Proliferation and Angiogenesis Using Immunohistochemistry in Prognosticating Multiple Myeloma

Sarah Grace Priyadarshini¹ · Debdatta Basu¹ · Rakhee Kar¹ · T. K. Dutta¹

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Abstract Multiple myeloma is a neoplasm of plasma cells characterised by the presence of M protein in serum and urine. Angiogenesis and proliferation play a major role in the pathogenesis of various neoplasms. The study evaluated proliferation and angiogenesis in 48 cases of myeloma, and correlated it with morphological and clinical parameters. The histomorphological features like plasma cell morphology, percentage of plasma cells and pattern of infiltration were studied in the bone marrow aspirate and trephine biopsy. Angiogenesis was assessed by calculating the microvessel density (MVD) using immunohistochemistry for CD34. Proliferation was assessed using Ki67 and CD38 highlighted the plasma cells. The mean Ki67 % was found to be significantly higher (19.6 % range 2–40 %) in poorly differentiated morphology compared to well differentiated morphology (4.06 % range 0.2–20 %) ($p = 0.003$). The mean MVD in the well differentiated morphology was 10.6 (range 1.2–47.4) compared to 20.3 (range 6.9–39.6) in the poorly differentiated morphology ($p = 0.04$). The mean MVD was 5.7 (range 1.2–12.8) in the interstitial pattern of infiltration compared to 20.04 (2.9–47.4) in the diffuse pattern ($p < 0.0001$). The mean

MVD was 6.4 in cases with serum albumin >3.5 gm/dl compared to 13.3 in cases with serum albumin <3.5 gm/dl ($p = 0.009$). Both the Ki67 and MVD showed an increasing trend with the clinical staging. Thus the study of proliferation and angiogenesis in bone marrow biopsy is useful for prognosticating patients with multiple myeloma.

Keywords Angiogenesis · Microvessel density · Proliferation · Myeloma

Introduction

Multiple myeloma is a heterogenous disease with varied clinical outcome [1]. Prognostication of Myeloma was earlier done by Durie Salmon staging system [2]. However currently the International staging system (ISS) based on serum $\beta 2$ microglobulin and serum albumin is preferred [3]. In a recent revised guidelines serum LDH and high risk chromosomal abnormalities have been incorporated for better risk stratification [4]. Novel prognostic methods like cytogenetics and FISH have been developed, but may be unavailable in many setups. Therefore alternative prognostic markers that serve as biological surrogates for the genetic changes in multiple myeloma may be of potential utility.

Bone marrow examination is done as a part of the initial workup as well as for follow up of myeloma patients. Early studies on myeloma have shown that histomorphological features like plasma cell morphology, pattern of infiltration and percentage of plasma cells have prognostic significance [5]. Studies have shown that angiogenesis and proliferation may carry prognostic significance [6]. We report our experience with these markers in a south Indian patient cohort.

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✉ Sarah Grace Priyadarshini
grace4ever03@gmail.com

¹ Departments of Pathology and Medicine, Jawaharlal Institute of Postgraduate Medical Education and Research, Puducherry, India

Materials and Methods

Forty eight cases of multiple myeloma diagnosed between January 2007 till April 2012 were enrolled after approval of the study design by the institutional Ethics committee. Bone marrow aspiration and biopsy was performed in all the cases. Biopsies were unilateral and were included irrespective of length. The bone marrow biopsies were fixed in 10 % neutral buffered formalin and decalcified using EDTA. Sections were stained with Hematoxylin and Eosin (H&E). The bone marrow aspirate slides were stained with Giemsa and Leishman stain.

The bone marrow aspirates and biopsies were studied to determine the following 3 morphological parameters:

1. Cytological grading of neoplastic cell differentiation into well, intermediate and poorly differentiated (Fig. 1) groups was done based on Sailer et al. criteria [7] using both the aspirates and biopsies. The '**well differentiated**' group included cases with more than 90 % mature plasma cell morphology. The '**poorly differentiated**' group included the cases with more than 30 % of plasmablasts and anaplastic morphology. The '**intermediate**' group included cases which did not fall in either of the criteria. This included those having plasma cells with less eccentric nucleus, less cytoplasm and not so prominent perinuclear hof.
2. The pattern of neoplastic growth was studied in the bone marrow biopsy. The '**interstitial**' pattern showed infiltration of the plasma cells in an otherwise preserved marrow space. The '**nodular**' pattern showed aggregate of more than 10 plasma cells in a

preserved marrow space. The '**diffuse**' pattern showed replacement of marrow space by plasma cells with loss of fat spaces [8].

3. The volume of infiltration as determined by percentage of plasma cells was assessed in both the aspiration and biopsy. It was divided into 3 groups: less than 20 %, 20–50 % and more than 50 %.

Immunohistochemical staining with anti CD34, CD38 and Ki67 was done in all cases. After deparaffinisation and rehydration the sections were treated with 3 % hydrogen peroxide. The antigen retrieval was done in a Decloaking chamber at a temperature of 125 °C for 40 s followed by 90 °C for 10 s. After washing with Tris buffer saline (TBS), the primary antibodies were added and incubated for 1 h. The primary antibodies used were anti CD34 (EP88), CD38 (EP135) and Ki67 (MIB-1), all from Dako. After second wash with TBS, the polymer (Dako envision K5007) was added and incubated for 40 min. The sections were stained with the chromogen (DAB) and then counterstained with hematoxylin.

The proliferation index was calculated as the percentage of plasma cells which were stained with Ki67. In cases with interstitial or scattered plasma cells, IHC with CD38 was cross-checked to identify the plasma cells on a separate slide. For calculating the microvessel density 'Hot spots' were selected, after staining with anti CD34, by examining under 10× and defined as areas showing maximum vascularisation. Both the vessels as well as the individual cells stained with anti-CD34 were counted in 10 high power fields (HPF) under 40× objective with a 20 mm eyepiece diameter and the average per HPF was taken as the 'Micro

Fig. 1 Bone marrow biopsies showing infiltration by well differentiated (a), intermediate (b), and poorly differentiated myeloma cells (c) (H&E 200×) (Inset showing the corresponding aspirates-Giemsa 200×)

vessel density' (MVD). The MVD was expressed as number of vessels per HPF. Thick walled blood vessels were not counted.

The statistical analysis was done using GraphPad InStat (GraphPad InStat version 3.06 for Windows 7, GraphPad Software, San Diego California USA) and a p value less than 0.05 was taken as statistically significant. The proliferation index was compared with the plasma cell morphology, pattern of infiltration and clinical stage using Kruskal–Wallis test. The MVD was compared with the morphological parameters and clinical stage using ANOVA test. The proliferation index and MVD was compared in those with and without hypoalbuminemia using Mann–Whitney and unpaired T test with Welch correction respectively. The MVD was correlated with the Ki67 using Pearson correlation.

Results

The age of the patients in the study ranged from 26 to 75 years with a median age of 51.5 years and a male:female ratio of 1.8:1. The most common clinical presentation was bone pain seen in 25 (52 %) cases. Nephrotic syndrome as the initial presentation was seen in one case and the renal biopsy of the same case showed amyloidosis. Pleural effusion was seen in one case along with lytic lesions in the vertebra. Pleural fluid cytology was done with a suspicion of metastatic malignancy, which revealed increase in plasma cells with plasmablastic morphology.

Serum albumin was less than 3.5 mg/dl in 63 % of cases. Hemoglobin was less than 8.5 gm/dl in 62 % of the cases. Thrombocytopenia ($<150 \times 10^9/l$) was seen in 39 % of the cases. Only 4 % of the cases presented with leucopenia. One case presented with pancytopenia. Based on the Durie–Salmon stage, there were 5 cases in stage I, 12 in stage II and 31 in stage III.

The morphological parameters including percentage of plasma cells, plasma cell morphology and pattern of infiltration were studied in the bone marrow aspirate and biopsy. The plasma cell percentage was more than 50 in 52 % of the cases. Well differentiated morphology was seen in 64.6 % ($n = 31$), intermediate in 16.7 % ($n = 8$)

and poorly differentiated in 18.7 % ($n = 9$) of the cases. Both the interstitial and diffuse pattern was seen in 40 % of cases each with the remaining 20 % showing a nodular pattern. There was a significant difference ($p = 0.0067$) in the pattern of infiltration between the morphological subgroups with plasmablastic (poorly differentiated) morphology associated with diffuse pattern of infiltration (Table 1). Morphology and pattern of infiltration also correlated with the clinical stage of the disease. In all the five stage I cases, there was no case with poorly differentiated morphology as compared to 7 of the 31 cases in stage III ($p = 0.3$). Also there were no cases with diffuse pattern of infiltration seen in stage I compared to 14 cases in stage III ($p = 0.05$). The clinical stage also correlated well with the percentage of plasma cell infiltration. In stage I there were no cases having more than 50 % cellular infiltration compared to 21 cases in stage III ($p < 0.0001$).

The proliferation index (Ki67 %) of the cases ranged from 0.1 to 40 % (Fig. 2) with a mean of 6.7 %. The mean Ki67 % in the well differentiated group was 4.06 (SD 5; Range 0.2–20 %; CI 2.2–5.9) compared to 19.6 (SD 11.9; Range 2–40 % CI 10.4–28.8) in the poorly differentiated group ($p = 0.003$) (Table 2). The mean Ki67 was also correlated with the pattern of infiltration. It was higher in the cases with diffuse infiltration (Mean 10.2; SD 12) compared to those with interstitial pattern of infiltration (Mean 4.7; SD 5.7) ($p = 0.18$) (Table 3). The mean Ki67 % in stages I, II and III were 3.34 (SD 4.1), 4.32 (SD 4.8) and 8.23 (SD 10.6), respectively ($p = 0.5$) (Table 4). The mean Ki67 % was compared in patients with normal (>3.5 gm/dl) as well as reduced (<3.5 gm/dl) serum albumin and was found to be higher in the latter (Mean 5.6; SD 5.9 vs. Mean 8.06; SD 9.3) ($p = 0.9$).

The microvessel density (MVD) ranged from 2.4 to 47.4 (Fig. 3) with a mean of 12.6. The difference in the mean MVD of well (Mean 10.6; SD 10.6; Range 1.2–47.4; CI 6.6–14.6) and poorly differentiated (Mean 20.3; SD 10.2; Range 6.9–39.6; CI 12.4–28.1) groups was found to be marginally statistically significant ($p = 0.0491$) (Table 2). The mean MVD was also compared with the pattern of infiltration (Table 3) and it was found that the diffuse pattern had a higher mean MVD (Mean 20.04; SD 12.4;

Table 1 Comparison of the plasma cell morphology with the pattern of infiltration

Pattern of infiltration	Plasma cell morphology			
	Well differentiated ($n = 31$)	Intermediate ($n = 8$)	Poorly differentiated ($n = 9$)	
Interstitial	17 (54.8 %)	2 (25 %)	0 (0 %)	$p = 0.0067^*$
Nodular	4 (12.9 %)	4 (50 %)	2 (22.2)	
Diffuse	10 (32.3 %)	2 (25 %)	7 (77.8 %)	

* χ^2 test

Fig. 2 Immunostaining with Ki67 showing low (a) and high proliferation index (b) (IHC 400×)

Table 2 Comparison of plasma cell morphology with proliferation and angiogenesis

Plasma cell morphology	Well differentiated (n = 31)	Intermediate (n = 8)	Poorly differentiated (n = 9)	<i>p</i> value
Mean Ki67 %	4.06	2.58	19.66	0.003*
Mean MVD	10.65	11.38	20.3	0.0491**

* Kruskal–Wallis test

** ANOVA test

Table 3 Comparison of pattern of infiltration with proliferation and angiogenesis

Pattern of infiltration	Interstitial (n = 19)	Nodular (n = 10)	Diffuse (n = 19)	<i>p</i> value
Mean Ki67 %	4.7	3.9	10.2	0.18*
Mean MVD	5.75	10.89	20.04	<0.0001**

* Kruskal–Wallis test

** ANOVA test

Table 4 Comparison of clinical staging with proliferation index and angiogenesis

Parameters	Stage I (n = 5)	Stage II (n = 12)	Stage III (n = 31)	<i>p</i> value
Mean Ki67 %	3.34	4.32	8.23	0.5*
Mean MVD	6.84	11.51	13.94	0.3**

* Kruskal–Wallis test

** ANOVA test

Range 2.9–47.4; CI 14–26) compared to the interstitial pattern (Mean 5.7; SD 2.8; Range 1.2–12.8; CI 4.3–7.1) and this was found to be statistically very significant ($p < 0.0001$).

The mean MVD of the patients with hypoalbuminemia was higher (Mean 13.3; SD 10.1) compared to those without hypoalbuminemia (Mean 6.4; SD 3.9) was

compared and was found to be statistically very significant ($p = 0.009$). The clinical staging was compared with the mean MVD and it showed an increasing trend with the mean MVD being 6.8 (SD 2), 11.5 (SD 9.9), 13.9 (SD 11.4) in stage I, II and III respectively ($p = 0.3$) (Table 4). The MVD was correlated with the Ki67 and showed a weak positive correlation ($r = 0.24$; $p = 0.09$).

Fig. 3 Immunostaining with CD34 showing low (a) and high microvessel density (b) (IHC 200×)

Discussion

Proliferation in myeloma has been assessed using proliferation markers like Ki67 and bromodeoxyuridine in different studies. Drach et al. [9] found that high Ki-67 values correlated significantly with higher clinical stage, beta-2-microglobulin, plasmablastic morphology, and diploid myeloma cell DNA-content. Thaler et al. [10] in their study also found a significant correlation between proliferation of myeloma cells and serum IL-6 values. In a recent study on 134 cases of plasmacytoma, Ghodke et al. [10] found the histologic grade and MIB1 proliferation index predicted an aggressive course with progression to myeloma.

In the present study, proliferation index was higher in the cases with poorly differentiated morphology and diffuse pattern of infiltration. Xu et al. [6] in a similar study also found a statistically significant difference in proliferation index between the morphological groups. Measuring Ki 67 may prove to be a useful parameter in prognostication of myeloma as it was significantly higher not only in poorly differentiated morphology but also correlated with prognostic features like the stage and low serum albumin levels in the present study. Assessing Ki67 in a highly proliferating organ like marrow may be associated with errors of observation as normal hematopoietic cells also show high proliferation. This was overcome in the present study by studying Ki 67 only in areas where the myeloma cells were present and cross checked by using CD 38 immunostaining.

Angiogenesis is an important phenomenon in neoplastic disorders. It has been studied in various solid

tumours as well as haematological malignancies [11]. It plays a key role in tumour progression and metastasis. It is regulated by the action of various pro-angiogenic as well as anti-angiogenic factors [11]. Vacca et al. [12] first studied angiogenesis in multiple myeloma in a group of 67 Caucasian patients. The microvessel area was found to be higher in patients with active disease than non-active disease. Rajkumar et al. [13] studied microvessel density (MVD) and found a significant correlation of MVD with plasma cell labelling index and the median survival. Alexandrakis et al. [14] had also performed MVD using CD34 monoclonal antibody and established a positive correlation with the stage of the disease and the percentage of plasma cell infiltration. In a recent study CD105 (endoglin) was used as marker of neoangiogenesis in myeloma which showed correlation with disease stage and decreased in response to treatment [15].

In the present study, MVD was found to be higher in the cases with poorly differentiated morphology, higher stage of disease and very significantly with a diffuse pattern of infiltration. This suggests that the increase in the angiogenesis is important for the infiltration of the marrow by the plasma cells. It is postulated that vascular endothelial growth factor (VEGF) produced by the tumour cells induces angiogenesis in marrow and the endothelial cells, in response to VEGF, secrete interleukin-6 which acts as a growth factor for myeloma cells.

Therefore both proliferation and angiogenesis are closely related factors in the progression of myeloma and play a major role in determining its prognosis.

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