Myelodysplasia-associated immunophenotypic alterations of bone marrow cells in myeloma: are they present at diagnosis or are they induced by lenalidomide?

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ABSTRACT

Increased risk of acute myeloid leukemia/myelodysplastic syndromes following treatment has been reported in multiple myeloma, but whether dysplastic features are already present at diagnosis remains to be investigated. Using multiparameter flow cytometry, we analyzed the distribution and phenotype of bone marrow hematopoietic cells from 47 multiple myeloma patients (15 symptomatic and 32 high-risk smoldering). From the 32 smoldering myeloma patients, 18 were studied at baseline and 22 after nine cycles of lenalidomide/dexamethasone treatment following the QUIREDEX trial (including 8 from baseline). Phenotypic alterations of bone marrow cells of 7 (47%) symptomatic and 6 (33%) smoldering myeloma patients were detected at baseline; there was no difference in the frequency and extent of phenotypic alterations between symptomatic *versus* smoldering cases. Likewise, no difference was seen between smoldering myeloma patients studied at baseline *versus* after lenalidomide/dexamethasone treatment. Our results suggest that phenotypic alterations of bone marrow hematopoietic cells are often present in newly diagnosed symptomatic and smoldering multiple myeloma patients.
QUIREDEX trial (NCT00480363)

Key words: multiple myeloma, phenotypic alterations, bone marrow cells, lenalidomide, diagnosis.

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Introduction

The association of alkylators with secondary hematologic malignancies, including myelodysplastic syndromes (MDS), is well recognized in multiple myeloma (MM).[1-5] In contrast, the potential association with lenalidomide has only recently emerged,[6-8] raising concern among physicians and patients. However, it is still not clear whether dysplastic features are already present at diagnosis. In fact, it has recently been reported that not only MM, but also patients with monoclonal gammopathy of undetermined significance (MGUS) have a higher risk of developing AML/MDS than age-matched controls.[9]

Multiparameter flow cytometry (MFC) immunophenotyping is currently considered a sensitive adjuvant test in the screening of MDS.[10,11] Herein, we report a comprehensive analysis of the distribution and immunophenotypic profile of bone marrow (BM) hematopoietic cells in newly diagnosed symptomatic MM and high-risk smoldering MM (SMM). In addition, the BM of high-risk SMM cases after nine courses of lenalidomide plus dexamethasone (Len/Dex) was also analyzed.

Design and Methods

A total of 47 myeloma (15 symptomatic and 32 SMM) patients were studied, all diagnosed according to the International Myeloma Working Group.[12] SMM patients were categorized as high-risk according to the presence of at least two of the three following criteria: BM plasma cell (PC) infiltration of 10% or over; high M-component (IgG ≥ 30 g/L or IgA ≥ 20 g/L or B-J Protein > 1 g/24 h); or 95% or over MM-PC/BMPC plus immunoparesis. SMM patients were included in the QUIREDEX trial (NCT00480363) that compared Len/Dex *versus* placebo. BM samples were studied at baseline in 33 (15 symptomatic MM and 18 SMM) cases. We also analyzed 22 SMM (including 8 from the

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baseline cohort) after nine cycles of Len/Dex (nine 4-week cycles of lenalidomide at daily doses of 25 mg on Days 1–21, plus 20 mg dexamethasone on Days 1-4 and 12-15). All BM samples were taken after obtaining informed consent in accordance with the recommendations of the local ethics committee. The study was approved by the relevant institutional review boards and ethics committee.

MFC immunophenotyping was performed as previously described[10,11] using a total of 27 MoAbs in 4-color combinations to identify and characterize BM hematopoietic cells, allowing 83 different phenotypic parameters to be assessed (*Online Supplementary Appendix*). Only those parameters with a deviation of 3 SD or more from reference values (defined in 40 normal/reactive BM samples) were considered to be altered. Accordingly, patients were classified as having "isolated alterations" (one alteration in one or more cell lineages) and "multiple alterations" (more than one alteration in one or more cell lineages). Patients with normal phenotypic profiles were categorized as "no alterations". The severity of the alterations was evaluated using a previously reported immunophenotypic score[10,11] that gives 1 or 2 points to each phenotypic parameter that has a deviation from normal reference values of between 3-4 SD or over 4 SD, respectively (*Online Supplementary Appendix*). The overall score for each sample was determined by the sum of all partial scores. In SMM patients included in the QUIREDEX trial, central morphological review of BM slides specifically for blast count and dysplasia was performed in 17 of 18 cases at diagnosis and in 12 cases after the nine induction cycles of Len/Dex. Response rates in the first group were as follows: 19% achieved (stringent and conventional) complete remission, 10% very good partial response, 57% partial response, and 14% remained with stable disease.

According to standard criteria, fluorescence *in situ* hybridization (FISH) analyses (and not conventional karyotyping) were performed on immunomagnetically enriched plasma cells.

Group differences were compared using the χ^2 and Mann-Whitney U tests for categorical and continuous variables, respectively (SPSS 18.0, Chicago, IL, USA).

Results and Discussion

The survival of MM has significantly increased in the last decade[13-15] but this extended survival has also alerted physicians to the potential risk of secondary MDS associated with some drugs/regimens, particularly alkylating-based schemes[1,2] and, more recently, lenalidomide treatment.[6-8] In turn, a predisposition to an overt MDS was recently observed in the overall MM (and MGUS) population.[9] However, a comprehensive screening of dysplastic features was not performed in newly diagnosed myeloma patients. In the present study. "multiple phenotypic alterations" similar to those observed in MDS patients were found at diagnosis in 2 of 15 (13%) symptomatic MM but not in SMM patients (Table 1). Therefore, the incidence of dysplastic features reported in MM after high-dose therapy/autologous stem cell transplantation (HDT/ASCT) (1-13%)[1-4,16,17] could have been overestimated since some of these may already be present at baseline.[18] Interestingly, up to 20% of all myeloma patients showed an immunophenotypic score that is found only in MDS and not in normal/reactive BM samples (*Online Supplementary Table S1*).

In addition to the aforementioned "multiple alterations", "isolated phenotypic alterations" at baseline were found in 34% of symptomatic MM (5 of 15) and 33% of high-risk SMM (6 of 18) cases (Table 1). It should be noted that, similarly to the HUMARA assay, gene chip profiling, or point mutation analysis, MFC is considered to be an adjuvant test for the screening of MDS that may provide additional information to the standard BM morphological and cytogenetic evaluation. In fact, we were able to investigate the presence of MDS-recurrent abnormalities through FISH analysis, or the presence of clonality by the HUMARA X-chromosome inactivation assay, on purified CD34$^+$ cells, neutrophils, erythroblasts and monocytes from 3 symptomatic MM patients showing either isolated or multiple phenotypic alterations (2 males and one female, respectively). Our results showed the presence of del(7q31) in both CD34$^+$ cells and erythroblasts in one of the 2 male patients, as well as the presence of clonality in neutrophils and monocytes purified from the female MM patient. Future studies are needed to replicate and expand on these observations.

Detection of multilineage BM dysplasia has been reported in non-clonal cytopenic conditions (e.g. vitamin B12 and folic acid deficiency).[11,19-21] In myeloma, it could be hypothesized that due to occupation of BM niches by clonal PC, normal BM cells are expelled from their natural microenvironment,[22] translating into deregulated hematopoiesis.[23] If this is the case, it would be expected that due to increasing tumor burden, the frequency and extent of phenotypic alterations would be slightly higher in newly diagnosed symptomatic patients than in high-risk SMM patients. However, detailed immunophenotypic analysis of the different hematopoietic BM cell compartments revealed no significant differences in the frequency of altered CD34$^+$ and/or CD117$^+$ hematopoietic precursor cells, or the neutrophil, monocytic, B-cell, erythroid, basophil and eosinophil lineages, except for a greater frequency of CD56$^+$ monocytes in symptomatic MM (Table 2). These data indi-

Table 1. Frequency of immunophenotypic alterations in bone marrow cell compartments (other than plasma cells) of patients with symptomatic multiple myeloma (MM) and smoldering multiple myeloma (SMM) at diagnosis, and SMM cases after induction therapy with nine courses of lenalidomide plus dexamethasone (Len/Dex).

Immunophenotypic characteristics of bone marrow cell compartments	Symptomatic MM at diagnosis (N = 15)	SMM at diagnosis (N = 18)	P*	SMM after Len/Dex (N = 22)	P**
No alterations	8 (53%)	12 (67%)	0.3	14 (64%)	0.5
Isolated alterations	5 (34%)	6 (33%)	0.7	7 (32%)	0.7
Unilineage	3	4	0.3	4	0.9
Multilineage	2	2		3	
Multiple alterations	2 (13%)	0 (0%)	0.1	1 (4%)	0.4

*Patients were classified as showing "isolated unilineage" (one alteration in a single cell lineage), "isolated multilineage" (one alteration in more than one cell lineage), and "multiple alterations" (more than one alteration in one or more cell lineages). Patients with normal phenotypic profiles were classified as having no alterations. BM: bone marrow; SMM: smoldering multiple myeloma; MM: multiple myeloma; MDS: myelodysplastic syndrome. * MM* vs. *SMM at diagnosis, ** SMM at diagnosis* vs. *SMM after induction.*

cate that, overall, there is no significant difference in maturation patterns of BM cell compartments between symptomatic and smoldering MM.

Due to recent concerns about the potential risk of secondary AML/MDS following lenalidomide treatment,[6-8] we also analyzed the relative distribution and phenotypic patterns of BM cells in SMM patients after nine courses of Len/Dex. Based on the use of lenalidomide to treat MDS,[24] we should also consider the alternative possibility of a reduction in the number of phenotypic alterations after treatment. Overall, no significant differences were noted between SMM patients after treatment and the baseline cohort with respect to the frequency and severity of the alterations detected (as evaluated by the immunophenotypic score), except for a trend towards increased frequency of phenotypically altered erythroblasts (P=0.08) (Tables 1-2 and *Online Supplementary Table S1*). Similarly, no differences were noted in the frequency of cases with mild morphological alterations before and after Len/Dex treatment (25% *vs.* 18%; P=0.6). Since MDS is also characterized by cytopenias, we quantified the distribution of the hematopoietic BM cell compartments by high-sensitivity MFC immunophenotyping. Only greater numbers of erythroblasts and basophils (P<0.05), and a lower (borderline significance) percentage of B cells (P=0.07) were detected after treatment (*Online Supplementary Table S2*). Therefore, our results do not suggest an association between Len/Dex and MDS development. Likewise, Mailankody *et al.* recently

Table 2. Specific phenotypic alterations detected in various cell populations in patients with symptomatic multiple myeloma (MM) and smoldering multiple myeloma (SMM) at diagnosis, as well as SMM cases after induction therapy with nine courses of lenalidomide plus dexamethasone (Len/Dex).

Cell populations and altered phenotypic parameters	N. patients with immunophenotypic aberrancies				
	Symptomatic MM at diagnosis (N = 7)	SMM at diagnosis (N = 6)	P *	SMM after Len/Dex (N = 8)	P**
CD34+ and/or CD117+ HPC	2 (29%)	0 (0%)	0.2	1 (11%)	0.4
Maturation blockade	1 (14%)	0	0.4	1 (11%)	0.4
SSClo	0	0	0.9	0	0.9
CD56+	2 (29%)	0	0.2	0	0.9
Neutrophil lineage	4 (80%)	4 (57%)	0.4	4 (44%)	0.2
Cy-MPOlo	0	1 (20%)	0.2	1 (11%)	0.6
SSClo	0	0	0.9	0	0.9
CD11b/CD13 pattern	2 (29%)	0	0.2	0	0.9
CD14+	2 (29%)	2 (40%)	0.7	0	0.2
CD15/CD16 pattern	2 (29%)	1 (20%)	0.7	1 (8%)	0.4
CD34+	0	0	0.9	0	0.9
CD56+	2 (29%)	0	0.2	0	0.9
CD65het	0	0	0.9	0	0.9
Monocytic lineage	5 (71%)	2 (40%)	0.3	2 (22%)	0.5
CD2+	0	1 (20%)	0.2	2 (22%)	0.9
CD11b/CD13 pattern	2 (29%)	1 (9%)	0.7	0	0.2
CD14/CD300e pattern	0	0	0.9	0	0.9
CD15/CD16 pattern	1 (14%)	0	0.4	0	0.9
CD36/CD64 pattern	0	0	0.9	0	0.9
CD56+	5 (71%)	1 (9%)	0.02	0	0.2
Erythroid lineage	2 (29%)	0	0.2	4 (50%)	0.08
CD36-	1 (14%)	0	0.4	3 (33%)	0.1
CD71-/lo	2 (29%)	0	0.2	2 (22%)	0.2
B-cell lineage	0 (0%)	0 (0%)	0.9	0 (0%)	0.9
Cy-CD79a-/lo	0	0	0.9	0	0.9
Basophil lineage	0 (0%)	0 (0%)	0.9	0	0.9
HLA-DR+	0	0	0.9	0	0.9
CD34+	0	0	0.9	0	0.9
Eosinophil lineage	0 (0%)	0 (0%)	0.9	0	0.9
CD15lo	0	0	0.9	0	0.9
CD33+	0	0	0.9	0	0.9
Median n. altered lineages	2 (1 - 4)	1 (1 - 2)	0.4	1 (1 - 2)	0.9

** SMM at diagnosis vs. SMM after induction; ** MM vs. SMM at diagnosis; -: not present; +: positive expression; het: heterogeneous expression. Results expressed as number and percentage (between brackets) of altered cases. SMM: smoldering multiple myeloma; MM: multiple myeloma; HPC: hematopoietic precursor cells; MPO: myeloperoxidase; SSC: sideward light scatter; Cy: cytoplasmic*

reported that the risk of AML/MDS in MM patients was similar before and after the introduction of immunomodulatory agents.[9]

Finally, in order to better assess any potential association between phenotypic alterations and Len/Dex treatment, we analyzed the 8 cases for which paired immunophenotypic studies had been performed at diagnosis and after the nine cycles of Len/Dex (*Online Supplementary Table S3*). Of these, 7 had no alterations at diagnosis: 4 of them remained without alterations after treatment while the other 3 developed isolated alterations. Finally, one patient with an isolated alteration up-front maintained the same phenotypic profile (MPO^{lo} expression in neutrophils) after treatment.

Our results suggest that, in a small proportion of MM and SMM patients, phenotypic alterations, detected by high-sensitivity MFC immunophenotyping, are already present in BM hematopoietic cell compartments at diagnosis. Whether these cells are more susceptible to further multistep accumulation of genetic defects remains to be clarified. Finally, our results do not support either a protective or a triggering effect between Len/Dex and MDS development.

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