The Relationship Between Prognosis and Plasma Cell Percentage, Infiltration Pattern, Fibrosis and Microvascular Density in Bone Marrow Biopsies of Plasma Cell Myeloma Patients

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ABSTRACT

Plasma cell myeloma is a monoclonal disease characterized by anemia, monoclonal protein in serum and/or urine, osteolytic lesions in bones, hypercalcemia, and renal failure. The study aims to evaluate the percentage of plasma cells in bone marrow, the pattern of bone marrow involvement, the intensity of fibrosis and angiogenesis in bone marrow, and to assess their relationship with clinical prognostic markers. In this study, bone marrow biopsies of 135 plasma cell myeloma cases were re-evaluated regarding plasma cell percentage and the pattern of bone marrow involvement. Fibrosis was assessed in 132 cases with reticulin staining and micro-vessel density (MVD) in 51 cases with CD34 staining. The relation of these morphological parameters with stage and survival status was analyzed. Plasma cell percentage, involvement pattern and micro-vessel density showed statistically significant correlation with stage, while bone marrow fibrosis was not significantly correlated ($p< 0.05$, $p= 0.001$, $p= 0.011$, $p> 0.05$, respectively). Elevation in percentage of plasma cells, diffuse pattern of bone marrow involvement, and increase in the degree of fibrosis was correlated with an increase in MVD ($p< 0.001$, $p< 0.001$, $p= 0.036$; respectively). The present study suggested that evaluation of plasma cell percentage, infiltration pattern, fibrosis and micro-vessel density in bone marrow biopsies of newly diagnosed plasma cell myeloma cases might be useful tools in the management and maintenance of the treatment.

Keywords: Plasma cell myeloma, Bone marrow, Angiogenesis, Fibrosis

INTRODUCTION

Plasma cell myeloma (PCM) is a multifocal neoplastic proliferation of plasma cells originating from the bone marrow (BM). BM is the origin of almost all PCMs, and most cases have extensive BM involvement where other organs may be involved secondarily. The diagnosis could be made based on clinical, morphological, immunological and radiological features.[1]

PCM constitutes 1% of all malignancies, 10-15% of hematological malignancies and 20% of deaths from hematological malignancies.[2,3] It is more common in males with a male/female ratio of 1.1:1 and occurs two-fold more in the black population than in white.[3,4] The disease is not seen in children and is very rare in those younger than 30 years of age.[5,6] The incidence of PCM increases along with the age in which more than 90% of cases are over 50 years. The median age at diagnosis is 70 years.

PCM is an incurable progressive disease for most patients, however recent therapeutic approaches have improved quality of life and survival.[7] The survival ranges from 6 months to 10 years (mean 5.5 years).[8]

Patients with PCM over 70 years with comorbidities or poor performance status have a worse prognosis. The International Staging System (ISS) is based on pre-treatment serum beta 2 microglobulin and albumin levels.[9] Treatment response determined by flow-cytometry, especially following autologous stem cell transplantation, might predict overall survival.[10,11]

Myelofibrosis is a cascade of events developed by cytokines in the BM stroma. Scoring the degree of myelofibrosis is based on the subjective assessment of the pathologist.[12] Detection of fibrosis in the BM before treatment could be found at a rate varying between 8.8% and 20.9% in patients with PCM, however it has been reported to be between 10 and 30% in different studies.[13-16] The degree of BM fibrosis is consistent with the amount of plasma cell infiltration and has prognostic significance. In PCM, interstitial fibrosis in the BM is usually focal and limited to areas of plasma cell infiltration.[15]

Angiogenesis is a multi-step process characterized by forming new vessels from existing ones. In recent years, increased angiogenesis in the BM has been demonstrated in several hematological malignancies including PCM. BM angiogenesis is associated with disease progression and prognosis in PCM patients.[17]

This study aims to evaluate the percentage of plasma cells, the infiltration pattern, the intensity of fibrosis and angiogenesis in BM, and to assess their relationship with clinical prognostic markers.

PATIENTS AND METHODS

BM biopsies of the patients diagnosed with PCM in our institution between 2015 and 2017 were enrolled in this study. The clinical information of the cases was obtained from the patient files. ISS was performed after analyzing beta 2 microglobulin levels in patients.

All hematoxylin and eosin (H&E) stained preparations of the cases were re-evaluated in terms of plasma cell infiltration pattern. The degree of fibrosis in the biopsies was determined by evaluating the Reticulin histochemical stain. The presence of fibrosis was divided into four grades as follows: Grade 0; BM without fiber increase, Grade 1; BM with mild fiber increase, Grade 2; BM with moderate fiber increase, Grade 3; BM with dense fiber increase.

Immunohistochemical Staining

For immunohistochemical staining of CD34 and CD38, 3 μm-thick sections were first cut in "Cell Conditioning 1" solution at 95^{0}C for 64 minutes; they were then incubated for 40 minutes with antibodies to CD34 (VENTANA anti-CD-34 QBEnd/10) and CD38 (CELL MARQUE anti-CD-38SP149). Staining was performed via the BenchMark ULTRA (Ventana Medical Systems, Tucson, AZ, USA) fully automated immunohistochemical staining system supported by the Ultraview universal DAB detection kit (Ventana Medical Systems). Tonsil tissue was used as a positive control of CD38 antibody.

Membrane staining with CD38 in plasma cells was considered as positive. Plasma cell infiltration rate and plasma cell infiltration pattern were evaluated in H&E and CD38 stained slides. The staining prevalence results were expressed as percentage (%) where the infiltration pattern as interstitial, nodular, or diffuse. According to the extent of CD38 staining, the cases were divided into three groups as follows: Group 1; stained below 20%, Group 2; stained between 20 and 50%, and Group 3; stained over 50%.

In BM biopsy stained with CD34, areas with the highest microvessel (capillaries or venules) density were selected at x100 magnification and these areas were determined as "hot spots". The arithmetic mean of the number of microvessels was calculated by counting the microvessels in 10 areas in the hot spots at ×400 magnification. The obtained value was stated as "microvessel density (MVD)". Microvessels were defined as endothelial cells that can be clearly separated from either tubules or larger groups formation or single or cluster formation. Lumen formation was not considered a mandatory condition. Large vessels in the periosteum or bone tissue were excluded from evaluation.

This retrospective study was approved by local ethics committee (Dokuz Eylul University Faculty of Medicine) with a grant number of 2019/21-23.

Table 1. The clinical and morphological features of the cases

		n (%)	Mean±SD (63.3±11.2)	Min-max (25-90)
Age (years) (n=135)				
Gender (n/%) (n= 135)	Male	93 (69%)		
	Female	42 (31%)		
Stage (n= 102)	1	27 (20%)		
	2	25 (19%)		
	3	50 (37%)		
Mortality (n= 135)	Alive	63 (47%)		
	Death	72 (53%)		
Follow-up duration (months)			26.3±16.4	1-57
CD38 (+) cell ratio (n= 135)	Group 1	8 (5.9%)		
	Group 2	30 (22.2%)		
	Group 3	97 (71.9%)		
Infiltration pattern (n= 135)	Interstitial	46 (34.1%)		
	Nodular	17 (12.6%)		
	Diffuse	72 (52.3%)		
Bone marrow fibrosis (n= 132)	0	16 (12.1%)		
	1	41 (31.1%)		
	2	54 (40.9%)		
	3	21 (15.9%)		
Microvessel density (n= 51)			7.96±7.3	1.0-36.3

Statistical Analysis

The variables were tested for normal distribution. Quantitative variables were presented as mean and standard deviation whereas qualitative variables as median and interquartile range (25%-75%) values. The analysis of the demographic characteristics was performed using the Chi-square test. Normally distributed variables were compared using the independent samples t test where abnormal ones by Mann-Whitney U test. Comparisons for there or more groups were performed using Kruskal-Wallis test. The relationship between two variables were analyzed by Kendall correlation analysis. The Kaplan-Meier method is used to analyze survival data. All analyses were performed using Statistical Package for Social Sciences version 22 program (SPSS Inc., Chi, IL). The significance level for analysis was set at $p< 0.05$.

RESULTS

A total of 135 cases were included in the study. The clinical and morphological features of the cases are presented in Table 1 and Table 2. BM biopsies of 42 (31%) females and 93 (69%) males were examined. The mean age was 63.3±11.2 ranging from 25 to 90 where 123 (91%) of the cases were ≥50 years old. For ISS; 27 (20%) of the cases were at Stage 1, 25 (19%) at Stage 2, and 50 (37%) at Stage 3. 63 (47%) of the cases were deceased. The overall survival of the cases ranged from 1 to 57 months, and the median survival was calculated as 34.5±6.6 months (Figure 1). CD38 (+) cell ratio was as follows: 8 cases (5.9%) in Group 1, 30 cases (22.2%) in Group 2, and 97 cases (71.9%) in Group 3. According to the pattern of BM involvement; interstitial involvement was observed in 46 (34.1%), nodular in 17 (12.6%), and diffuse in 72 patients (53.3%). BM fibrosis was detected in 16 (11.9%) of the cases as Grade 0, 41 (30.4%) as Grade 1, 54 (40%) as Grade 2, and 21 (%15.6) as Grade 3. The mean MVD was 7.96 ±7.3. The frequency of diffuse patterns of BM involvement and Stage 3 cases was significantly higher than the other stages and patterns ($p= 0.001$). There was a mild positive correlation between CD38 (+) cell groups and stages ($p= 0.001$; $r= 0.353$). The frequency of CD38 (+) cell ratio in Group 3 and Stage 3 were higher compared to the other stages and groups ($p< 0.05$). In

Table 2. Morphological and clinical evaluation of the cases.

The pattern of bone marrow involvement					
	Interstitial (n= 35)	**Nodular (n= 11)**	**Diffuse (n= 56)**		**p**
Stage 1	11 (40.7%)	7 (25.9%)	9 (33.3%)		0.001*
Stage 2	13 (52.0%)	2 (8.0%)	10 (40.0%)		
Stage 3	11 (22.0%)	2 (4.0%)	37 (74.0%)		
CD38 (+) cell groups					
	Group 1 and 2 (n= 26)		Group 3 (n= 76)		
Stage 1	13 (48.1%)		14 (51.9%)		<0.05*
Stage 2	8 (32.0%)		17 (68.0%)		
Stage 3	5 (10.0%)		45 (90.0%)		
CD38 (+) cell groups					
	Group 1 and 2 (n= 38)		Group 3 (n= 97)		
Interstitial	29 (63.0%)		17 (37.0%)		<0.05*
Nodular	6 (35.3%)		11 (64.7%)		
Diffuse	3 (4.2%)		69 (95.8%)		
Bone marrow fibrosis					
	BMF 0 (n= 16)	BMF 1 (n= 41)	BMF 2 (n= 54)	BMF 3 (n= 21)	
Interstitial	8 (50%)	23 (56.1%)	13 (24.1%)	1 (4.8%)	<0.05*
Nodular	2 (12.5%)	0 (0%)	10 (18.5%)	5 (23.8%)	
Diffuse	6 (37.5%)	18 (43.9%)	31 (57.4%)	15(71.4%)	

**p< 0.05 Chi-square test BMF= bone marrow fibrosis*

addition, the frequency of diffuse pattern of BM involvement and Group 3 of CD38 (+) cells were significantly higher than in other patterns and groups (p< 0.05). There was a mild positive correlation intensity of BM fibrosis and the pattern of BM involvement (p< 0.05; r= 0.263). The frequency of CD38 (+) cell ratio in Group 3 and BM fibrosis Group 3 were higher compared to the other groups (p= 0.00). CD38 (+) staining changes were showed on Figure 2. The MVD values showed a significance while grouped according to the intensity of BM fibrosis (p=0.036). The MVD values in Group 1 was significantly lower than in Group 2 (p< 0.05; Table 3). The MVD values demonstrated a significance while grouped according to the pattern of BM involvement (p< 0.05; Table 3). The MVD values with an interstitial pattern of BM involvement was significantly lower than in diffuse (p< 0.05; Table 3). The MVD values showed a significance while grouped according to the CD38 (+) cell groups (p< 0.05; Table 3). The MVD values in Group 2 was significantly lower than in Group 3 (p< 0.05; Table 3). The MVD values were revealed a significance while grouped according to the ISS (p= 0.011; Table 3). The MVD values in Stage 2 was significantly lower than in Stage 3 (p= 0.008; Table 3).

DISCUSSION

The present study revealed that increased BM angiogenesis in PCM is correlated with increased disease stage. In addition, it has been shown that high plasma cell ratio in the BM, diffuse plasma cell infiltration pattern and fibrosis, which are known to be poor prognostic factors in myeloma, are also associated with increased angiogenesis. However, angiogenesis and fibrosis were not associated with survival status.

The prognostic factors should be determined before the treatment starts to guide physician to plan the treatment. In addition to other clinical and laboratory findings, BM examination is a requirement for diagnosis and follow-up.

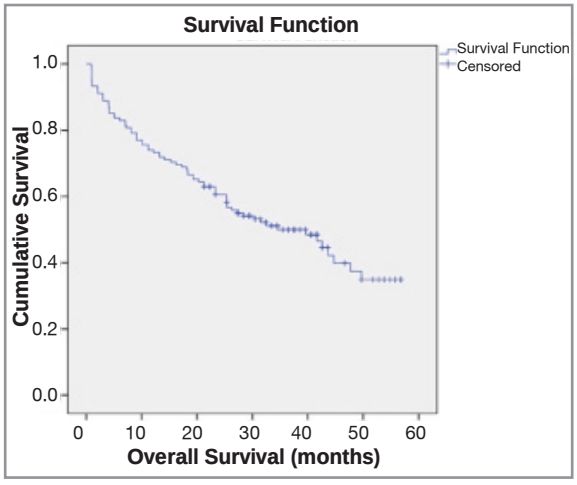

Figure 1. Overall survival curve

In the present study, while BM fibrosis was not associated with survival status and stage; as BM fibrosis increased, CD38 positive plasma cells were also observed to increase, but these two parameters were found to be weakly correlated with each other. 90% of stage 3 cases had more than 50% CD38 positive plasma cells. In relation, 59.2% of the cases those had more than 50% CD38 positive plasma cell were included in Stage 3 group. Based on these findings, it can be suggested that the rate of CD38 positive plasma cells is associated with advanced disease and it is a poor prognostic marker.

In addition to the presence of BM fibrosis and plasma cell percentage in BM biopsies in PCM, the infiltration pattern should also be evaluated. Patients with a plasma cell percentage greater than 50% show often diffuse infiltration pattern.[13] Singhal et al. found diffuse infiltration in 22% of 49 patients. Cases with interstitial patterns, with a plasma cell percentage of less than 20%, and without fibrosis showed a survival longer than 5 years.[16] In contrast, Bartl et al. demonstrated that increased fibrosis was associated with a diffuse infiltration pattern.[18]

In the current study, consistent with the literature, the majority of stage 3 cases (74%) showed diffuse infiltration pattern. BM fibrosis was grade 3 in most of the cases with diffuse infiltration pattern, while the percentage of plasma cells was more than 50%.

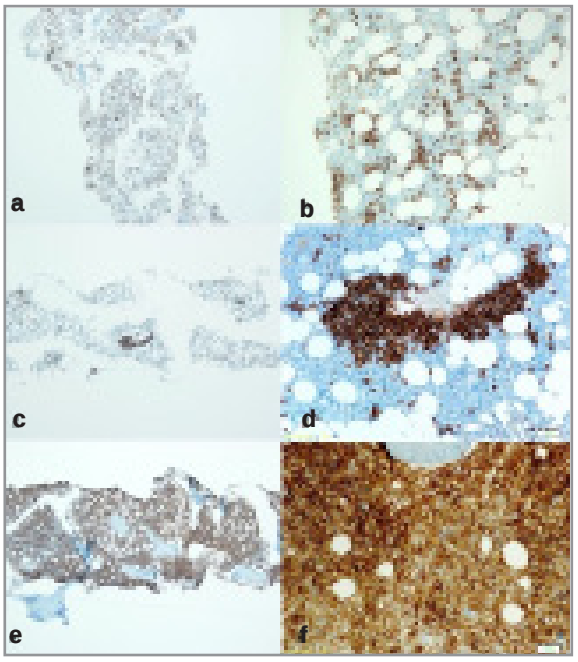

Figure 2. CD38 (+) staining **(a)** Interstitial involvement pattern (x40), **(b)** Interstitial involvement pattern (x200), **(c)** Nodular involvement pattern (x40), **(d)** Nodular involvement pattern (x200), **(e)** Diffuse involvement pattern (x40), **(f)** Diffuse involvement pattern (x200).

In contrast to studies in the literature.[14,16], this study revealed that BM fibrosis was not associated with stage and survival status. The percentage of CD38 positive cells and the pattern of infiltration were closely related with stage.

Angiogenesis is an important process in neoplastic diseases. It plays a key role in tumor progression and metastasis, and regulated by proangiogenic and antiangiogenic factors.[19] In our study, BM biopsies of 51 patients were examined with CD34 immunohistochemistry for MVD. In accordance with the literature[20], MVD is higher in cases with a diffuse pattern and a CD38 positive cell rate of more than 50%. In addition, when MVD was compared between stages, it was observed that there was a significant difference between stage 2 and 3 cases.

Sezer et al. showed that MVD could be a significant prognostic factor such as beta 2 microglobulin, CRP, and age, by performing MVD with CD34 immunohistochemistry.[21] In different studies, to determine the MVD different antibodies have been used and similar results have been obtained.[22-24]

Table 3. The relationship between MVD and other prognostic features.

Bone marrow fibrosis					
	BMF 0 (n= 9)	**BMF 1 (n= 14)**	**BMF 2 (n= 18)**	**BMF 3 (n= 9)**	**p**
	Mean±SD (min-max)	Mean±SD (min-max)	Mean±SD (min-max)	Mean±SD (min-max)	
MVD	5.9±3.6 (2.2-11.2)	4.7±4.0 (1.0-13.7)	11.6±10.2 (1.7-36.3)	8.0±3.7 (4-15.7)	0.036*
The pattern of bone marrow involvement					
	Interstitial (n=25)	Nodular (n= 5)	Diffuse (n= 21)		
MVD	4.2±2.7 (1.0-10.4)	6.9±3.1 (4.1-11.2)	12.7±8.9 (3.2-36.3)	<0.05*	
CD38 (+) cell groups					
	Group 1 (n= 3)	Group 2 (n= 13)	Group 3 (n= 35)		
MVD	3.7±2.1 (1.7-5.8)	3.1±0.9 (1.8-4.3)	10.1±7.8 (1-36.3)	<0.05*	
ISS					
	Stage 1 (n= 10)	Stage 2 (n= 7)	Stage 3 (n= 17)		
MVD	7.1±6.8 (2.0-24.2)	3.3±2.4 (1.0-8.1)	10.1±7.1 (1.8-33.1)	0.011*	

* p< 0.05 Kruskal-Wallis test MVD= microvessel density; BMF= bone marrow fibrosis; ISS= international staging system

It has been reported angiogenesis was higher in relapsed PCM cases than in newly diagnosed PCM cases. Therefore, it has been suggested that angiogenesis plays a role in the progression of asymptomatic PCM to symptomatic PCM.[25] Moreover, Sucak et al. analyzed BM biopsies of 29 PCM patients undergoing autologous stem cell transplantation for MVD, and they found that MVD was positively correlated with serum monoclonal protein levels.[26] Priyadarshini et al. evaluated BM biopsies of 48 myeloma patients in terms of plasma cell percentage, infiltration pattern, cytological grade, proliferation index and MVD. Their results were similar to ours.[27] In a case series of 42 cases conducted by Babarovic et al., BM biopsies at diagnosis and after treatment were evaluated for fibrosis, angiogenesis and plasma cell infiltrates. MVD and plasma cell infiltrate were higher in patients with fibrosis in pre-treatment BM biopsies. In post-treatment biopsies; fibrosis, MVD, and plasma cell infiltrate were higher in those who did not respond to treatment. Overall survival was significantly shorter in those who did not respond to treatment and those with fibrosis. It was emphasized that detailed morphological examination of BM biopsies is very important in monitoring PCM patients.[28] Rao et al. stated that in PCM patients epidermal growth factor receptor (EGFR) and heparin-binding EGF-like growth factor (HB-EGF) levels are in parallel with the number of plasma cells, and HB-EGF is a strong inducer of angiogenesis. Therefore, EGFR inhibitors, which inhibit the HB-EGF-EGFR signaling pathway, can be used in combination with conventional cytotoxic drugs.[29]

Nevertheless, Ribatti et al. stated that mast cells were increased with angiogenesis in PCM and suggested that this may be a new target therapy area.[30] In another study, it was determined that relapsed extramedullary disease was associated with MVD and as a result, increased MVD can be used to predict relapsed extramedullary disease.[31] Chung et al. stated that MVD is an important prognostic parameter in PCM patients and the measurement of MVD with automated methods can save time and provide more objective results.[32]

Some studies indicated that MVD was increased in cases with a high plasma cell infiltration rate whereas others did not.[20,21,27] For now, no consensus has been reached on this issue. Rana et al. stated that angiogenesis might be more valuable than the percentage of plasma cell infiltration in predicting the response to treatment. In this study, angiogenesis in PCM cases has also been shown to increase in patients with residual disease compared to those who developed a complete response after treatment. Accordingly, the evaluation of angiogenesis in newly diagnosed PCM cases may be

useful in predicting the course of the disease. They also stated angiogenesis is associated with other prognostic factors and can be evaluated as an independent prognostic factor.[33]

Considering that there is currently no curative treatment for PCM and of those also have several side effects, it could be suggested that the development of treatment methods targeting angiogenesis may increase disease survival. In addition, routine evaluation of plasma cell percentage, infiltration pattern, fibrosis and MVD in BM biopsies of newly diagnosed cases could provide alternative treatment modalities for the prognosis of the disease.

Conclusion

The findings of the present study suggested that evaluation of plasma cell percentage, infiltration pattern, fibrosis and MVD in BM biopsies of newly diagnosed PCM cases might be useful tools in the management and maintenance of the treatment. To the best of our knowledge, this is the first study to evaluate angiogenesis and fibrosis in the BM together.

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