function, and chromosomal segregation. We will discuss the mechanisms by which genetic aberrations give rise to multiple pathogenic events required for myelomagenesis and conclude with a discussion of the clinical applications of these findings in MM patients.

1. Introduction

Multiple myeloma (MM) is a clonal B-cell malignancy characterized by excessive bone marrow plasma cells in association with monoclonal protein [1, 2]. The therapeutics currently available improve patients' survival and quality of life, but resistance to therapy and disease progression remain unsolved issues [3, 4]. Therefore, the definition of novel targeted vulnerabilities in MM biology remains a major basic and clinical research goal. Recent studies have demonstrated that MM is characterized by a significant heterogeneity, which is mainly related to molecular characteristics of the tumor clone [5]. Such feature, occurring also at early stages, makes MM quite different from other hematologic diseases such as leukemia and lymphomas that harbor a restricted number of genetic changes. By contrast, a wide variety of chromosomal and genomic rearrangements are frequently observed in solid tumors. Thus, MM is considered in between these two genetic landscapes with a complex oncogenic network deregulation [6].

Genome instability, defined by higher rate of genomic changes acquisition per cell division compared to normal cells, represents a prominent feature of MM cells [7]. There are various forms of genetic instability such as chromosomal instability (CIN), microsatellite instability (MSI), and base-pair mutations. CIN refers to the high rate by which chromosome structure and number changes in MM cells compared with normal cells. Numerical chromosome abnormalities may be generated by centrosome amplification or alterations in the spindle assembly checkpoint [8]. In contrast, structural alterations, such as chromosomal deletions or translocations, might arise from alterations in the repairing of DNA double strand breaks (DSBs). The specific contribution of each event in MM tumorigenesis is not fully understood, but the most frequently observed changes include hyperdiploidy [9], loss of chromosome 13 [10, 11], and specific translocation like t(11;14) (q13;q32); t(4;14)(p16;q32); or t(14;16)(q23;q32) [12–15]. Such aneuploidy can be interpreted as a consequence of the general chaos that progressively envelops cancer cells

as they advance toward highly malignant states, or it is an inherent element of tumorigenesis. Indeed, in absence of the increased mutability associated with aneuploidy, most clones of incipient tumor cells could never succeed in acquiring all genetic alterations needed to complete multistep tumorigenesis. Therefore, cancer cells by changing their genomes through chromosome instability create promising configurations that allow growth of neoplastic cells. Although CIN represents the most common form of genomic instability, others have also been described including microsatellite instability, characterized by the expansion or contraction of the number of oligonucleotide repeats present in microsatellite sequences, and the base-pair mutations which refer to increased frequencies of base-pair mutations in tumor cells [7]. Overall, the comprehensive karyotypic analysis provides insights into molecular mechanisms and clinical management of MM. Indeed, chromosomal aberrations allow identifying two broad subtypes of disease, one characterized by chromosomal gains (hyperdiploidy) and the other by structural changes (nonhyperdiploidy), leading to different results in terms of prognosis [9].

However, causes of genomic instability remain to date unclear thus failing identification of universal driver event in MM cells. An increased c-MYC expression, K-RAS mutations and fibroblast growth factor receptor-3 (FGFR3) overexpression seem to be the most frequently genetic aberration observed during disease progression [16]; nevertheless additional genetic abnormalities further contribute to increase genetic complexity of such a tumor. It follows that MM genome is extremely heterogeneous with marked changes affecting both prognostic stratification and therapeutic approaches. In addition to this inter-MM heterogeneity, deep genome sequencing studies proved existence of intraclonal diversity affecting MM patients individually with altered clones present at diagnosis and during disease evolution [17–19]. Accordingly, genetic instability by supporting mutations development hugely increases complexity of MM, by allowing survival advantage and progression.

Based on these findings, here we will review the significance of this heterogeneity in MM cells, by focusing on biological relevance of genomic instability, and examining how the currently available therapeutic strategies can exploit this feature.

2. Heterogeneity of MM

A hallmark of almost all human cancers is represented by aberrations in their genomic architecture, which refers to permanent or temporary changes [18]. Among these alterations, CIN (gain or loss of whole chromosomes as well as inversions, deletions, duplications, and translocations of large fragments of chromosomes) is frequently observed in numerous solid tumors. As such this abnormality results in large-scale changes of genes, which are involved in cellular processes critical for maintenance of genome integrity during disease progression [20]. Based on these findings the two categories identified, *hyperdiploid* and *nonhyperdiploid*, show different prognostic significance with the latter associated with poorer overall survival (Table 1). Specifically, trisomies of chromosomes 3, 5, 7, 11, 15, 19, and 21 define hyperdiploid karyotype (50–60% of MM patients); otherwise the nonhyperdiploid karyotype is frequently characterized by translocations affecting immunoglobulin heavy chain (IGV) locus at 14q32, including t(11;14) and t(4;14), which are the most clinically relevant. Indeed t(11;14) is observed in 20% of MM patients and confers favorable outcome [11, 21]. Differently, t(4;14) occurs in 15% of MM patients and is associated with very poor prognosis with its presence requiring specific therapeutic approaches such as proteasome inhibitors or immunomodulatory agents [5]. Molecularly t(4;14) results in simultaneous overexpression of two genes located on 4p: the multiple myeloma SET domain (MMSET), which is a homologous of histone methyltransferase, and the fibroblast growth factor receptor 3 (FGFR3), which is an oncogenic receptor tyrosine kinase. As both genes have potential oncogenic activity, their deregulation triggered by chromosomal aberration is associated with poor survival. In general t(4;14), t(14;16), chromosome 13 deletion, and loss of 17p13 are associated with poor prognosis in patients undergoing high-dose therapy, whereas hyperdiploidy and t(11;14) translocations are associated with better outcome. One less frequent (6-7% of MM patients) but clinically relevant translocation is t(14;16), which involves MAF genes and confers poor outcome [22]. del(17p) is carried by 8–10% of patients and represents the most important aberration for prognosis since its presence is associated with a remarkable short survival irrespective of treatment [23]. Finally, several reports have shown additional abnormalities such as amp(1q), del(1p), del(12p), del(16q), and del(6q) having prognostic relevance, with the latter associated with worse prognosis then del(17p) [24, 25].

Table 1: Recurrent chromosomal aberrations observed in MM and their prognostic relevance.

Aberration	Incidence	Outcome
Trisomies of chromosomes 3, 5, 7, 11, 15, 19, and 21	60%	Favorable
t(4;14)	15%	Poor
t(11;14)	20%	Favorable
t(14;16)	6-7%	Poor
del(17p)	8–10%	Poor

A large number of chromosomal changes including MYC translocations, loss or deletion of chromosome 13, deletions and/or amplifications of chromosome 1, and deletion of chromosome 17, are observed also during MM progression [18]. Indeed, about 45% of MM patients with advanced disease carry translocations and/or amplifications of the oncogene MYC that is associated with more aggressive disease. Also deletion of 17p13 is a late event occurring in 10% of MM patients and results in TP53 inactivation with poor prognosis.

Based on such karyotypic complexity, several attempts have been done to provide clues on the molecular basis of instability by using different approaches (gene expression analysis, DNA-based techniques, and deep genome sequencing). Zhan et al. [26] in 2006 first made a molecular classification, by identifying 7 subclasses of MM. In this model,

the first class (MS class) was defined by the overexpression of the MMSET and/or FGFR3 genes resulting from translocation t(4;14). The second class (MF class) showed upregulation of MAF genes following translocations t(14;16) or t(14;20). The overexpression of CCND1 or CCND3, triggered by the translocations t(11;14) or t(6;14), identified the third and the fourth group CD1 and CD2, respectively. The fifth group (HY class) was represented by hyperdiploidy. The last two groups were characterized by a low incidence of bone disease with low levels of genes involved in bone disease (LB class), whereas the last group (PR class) was identified by high levels of genes involved in progression and proliferation. This molecular heterogeneity has been further confirmed and improved in several subsequent gene expression-based studies [27]. Moreover, copy numbers changes analysis by high-density single nucleotide polymorphism (SNP) array has identified other levels of molecular heterogeneity, which result in significant outcomes differences [28, 29]. Therefore, combining gene expression with copy number leads to more accurate analysis of this heterogeneity, which is related to the uncontrolled recombination mechanisms existing in this tumor. Remarkably, such knowledge can be exploited in both understanding MM biology and developing effective therapeutic strategies.

More recently, several efforts in deciphering molecular events driving MM progression have been made using genome sequencing analysis. This approach, by showing the complex subclonal structure of MM patients at diagnosis, which dynamically evolves over time, suggests the marked intertumor heterogeneity. Indeed, the mutational repertoire affecting genes of likely pathogenetic significance such as NRAS, KRAS, BRAF, p53, FAM46C, DIS3, SP140, LTB, ROBO1, and EGR1 indicates a cooperative role for multiple molecular pathways in supporting disease progression [17, 30–32]. Overall these studies demonstrate the existence of a multistep transformation process that changes MM genetic landscape over time (due to somatic mutations, epigenetic and chromosomal copy number variations).

Based on these findings, a clonal evolution has been proposed with progression disease achieved through branching, nonlinear pathways, which is a typical pattern of a complex ecosystem of clones competing for evolution [17, 33–35]. Therefore, all these studies suggest a disease landscape with complex pattern of genetic mutations at diagnosis aside from a Darwinian branching model of tumor evolution driving the alternating dominance of competing or collaborating clones present at diagnosis, over time. In such a scenario, the quantitative nature of next generation sequencing (NGS) data allows for higher resolution of the subclonal architecture of cancers and its monitoring over time with implication for prognostic stratification, tumor monitoring, and emergence of chemoresistance [17].

Overall, the tremendous knowledge achieved in MM molecular description with identification of high variability in its genomic architecture further underscores substantial heterogeneity of this hematologic malignancy and highlights the need for therapeutic interventions directed at multiple targets rather than a single genomic anomaly, as exemplified by success of combination therapies.

3. DNA Damage Response Mechanisms in MM Cells

Maintenance of genome integrity is crucial for tumor suppression and for the propagation of genomic information to subsequent generations. However, DNA integrity is persistently challenged by metabolism, errors in DNA replication and recombination, and exogenous genotoxic agents (ultraviolet light, oxidative stress, and chemical mutagens) that can lead to a range of DNA breaks. Indeed, these lesions can block genome integrity and if not repaired or repaired incorrectly lead to mutations or aberrations threatening cell viability. Thus, to counteract these attacks, cells use a sophisticate response system that, by inducing cell cycle arrest, allows DNA repair. Namely, to combat the constant threats posed to genome integrity, cells have evolved mechanisms—collectively termed the DNA damage response (DDR)—to detect DNA lesions and promote repair [36]. Such machinery is a complex and intertwined network of several proteins that enable proper DNA replication and that correct and repair breaches in the integrity and fidelity of the genetic code [37]. Cells defective in these mechanisms generally display an enhanced sensitivity towards DNA damaging agents and many of these defects cause human disease. Current studies have significantly increased our understanding on DNA damage response systems, allowing a better knowledge of such a complex feature orchestrated by tumor cells.

MM as well as most cancers has a striking genetic instability, which in turn leads to accumulation of mutational changes, some of which underlie tumor progression, drug resistance, and metastasis [19, 38]. Therefore the molecular basis causing this genetic diversity in cancer cells has important implications in understanding cancer progression. It is also noteworthy that most carcinogens operate by generating DNA damage and causing mutations [39]; consequently DNA repair provides a common mechanism for cancer-therapy resistance. A paradigm is the success of PARP inhibitors in those breast tumors, which lack functional BRCA1 or BRCA2 [40]. Namely, tumor cells with any DDR deficiency or "BRCAness" are likely to be particularly sensitive to PARP inhibitors because they are unable to cope effectively with the increase in lethal DSBs associated with replication fork collapse [41, 42]. However, BRCA-deficient tumors represent only a small percentage of cancer, restricting therefore the therapeutic utility of this synthetic lethal phenotype (SLI). In MM cells, direct evidence of homozygous loss or mutations in BRCA1/2 or other DDR genes is lacking, but an increased DNA repair activity capable of coping with a higher number of ongoing mutations has been previously reported. Overall, great progress has been made towards understanding the DDR but much remains to be learned.

In general, DDR mechanisms can be divided into single strand (SSB) or double strand (DDB) break repair, according to their specific activity. Namely, in presence of DNA single strand damage, the repair involves mismatch (MMR), base excision (BER), or nucleotide excision (NER) repair pathway. Importantly BER requires poly-ADP-ribose polymerase (PARP) which following DNA SSBs binding catalyzes synthesis and addition of large chains of poly-ADP-ribose (PAR)

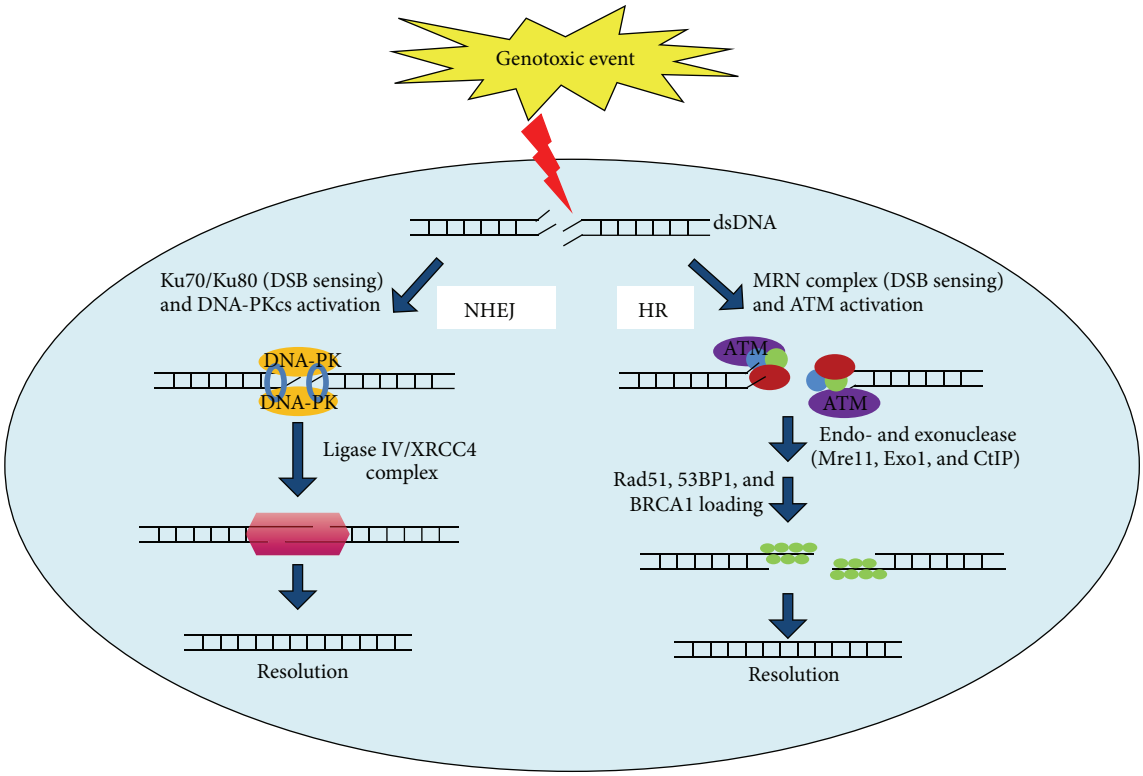

Figure 1: DSBs repair mechanisms. On the *left* part NHEJ. DSBs are identified by the ring-shape heterodimer Ku70/Ku80 which binds DNA broken ends and recruits the DNA-PKcs (DNA-dependent protein kinase catalytic subunit). This complex stabilizes DNA ends allowing a ligation carried out by XRCC4 and Ligase IV complex that finally reattaches the broken DNA. On the *right* part HR. The ATM kinase is recruited to DSB via an interaction with the MRN (Mre11-Rad50-Nbs1) complex, RNF8 and RNF168. Once ATM becomes activated, it phosphorylates multiple substrates including endo- and exonuclease (such as Mre11, Exo1, and CtIP) that are coated with ssDNA. Moreover ssDNA regions attract also Rad51 and other associated proteins (53BP1, BRCA1, etc.) which collectively assure new DNA synthesis. Defects of these mechanisms/cooperation lead to genomic instability, which in turn mediates tumor cell growth and progression.

polymers on target proteins, including histones H1, H2B, and PARP1 itself [18]. If persistent or left unrepaired, SSBs result in potentially lethal double strand DNA breaks. Although DSBs do not occur as frequently as the SSBs lesions, they are difficult to repair and extremely toxic [43]. To handle this warning, cells employ several DSBs repair mechanisms: Non-Homologous end joining (NHEJ) and Homologous Recombination (HR) [44]. Whilst NHEJ is considered highly mutagenic pathway with its activity resulting in small insertions or deletions at the junction site, HR is error-free mechanism. NHEJ works primarily during G0-G1 phases of cell cycle. It promotes DNA broken ends bridging without using a specific template resulting therefore in a less accurate repair of DSB. Specifically, following DSBs Ku70/80 heterodimes binds DNA broken ends and recruits the DNA-PKcs (DNA-dependent protein kinase catalytic subunit). This complex stabilizes DNA ends allowing a ligation carried out by XRCC4 and Ligase IV complex [17]. By contrast, DSBs during S/G2 phase triggers repair activity via HR pathway, in which MRN complex acts as major player and the sister chromatid is used as template to copy the missing information into the broken locus. Such process begins with H2AX phosphorylation by PI3-K family members ATM (ataxia telangiectasia) and ATR (Rad3 related), after their recruitment to DSBs regions. Such event initiates a dynamic recruitment of MDC1 along with its binding partners (MRN complex, RNF8 and RNF168, etc.) at sites of DNA damage. Next a second wave involves proteins playing key roles in repair and maintenance of genomic integrity, including 53BP1 and BRCA1. Overall, a complex processes network preserves genome integrity in mammalian cells with its impairment that fuels instability (Figure 1).

In MM cells elevated HR activity supports the increased rate of mutation and progressive accumulation of genetic variation observed over time, as reported by Shammas et al. [45]. Likewise, also NHEJ impaired activity contributes to genomic instability of such a tumor. Specifically, defects in XRCC4 or Ku70 have been described in U266 and RPMI8226 cell lines by Herrero et al. [46]. These authors found an upregulation of both DSBs repair mechanisms in MM cells, suggesting that HR and NHEJ contribute equally to the enormous genomic instability featuring these cells. Genome sequencing analyses revealed mutations in several genes involved in these pathways including ATM, ATR, MRN complex, XRCC3-4, RNF168, and BRCA1 [17, 30, 31, 35, 47, 48] (Table 2). In line with these data, we have recently demonstrated that MM cells exhibit high levels of NAD^+-dependent deacetylases SIRT6, which plays a key role in DSBs repair mechanisms and positively correlates with HR

Table 2: Summary of molecules involved in DNA damage and frequencies of their mutations.

Genes	Walker et al. (n = 463)	Lohr et al. (n = 203)	Bolli et al. (n = 67)	Chapman et al. (n = 38)	*Cifola et al. (n = 12)
ATM	18 (3%)	8 (3.9%)	2 (3%)	1 (2.6%)	4 (34%)
ATR	6 (1.3%)	2 (1%)	1 (1.5%)	0	3 (25%)
XRCC4	0	0	1 (1.5%)	0	0
RNF168	0	2 (1%)	2 (3%)	1 (2.6%)	0
BRCA1/2	0	2 (1%)	1 (1.5%)	1 (2.6%)	2 (34%)

*Plasma cell leukemias.

and NHEJ activities. We therefore propose this protein as crucial in preserving genome integrity of MM cells with its targeting as able to enhance chemotherapeutic response of DNA damaging Agents (Cea et al. manuscript submitted).

Overall, an imbalance between these two DSBs repair mechanism represents a hallmark of MM cells and contributes to its karyotypic instability.

4. Role of Epigenetic Changes and Telomeres in Genetic Instability of MM Cells

Likewise sequence alteration and chromosomal aberrations, also posttranslational processes, are common features of MM cells influencing gene expression and genome stability [18, 49, 50]. These are inheritable gene expression changes, named epigenetic process, which do not affect the genetic code [51]. Among such events, CpG islands methylation achieved by DNA methyltransferases (Dnmts), and histone modifications resulting from histone acetyltransferases (HATs) or histone deacetylases (HDACs), are frequently observed in MM cells [52, 53]. Indeed, genome-wide methylation microarrays have revealed specific changes in DNA methylation of MM cells according to clinical stages with progressive hypermethylation observed during disease progression [49, 54]. Of interest, since hypermethylation is associated with transcriptional silencing [55–57], whilst DNA hypomethylation is implicated in the genetic instability seen in many cancers [58], few authors suggest demethylating agents as alternative option to treat MM patients [30]. Importantly, 15–20% of MM patients harbor t(4;14) translocation, which results in increased expression of a histone methyltransferase gene, MMSET. Such event globally changes histone modifications, by supporting genomic instability in these patients [59–61]. Epigenetic changes in specific DNA damage repair genes have been also observed in MM patients as represented by the human thymine DNA glycosylate (TDG) that is involved in BER mechanisms. Methylation of this gene results in detrimental effect on DNA repair efficacy, further increasing genomic instability [62].

Also histone signature changes modulate gene expression and have been associated with cancer development. Specifically, HATs by catalyzing acetyl groups addition to lysine residues of histone tails allows relaxed chromatin state making DNA elements more accessible to transcription factors. On the other hand, HDACs, by removing acetyl groups from histone tails, make chromatin condensed and reduce activity of transcription factors [63]. Importantly, MM cells exhibit an imbalance of these two enzymes in favor of HDACs, which results in enhanced activity of key transcriptional factors and oncogenes [64–67].

A further regulator of genomic stability surveillance is represented by telomeres. These are nucleoprotein structures that cap the ends of chromosomes in order to prevent loss of genomic sequence during replication [39], and chromosomes fusion at the end [68]. Specific enzymes, named telomerase preserve telomere length and counterbalance their shortening following cell proliferation [69]. Opposite to normal cells, tumors reactivate telomerase, inducing tumorigenic phenotype [68]. Moreover, recent reports suggest one further mechanism of telomerase adopted by tumor cells to preserve telomere length, the alternative lengthening of telomeres (ALT) [70, 71]. Thus, based on these features, it is clear that although numerous mechanisms employed by tumor cells to maintain telomere length, it results critical for tumorigenesis and represents a cause or a consequence of genetic instability [18, 72, 73].

5. Clinical Implications of Genomic Instability in MM Cells

The constitutive ongoing DNA damage represents a trait of hematologic tumors [6], which leads to genomic instability and ultimately to more aggressive disease, often resistant to current therapies. Thus, genomic instability has important clinical implications with identified genomic alteration patterns providing basis for improved MM classification and prognostication. Example of such significance include the stimulated activity of DNA DSBs repair mechanisms NHEJ and HR in MM cells, in line with other hematologic malignancies. Indeed, hyperactivity associated with putative imbalance of these pathways observed in MM cells results in emergence of genetic changes responsible for disease progression and acquisition of drug resistances [46].

Whereas on one hand genomic instability is largely useful to transformed cells by providing a progressive growth advantage and development of drug resistance; on the other hand it may create exploitable vulnerabilities. Indeed, current therapeutic efforts aim to create synthetic lethal interactions in MM cells by targeting presumptive DNA repair defects of tumor cells specifically [74]. An example of this strategy is represented by marked sensitivity of MM cells to poly(ADP-ribose) polymerase (PARP) inhibitors triggered by 26S proteasome inhibition. Specifically this approach by impairing BRCA1 and RAD51 recruitment at DNA damaged

sites makes a functional *BRCAness* state in MM cells. As such cotreatment with proteasome and PARP inhibitors does result in contextual synthetic lethality and leads to striking MM cell death [75]. A clinical phase I trial supporting efficacy of this strategy in MM patients is currently ongoing.

In addition to direct targeting genetic vulnerabilities, the acquired epigenetics knowledge has provided further valuable therapeutic insights as observed with recent using of HDAC and DNMTs inhibitors for the treatment of MM [76–82].

In summary, presumptive DNA repair defect(s) in complex pattern might result in selective sensitivity to certain classes of anti-MM therapeutics including DNA damaging agents, bortezomib and IMiDs.

6. Future Directions

The knowledge of biological MM features is evolving rapidly but much remains to be learned. It is a very largely heterogeneous disease but basis for its phenotypic and genomic multiplicity remains uncertain, though continued proteomic and sequence-level analysis of its architecture is likely to provide insight. Mechanisms whereby ongoing heterogeneity shapes tumor genomes still remains unclear. Future studies should clarify the nature of a possible defect in DSB repair (and, more broadly, in the DNA damage response), as well as the functionality of each repair-pathway component. Such expertise would be exploited to identify selective vulnerabilities created by underlying genomic instability, which may be tested by unbiased drug screens. Therefore, future efforts should focus not only on the identification of mechanisms causing genomic instability, but also on clinical translation of these information, which should eventually lead to new treatment options for patients with MM.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Inhibition of P-Selectin and PSGL-1 Using Humanized Monoclonal Antibodies Increases the Sensitivity of Multiple Myeloma Cells to Bortezomib

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Multiple myeloma (MM) is a plasma cell malignancy localized in the bone marrow. Despite the introduction of novel therapies majority of MM patients relapse. We have previously shown that inhibition of P-selectin and P-selectin glycoprotein ligand-1 (PSGL-1) play a key role in proliferation of MM and using small-molecule inhibitors of P-selectin/PSGL-1 sensitized MM cells to therapy. However, these small-molecule inhibitors had low specificity to P-selectin and showed poor pharmacokinetics. Therefore, we tested blocking of P-selectin and PSGL-1 using functional monoclonal antibodies in order to sensitize MM cells to therapy. We have demonstrated that inhibiting the interaction between MM cells and endothelial and stromal cells decreased proliferation in MM cells and in parallel induced loose-adhesion to the primary tumor site to facilitate egress. At the same time, blocking this interaction *in vivo* led to MM cells retention in the circulation and delayed homing to the bone marrow, thus exposing MM cells to bortezomib which contributed to reduced tumor growth and better mice survival. This study provides a better understanding of the biology of P-selectin and PSGL-1 and their roles in dissemination and resensitization of MM to treatment.

1. Introduction

Multiple myeloma (MM) is a plasma cell malignancy located mainly in the bone marrow (BM), characterized by continuous dissemination of cancer cells [1, 2]. Accumulating evidence indicates that egress of MM cells from one site of the BM to a new site is a complex process that involves cellular and acellular interactions with endothelial cells, stromal cells, soluble growth factors, and extracellular matrix. Molecular mechanisms of cell adhesion and cell trafficking and thus metastasis in MM have been intensively investigated [3, 4]. The interactions of MM cells with the BM microenvironment play a crucial role in cell survival, cell trafficking, and drug resistance in MM; and interrupting these interactions enhances MM cells sensitivity to chemotherapy [3–7].

Selectins (CD62) are cell surface lectin-like adhesion molecules which bind sugar polymers and are involved in lymphocyte extravasation, especially during inflammation and cancer metastasis [8]. Selectin family consists of E-selectin, L-selectin, and P-selectin, expressed on endothelium, leukocytes, and platelets, respectively [8]. When endothelium is activated, P-selectin travels to the cell surface and can bind to ligands expressed on both leukocytes and cancer cells. The selectins and ligands interact rapidly in order to facilitate tethering, followed by rapid dissociation to enable rolling on the endothelium and ultimately cell extravasation [9]. P-selectin glycoprotein ligand-1 (PSGL-1, CD162) is the best characterized ligand for all three types of selectins and is expressed on myeloid, lymphoid, and dendritic cells [10]. PSGL-1 undergoes posttranslational modifications which are required to bind selectins and are similar for binding P-selectin and L-selectin [11]. PSGL-1 has especially high affinity for P-selectin on intact leukocytes compared to other ligands and is essential for adhesion to P-selectin [12, 13].

During cancer metastasis, cell adhesion and cell migration are frequently malfunctioning. Since cancer cells mimic leukocytes exploiting selectin-dependent mechanisms to extravasate, there is a growing interest in blocking selectins and their ligands in inflammation, tumor progression, and metastasis [14–16]. In solid tumors, it was demonstrated that absence or blocking of P-selectin with antibody decreased tumor cell adhesion and metastasis in rat lungs [17], gastric cancer in mice [18], and colorectal cancer [19].

Both P-selectin and PSGL-1 were also suggested as new targets in MM [6, 20, 21]. Expression of PSGL-1 was reported in normal plasma cells, with higher levels of PSGL-1 indicating plasma cell differentiation [6, 22]. PSGL-1 was shown to be highly expressed in MM biopsies and MM cell lines [5, 6, 23], and PSGL-1 gene expression increased in the course of MM progression [6]. Another study performed on MM biopsies demonstrated a significant correlation between the degree of PSGL-1 expression and the Durie-Salmon stage; thus PSGL-1 could be used as a diagnostic marker in MM [21]. It was previously demonstrated that knocking down PSGL-1 with siRNA in MM cells delayed tumor initiation *in vivo* [6]. Moreover, blocking selectins with pan-inhibitor GMI-1070 in MM mouse model in combination with bortezomib inhibited tumor growth during treatment and delayed tumor progression after halting the therapy significantly improving mice survival [6]. However, this inhibitor was previously shown to be a potent inhibitor of E-selectin and a nonpotent inhibitor of P-selectin, with high concentrations needed to inhibit P-selectin [24]. The necessity of using very high concentrations of GMI-1070 to achieve inhibition of P-selectin-mediated interactions of MM cells with the BM microenvironment limits the possibility to translate it into clinical settings. Thus, there is an urgent need to use novel, specific, and potent P-selectin/PSGL-1 interaction inhibitors.

In this study, we focused on the role of blocking P-selectin and PSGL-1 to inhibit MM progression and dissemination using specific humanized blocking antibodies for P-selectin and PSGL-1. We tested MM cell adhesion and proliferation *in vitro*, as well as MM cells extravasation, homing, tumor growth, and mice survival *in vivo*. These studies emphasize the importance of targeting P-selectin and PSGL-1, in combination with bortezomib, in the context of BM microenvironment, as a promising therapy for MM patients.

2. Materials and Methods

2.1. Cell Culture. The MM cell lines (MM1.s and H929, mycoplasma-negative) were obtained from American Type Culture Collection (ATCC, Rockville, MD). MM cell lines were cultured in RPMI-1640 media (Corning CellGro, Mediatech, Manassas, VA) supplemented with 10% fetal bovine serum (FBS, Gibco, Life Technologies, Grand Island, NY), 2 mmol/L of L-glutamine, 100 U/mL Penicillin, and 100 μg/mL Streptomycin (CellGro, Mediatech, Manassas, VA). Human umbilical vein endothelial cells were purchased from Lonza, Allendale, NJ. Human stromal cells were obtained from MM patients' BM biopsies depleted of CD138-positive myeloma plasma cells. Endothelial cells were cultured in endothelial cell growth media (EGM-2, Lonza) and stromal cells were cultured in 20% FBS Dulbecco's Modified Eagle's Medium (Corning CellGro, Mediatech, Manassas, VA) containing L-glutamine, Penicillin/Streptomycin. Cells were cultured at 37°C (5% CO_2) in the NuAire water jacket incubator (Plymouth, MN).

2.2. Animals. SCID-beige mice (females, 8-week old) and Balb/C mice (females, 9-week old) were obtained from Charles Rivers Laboratories (Wilmington, MD). Approval for these studies was obtained from the Ethical Committee for Animal Experiments at Washington University in St. Louis Medical School.

2.3. Effect of SelG1 and SelK2 on MM Cell Adhesion and Proliferation. The humanized monoclonal antibodies anti-P-selectin (SelG1) and anti-PSGL-1 (SelK2) were obtained from Selexys Pharmaceuticals (Oklahoma City, OK). For adhesion assay, endothelial and stromal cells (3×10^3 cells per well in 96-well plate) were incubated with SelG1 (10 μg/mL) and MM cells prelabeled with calcein-AM of final concentration 1 μg/mL (Invitrogen, Life Technologies, Grand Island, NY) were incubated with SelK2 (10 μg/mL). MM cells were applied to unlabeled endothelial cells or stromal cells for 1 hr, nonadherent cells were aspirated, and adherent cells were measured by detecting the fluorescent intensity signal using fluorescent reader (excitation/emission = 485/520 nm). For proliferation assay, H929 prelabeled with DiD (Invitrogen) were cultured alone, with endothelial or stromal cells, and were treated with or without bortezomib (Selleck Chem, Houston, Texas), in presence or absence of SelG1 and SelK2 antibodies, and cell proliferation was determined by flow cytometry.

2.4. Effect of SelG1 and SelK2 on MM Cell Extravasation and Homing. MM1.s labeled with calcein-AM were injected intravenously (IV) into Balb/C mice (3×10^6 cells/mouse) creating 3 groups: (1) mice treated with anti-mouse P-selectin antibody injected intraperitoneally (IP) the day before ($n = 3$); (2) mice treated with anti-mouse PSGL-1 antibody (rat anti-mouse CD162 antibody, catalog number 557787, BD Pharmingen, San Jose, CA) injected IP the day before and MM1.s treated with SelK2 antibody (anti-human PSGL-1) for 1 hr prior to the injection ($n = 3$); or (3) untreated MM1.s ($n = 3$). 50 μL of blood was collected from the portal vein at 50 min after injection, red blood cells were lysed with a buffer (BioLegend, San Diego, CA), and the circulating calcein-AM-positive MM cells were counted by flow cytometry. The presence of MM cells in the circulation signified retention, or in other words the absence of these cells in the circulation signified extravasation of the MM cells. After the last blood aspiration, mice were sacrificed; mononuclear cells were isolated from femurs, washed, and analyzed by the flow cytometry. The number of calcein-AM-positive cells was analyzed in each mouse and reflected the number of MM cells which homed to the BM.

2.5. Tumor Progression and Survival Study. MM1.s cells were genetically engineered to express green fluorescent protein

(GFP) and luciferase (Luc), as described previously [25]. In the first experiment, human MM1.s-GFP-Luc cells were injected into 24 SCID mice IV at a concentration of 2×10^6 cells per mouse and allowed to grow for 3 weeks. The mice were then divided randomly into 3 groups (8 mice/group) and treated as follows: (1) vehicle control which received PBS as vehicle; (2) anti-mouse P-selectin antibody (5 mg/kg) (rat anti-mouse CD62P antibody, catalog number 553741, BD Pharmingen, San Jose, CA) to inhibit P-selectin in the mouse stroma and endothelium; and (3) SelK2 and anti-mouse PSGL-1 (5 mg/kg) (catalog number 557787, BD Pharmingen) to inhibit PSGL-1 on human MM cells and in the mouse microenvironment, respectively. Tumor progression was monitored by bioluminescence imaging (BLI) once a week for 4 weeks (week 3 = time 0). In the second experiment, human MM1.s-GFP-Luc cells were injected into 32 SCID mice IV at a concentration of 2×10^6 cells per mouse and allowed to grow for 2 weeks. The mice were then divided randomly into 4 groups (8 mice/group) and treated as follows: (1) vehicle control; (2) bortezomib alone (1 mg/kg); (3) bortezomib (1 mg/kg) + anti-mouse P-selectin antibody (5 mg/kg); and (4) bortezomib (1 mg/kg) + SelK2 and anti-mouse PSGL-1 (5 mg/kg). Tumor progression was monitored by BLI twice a week for 4 weeks (week 2 = time 0). In both experiments, the vehicle, bortezomib, and antibodies were injected IP twice a week. Bortezomib and antibodies were administered IP sequentially twice a week. Tumor progression was followed twice a week using bioluminescence imaging. Survival of mice was followed every day by investigator, with no blinding.

2.6. Statistical Analysis. The *in vitro* experiments shown on Figures 1 and 3 were performed in quadruples and replicated independently two more times. Results are shown as mean ± s.d. The *in vivo* experiments, also depicted as mean ± s.d., were analyzed using student t-test (Figure 2) or chi-squared test (Figure 4) for independence for statistical significance, with the data meeting the assumption of the tests such as normal distribution. Variation within each group was equally variant and similar between the groups that were statistically compared. Values were considered significantly different for p value less than 0.05.

3. Results

3.1. P-Selectin and PSGL-1 Regulate Adhesion of MM Cells to Endothelial and Stromal Cells. First, we tested different concentrations (2.5, 5.0, and 10 μg/mL) of SelG1 (Figure 1(a)) or SelK2 (Figure 1(b)) on MM cell adhesion to endothelial and stromal cells and we found a dose-dependent effect of these monoclonal antibodies. Next, we examined the adhesion of MM cells (H929 and MM1.s) to stromal and endothelial cells. We found that after blocking P-selectin using a single concentration of SelG1 (10 μg/mL) on stromal cells H929 adhesion was decreased by 60% and MM1.s by 20% (Figure 1(c)); or on endothelial cells H929 adhesion was decreased by 43% and MM1.s by 23% (Figure 1(d)). Likewise, after blocking PSGL-1 on MM cells using a single concentration of SelK2 (10 μg/mL), H929 cell adhesion was decreased by 50% and MM1.s by 12% in coculture with stromal cells (Figure 1(e)), or H929 cell adhesion was decreased by 28% and MM1.s by 40% in coculture with endothelial cells (Figure 1(f)).

3.2. Blocking P-Selectin and PSGL-1 Decrease Extravasation and Decrease Homing of MM Cells to the BM In Vivo. To examine the role of P-selectin and PSGL-1 interaction on extravasation and homing of MM cancer cells to the BM *in vivo*, we injected MM cells labeled with calcein-AM and detected the number of calcein-AM-positive cells both in the blood and the BM samples 50 minutes after injection, indicating extravasation and homing, respectively. In mice pretreated with anti-mouse P-selectin antibody, MM cells displayed delayed extravasation with approximately 2.7-fold more MM cells still present in the circulation, whereas, in mice pretreated with anti-mouse PSGL-1 antibody and anti-human SelK2, there were 1.4-fold more MM cells in the circulation compared to untreated mice at time 50 minutes (Figure 2(a)). In case of homing, pretreatment with anti-mouse P-selectin antibody decreased the number of MM cells that had homed to the BM by 82%, whereas in mice pretreated with anti-mouse PSGL-1 antibody and SelK2 homing was decreased by 42%, compared to untreated mice (Figure 2(b)).

3.3. P-Selectin and PSGL-1 Affect Proliferation of MM Cells Cocultured with Endothelial and Stromal Cells. It was demonstrated before that the interaction between BM microenvironment and MM cells contributes to drug resistance [4]. Here we investigated the effect of inhibiting the interaction between P-selectin and PSGL-1 on MM cell proliferation detected by flow cytometry. The stroma and endothelial cell-induced proliferation of H929 was decreased using SelG1 or SelK2. Similar effects were observed when combining the antibodies with bortezomib, in which SelG1 or SelK2 enhanced the effect of bortezomib on H929 proliferation when cocultured with endothelial cells (Figure 3(a)) and stromal cells (Figure 3(b)).

3.4. Inhibition of P-Selectin in Combination with Bortezomib Decreases Tumor Size and Improves Survival in MM Mouse Model In Vivo. Finally, we studied the effect of blocking P-selectin and PSGL-1 on MM tumor progression in MM xenograft mouse model in combination with bortezomib. MM tumors were established in SCID mice and one week before bortezomib initiation, two groups of mice were pretreated twice a week with P-selectin and PSGL-1 antibodies. The combination of P-selectin antibody with bortezomib inhibited tumor growth significantly, compared to vehicle control and bortezomib alone treated mice. On the other hand, combination of PSGL-1 antibody with bortezomib delayed tumor growth to similar extent as bortezomib alone (Figure 4(a)). Survival study revealed that mice treated with P-selectin antibody (but not PSGL-1 antibody) in combination with bortezomib had significantly prolonged survival compared to other groups (Figure 4(b)). Moreover, we found that inhibiting P-selectin or PSGL-1 alone does not

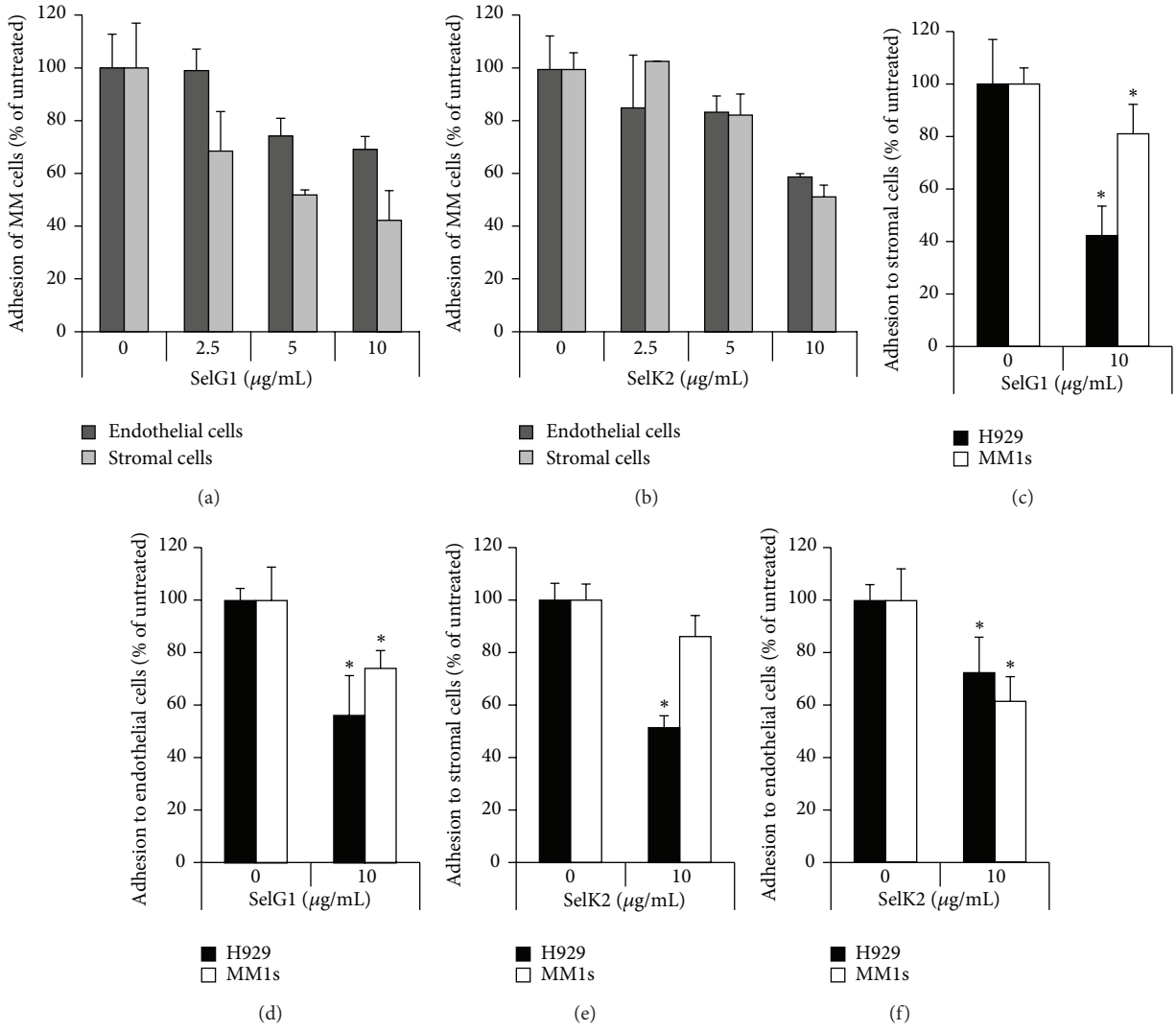

Figure 1: P-selectin and PSGL-1 regulate adhesion of MM cells to endothelial and stromal cells *in vitro*. Endothelial and stromal cells were treated with increasing concentrations of SelG1 antibody (2.5, 5, and 10 μg/mL) followed by adhesion of H929 or MM1.s cells labeled with calcein-AM. MM cell adhesion was assessed as a signal of adherent calcein-AM-positive MM cells measured by fluorescent reader and normalized to untreated cells (a). Likewise, H929 or MM1.s cells labeled with calcein-AM were incubated with increasing concentrations of SelK2 antibody (2.5, 5, and 10 μg/mL) and plated on untreated endothelial and stromal cells, and the cell adhesion was measured as above (b). Stromal (c) and endothelial cells (d) were treated with SelG1 (10 μg/mL) for 1 hr followed by plating MM1.s and H929 labeled with calcein-AM, and the adhesion was assessed as a signal of adherent calcein-AM-positive MM cells measured by fluorescent reader and normalized to untreated cells. Likewise, MM1.s and H929 were treated with SelK2 (10 μg/mL) for 1 hr and plated on stromal (e) or endothelial cells (f), and the cell adhesion was measured as above. Values were considered significant for $^{*}P < 0.05$.

influence tumor progression (Figure 4(c)) and mice survival (Figure 4(d)), compared to untreated mice.

4. Discussion

MM is characterized by continuous dissemination of cancer cells throughout the BM [1, 2]. During metastasis in MM, adhesion of cancer cells to vascular wall requires the presence of P-selectin on the endothelium and stroma and PSGL-1 on cancer cells [6]. It was demonstrated that PSGL-1 is highly expressed on MM cells and regulates adhesion and cell trafficking in MM; these interactions involve both endothelial and BM stroma cells which express high levels of P-selectin [6]. PSGL-1 was previously suggested as a novel target for immunotherapy in MM using monoclonal antibody, where anti-PSGL-1 antibody increased cell death of MM cells in a time- and dose-dependent manner [26]. Loss-of-function study and a small molecule pan-selectin inhibitor GMI-1070 demonstrated that PSGL-1 and P-selectin regulate the activation of integrins, adhesion, and proliferation, as well as downstream signaling. The anti-pan-selectin treatment using GMI-1070 sensitized MM cells to bortezomib *in vitro* and *in vivo*, controlling growth, dissemination, and drug resistance of MM in the context of the BM microenvironment.

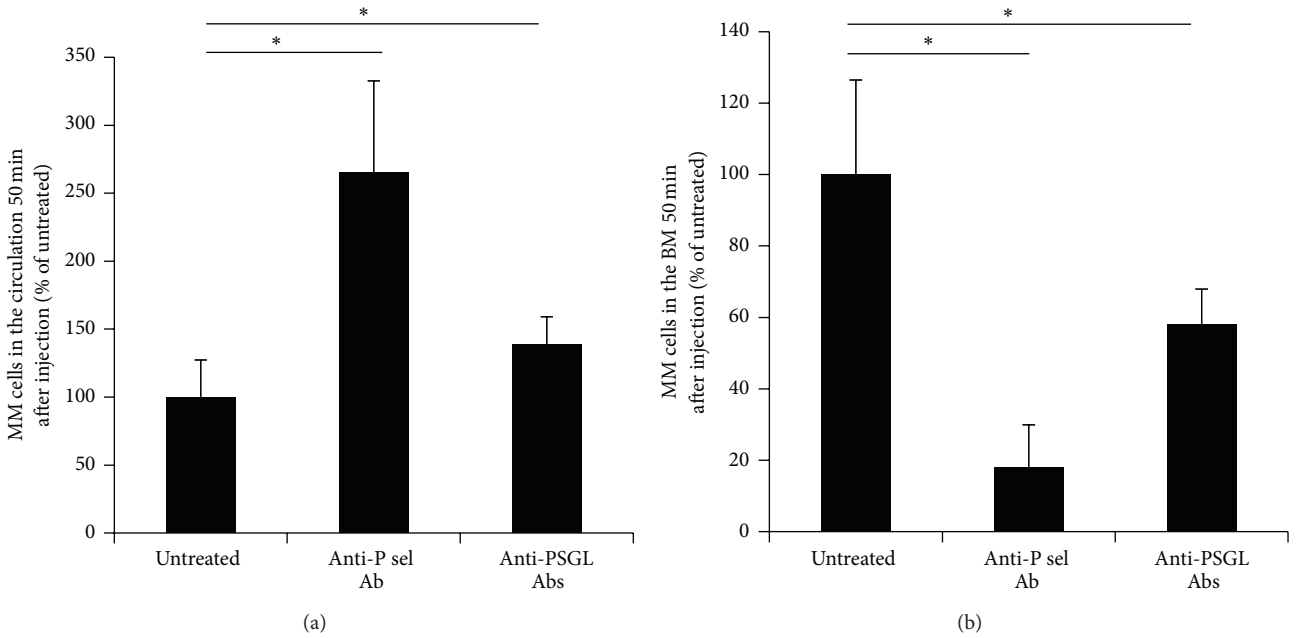

Figure 2: Blocking P-selectin and PSGL-1 decrease extravasation and decrease homing of MM cells to the BM *in vivo*. The effect of P-selectin- and PSGL-1-blocking antibodies on the number of circulating MM cells detected as calcein-AM-positive MM cells detected by flow cytometry at 50 minutes of blood aspiration after injection (a). The effect of blocking P-selectin and PSGL-1 on MM cell homing to the BM shown as the number of calcein-AM-positive cells detected in the BM, analyzed by flow cytometry, and normalized to untreated cells (b). Results are depicted as mean ± s.d. and statistical significance was analyzed by student t-test ($n = 5$ mice per group). Values were considered significant for $^{*}p < 0.05$.

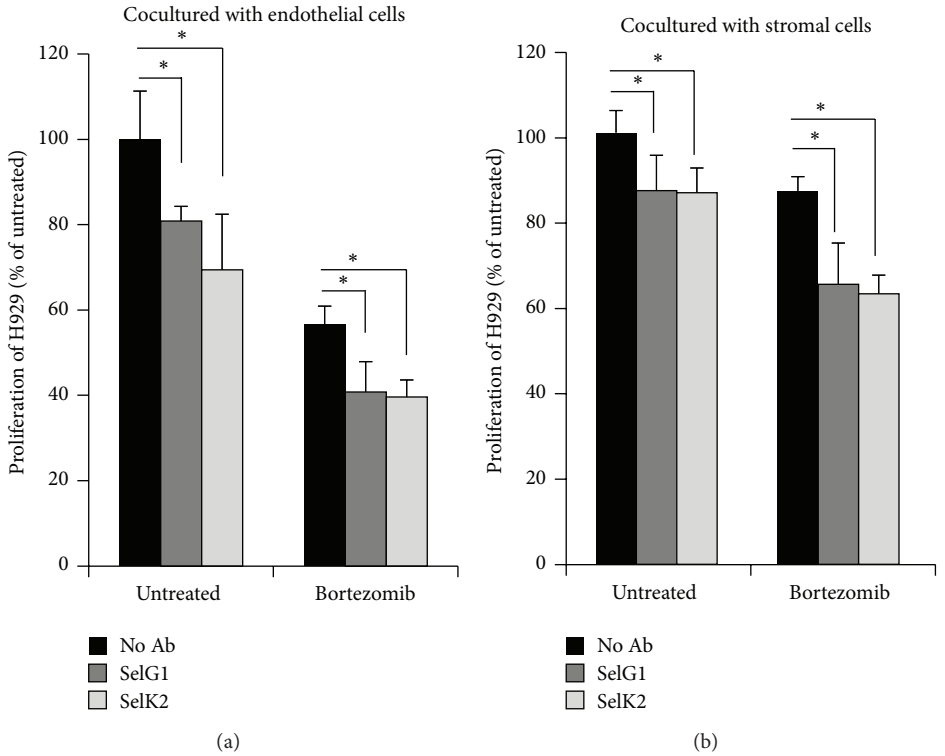

Figure 3: P-selectin and PSGL-1 affect proliferation of MM cells cocultured with endothelial and stromal cells *in vitro*. The effect of SelG1 antibody (10 μg/mL) used on endothelial and stromal cells and SelK2 (10 μg/mL) used on H929 cells on MM cell proliferation, with or without bortezomib (5 nM) treatment, analyzed by flow cytometry and normalized to untreated cells. Values were considered significant for $^{*}p < 0.05$.

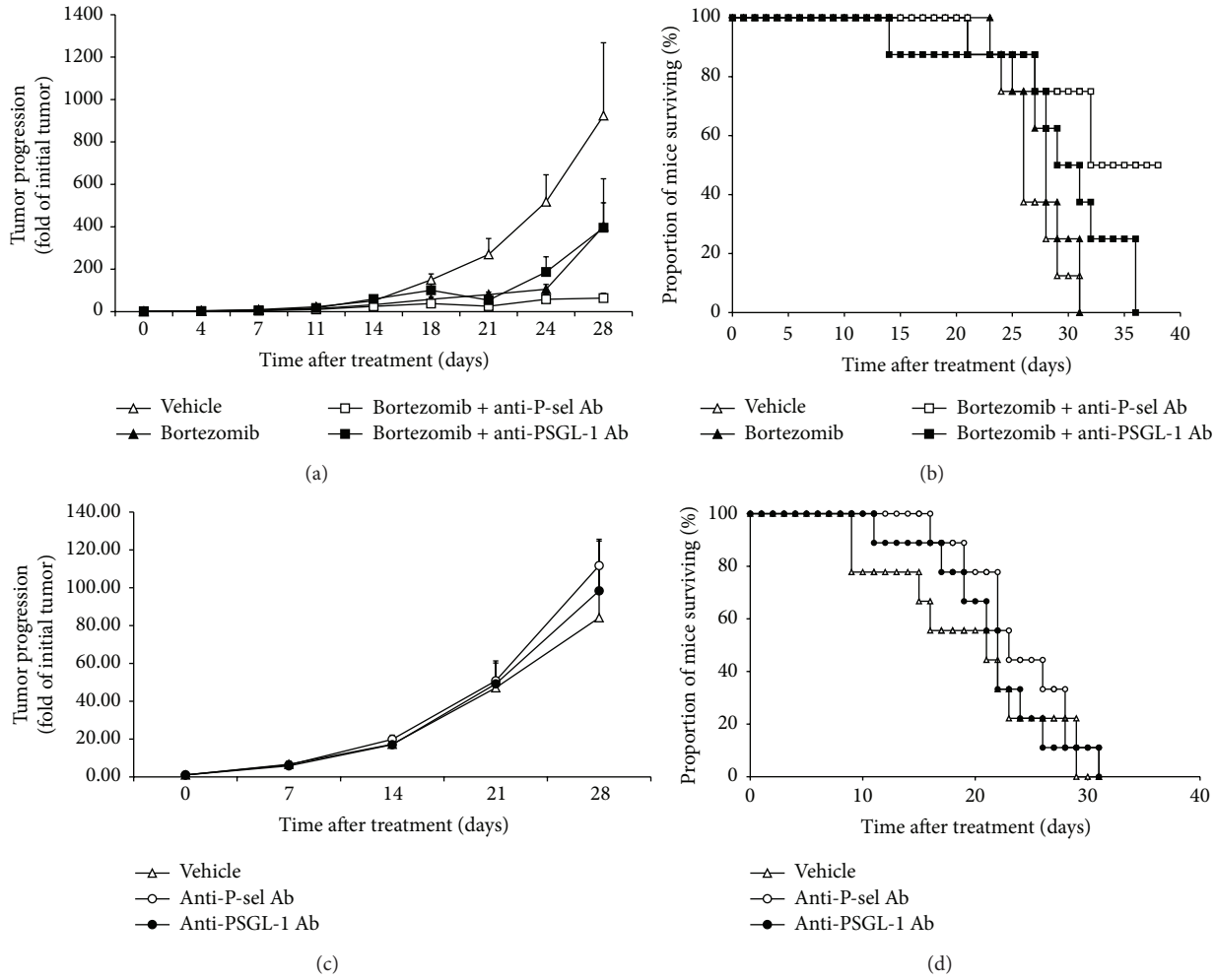

Figure 4: Inhibition of P-selectin in combination with bortezomib decreases tumor size and improves survival in MM mouse model *in vivo*. The effect of P-selectin and PSGL-1 inhibition on sensitivity to bortezomib of the MM-bearing mice. SCID mice ($n = 8$ per group) were injected with MM1.s-GFP-Luc and tumor growth was determined by bioluminescence imaging (BLI). In the first experiment (c and d), tumor was allowed to grow for 3 weeks. The mice were then divided into 3 groups: (1) vehicle control; (2) anti-mouse P-selectin antibody (5 mg/kg); and (3) SelK2 and anti-mouse PSGL-1 (5 mg/kg). Tumor progression was monitored by BLI once a week for 4 weeks (week 3 = time 0). In the second experiment (a and b), tumor was allowed to grow for 2 weeks and then the mice were divided randomly into 4 groups: (1) vehicle control; (2) bortezomib alone (1 mg/kg); (3) bortezomib (1 mg/kg) + anti-mouse P-selectin antibody (5 mg/kg); and (4) bortezomib (1 mg/kg) + SelK2 and anti-mouse PSGL-1 (5 mg/kg). Tumor progression was monitored by BLI twice a week for 4 weeks (week 2 = time 0). Tumor progression was detected shown as region of interest (ROI) normalized to initial tumor size in each group; the statistical significance was assessed by student t-test. The statistical significance was present between groups: vehicle versus bortezomib ($p = 0.0025$), vehicle versus P-sel + bortezomib ($p = 0.0023$), vehicle versus PSGL-1 + bortezomib ($p = 0.01$), bortezomib versus P-sel + bortezomib ($p = 0.0018$), and P-sel + bortezomib versus PSGL-1 + bortezomib ($p = 0.0172$) (a). Survival of mice was followed 40 days after starting the treatment and depicted as Kaplan-Meier curve. The p values were calculated from the chi-squared test for independence. The statistical significance was present between groups: vehicle versus P-sel + bortezomib ($p = 0.005$) and bortezomib versus P-sel + bortezomib ($p = 0.01$) (b).

However, low specificity of GMI-1070 to P-selectin and its pharmacokinetic profile with a very short half-life may hold up further usage of this drug [6].

In the present study, we examined the effect of potent humanized monoclonal antibodies targeting P-selectin and PSGL-1, and we confirmed that both antibodies decreased MM cell adhesion to endothelial and stromal cells. Since the MM cells in the presence of SelG1 and SelK2 were less adhesive *in vitro*, we tested the blocking effect of these antibodies on cell extravasation *in vivo* and we found that inhibiting the interaction of P-selectin and PSGL-1 prevented MM cell extravasation and increased their circulation time *in vivo*. At the same time, blocking P-selectin and PSGL-1 significantly decreased the capability of cancer cells to home to the BM *in vivo*. These results are in agreement with our previous studies showing that inhibition of the interaction using siRNA downregulation of the genes or the small molecule inhibitor reduced the adhesion of MM cells to endothelial and stromal cells *in vitro* and prevented extravasation and homing to the BM *in vivo* [6].

Next, we studied the effect of P-selectin and PSGL-1 antibodies on proliferation of MM cells when cocultured with endothelial or stromal cells. Results showed that inhibition of the interaction of MM cells with stromal and endothelial cells using the anti-P-selectin (SelG1) and the anti-PSGL-1 (SelK2) antibodies reversed the stromal and endothelial cell-induced proliferation of MM cells. Similarly, the antibodies increased the sensitivity of MM cells to bortezomib when these were cocultured with stromal and endothelial cells. These results confirm that blocking the P-selectin/PSGL-1 sensitizes MM cells to chemotherapy.

We then tested the effect of blocking P-selectin on the BM microenvironment and blocking PSGL-1 on MM cells on proliferation and drug resistance of MM cells *in vivo*. The antibodies alone did affect neither tumor growth nor mice survival; this is in agreement with our previous findings that GMI-1070 alone did not induce any delay in tumor progression *in vivo*. However, we found that inhibition of the interaction between PSGL-1 and P-selectin using the humanized antibodies, SelG1 and SelK2, induced sensitization of MM cells to bortezomib, increased the survival of animals with MM, and delayed tumor progression. The combination of bortezomib with the anti-P-selectin antibody SelG1 was more effective than the combination of bortezomib with the anti-PSGL-1 antibody SelK2. The possible mechanism for delayed tumor growth could be prolonged circulation and exposure of cancer cells in the peripheral blood to bortezomib due to increased cell retention and reduced cell homing. In addition, it was demonstrated previously that PSGL-1 and selectins are involved in macrophage-mediated drug resistance in MM; by blocking PSGL-1 in MM cells with antibody or by silencing PSGL-1 with shRNA using lentiviral vector, MM cells were resensitized to melphalan when cocultured with macrophages, which was driven by Erk1/2 pathway activation and c-myc upregulation [23].

In conclusion, our results demonstrate that inhibition of P-selectin/PSGL-1 axis using humanized monoclonal antibodies, SelG1 and SelK2, is promising as a treatment for MM and that these antibodies were potent (only 5 ug/mL was needed) with a very good pharmacokinetics (antibodies were injected twice a week only). The use of the antibodies disrupted the interaction between MM cells and BM microenvironment, decreased proliferation and adhesion of MM cells *in vitro*, and delayed tumor growth and extended survival in MM xenograft mouse model. This data provides a basis for future clinical trials for sensitization of refractory MM patients to therapy by blocking the P-selectin/PSGL-1 axis using the humanized monoclonal antibodies SelG1 and SelK2.

Conflict of Interests

Abdel Kareem Azab reports receiving commercial research funding from Selexys Pharmaceuticals; Scott Rollins, Richard Alvarez, and Ziad Kawar are the employees of Selexys Pharmaceuticals. No potential conflict of interests was disclosed by Barbara Muz, Feda Azab, and Pilar de la Puente.

Authors' Contribution

Barbara Muz designed the study, performed experiments, analyzed and interpreted the data, and wrote the paper; Pilar de la Puente and Feda Azab performed experiments, evaluated the data, and reviewed the paper; Scott Rollins, Richard Alvarez, and Ziad Kawar provided the antibodies and reviewed the paper. Abdel Kareem Azab designed the study, analyzed and interpreted the data, wrote the paper, and supervised the study.

Acknowledgment

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