Journal of the Egyptian Society of Haematology & Research, Vol. 9, No. 1, March: 33-38, 2013

Bone Marrow Biopsy Angiogenesis in Multiple Myeloma: Computerized Image Analysis and Correlation with Clinico-Pathologic and Laboratory Prognostic Factors

HEBA N. RASLAN, M.D. and BASMA M. ELGAMAL, M.D.

The Department of Clinical Pathology, National Cancer Institute, Cairo University

ABSTRACT

Background: Increased angiogenesis has been found to be an adverse prognostic factor in solid tumors. Evidence shows that angiogenesis plays an important role in hematological malignancies including multiple myeloma (MM) as well.

Aim of the Study: We aimed in the present study to investigate the various angiogenesis parameters; namely microvessel density (MVD) and total vascular area (TVA), using a standardized computer assay instead of the routinely used hot-spot technique, on bone marrow biopsy (BMB) of de novo MM patients. We also correlated BMB angiogenesis with clinico-pathologic and laboratory parameters established to have prognostic impact on MM.

Patients and Methods: BMB from thirty eight newly diagnosed cases of MM and twenty morphologically and immunohistochemically negative Hodgkin's lymphoma de novo patients as a control group, were examined using a computerized image analyzer. Bone marrow biopsies of test cases (n=38) were immunohistochemically stained with CD34 for visualization of microvessels, and MVD and TVA were measured.

Results: MVD and TVA were significantly increased in MM patients Vs controls (p<.0.001 for both). Angiogenesis was correlated with higher tumor burden, higher B_2 microglobulin level, higher M protein level and diffuse pattern of marrow infiltration.

Conclusion: Angiogenesis in BMB of MM cases is correlated to all other prognostic parameters which qualify it as a potential prognostic parameter. Standardization of the method is mandatory before testing it as an indicator of treatment outcome.

Key Words: *Angiogenesis – Multiple myeloma – Bone marrow biopsy – Microvessel density – Total vascular area – Computerized image analysis.*

INTRODUCTION

Multiple myeloma (MM) accounts for 1% of all malignancies and >10% of malignant hematological neoplasms **[1]**. It is characterized by the presence of a monoclonal (M) protein, lytic bone lesions, and increased plasma cells in the bone marrow and may be associated with anemia, renal failure, and hypercalemia **[2]**. Angiogenesis, the formation of new blood vessels, occurs physiologically during embryonal growth, wound healing, and in the female genital system during the menstrual cycle. It is important for the proliferation and metastasis of most malignant neoplasms. In the absence of angiogenesis, tumors cannot grow beyond 1-2mm in size **[3]**. Increased angiogenesis has been found to be an adverse prognostic factor in solid tumors but evidences show that angiogenesis also plays an important role in hematological malignancies including multiple myeloma **[4]**.

The limited success achieved by targeting only myeloma cells with the existing conventional and/or high-dose chemotherapy highlights the importance of understanding the role of the bone marrow microenvironment and its contribution to myeloma genesis. The microenvironment in multiple myeloma is composed of clonal plasma cells, bone marrow stromal cells, extracellular matrix proteins, inflammatory cells, and microvessels **[5]**. There is substantial evidence that interactions between these components have a crucial role in the proliferation and survival of myeloma cells and their acquisition of drug resistance and disease progression **[6]**.

There is growing evidence that not only increased bone marrow angiogenesis occurs in multiple myeloma, but also it is related to disease activity. The mechanism behind the increased angiogenesis in myeloma is not fully understood. There are data that myeloma cells express the potent angiogenic cytokines, VEGF and basic fibroblast growth factor **[7]**. Preliminary data using reverse transcription-PCR techniques indicate that VEGF isoforms, VEGF121 and VEGF165, are expressed by myeloma cells both in studies of bone marrow samples from patients with myeloma and on various myeloma cell lines **[4,8]**. Targeting angiogenesis with antiangiogenic agents is a promising and exciting therapeutic approach and is the subject of intense investigation **[9]**.

The aim of our study is to investigate the extent of angiogenesis in bone marrow biopsy samples of newly diagnosed multiple myeloma patients compared to controls, using a computer based image analyzer soft ware, and to find out its correlation with other established clinicopathologic and laboratory prognostic parameters.

PATIENTS AND METHODS

Thirty eight newly diagnosed multiple myeloma patients were enrolled in our study. Patients were diagnosed to have multiple myeloma according to the WHO criteria such as bone marrow aspirate plasma cell percentage, lytic bone lesions on skeletal survey, anemia, hypercalcemia, renal insufficiency, Immunophenotyping, monoclonal gammopathy and Bence Jones proteinuria **[10]**. Pretreatment bone marrow trephine biopsy samples were available from all the patients.

Information on prognostic factors included clinical staging according to the Durie and Salmon staging system **[11]**, serum M-protein levels, percentage of bone marrow aspirate plasma cells, B_2 microglobulin levels (B_2M), pattern of bone marrow biopsy plasma cell infiltration, and extent of marrow fibrosis; they were correlated with the degree of bone marrow angiogenesis. Twenty age- and sex-matched bone marrow biopsies performed for staging of Hodgkin's lymphoma for patients who proved to have no evidence of infiltration, based on morphology and immunohistochemistry for CD30, and who received no previous chemotherapy were included in the study as a control group.

Bone marrow biopsy preparation, immunohistochemical staining and assessment:

Biopsies were fixed in 10% formalin, decalcified in 10% EDTA for 48 hours, and embedded in Paramat extra (BDH, Poole, Dorset, UK). Initially, haematoxylin and eosin stained, 3μm thick sections were examined by light microscopy. All slides (H & E) were evaluated for confirmation of the original diagnosis. The pattern of infiltration of the bone marrow by MM was highlighted by immunostaining the neoplastic plasma cells with a monoclonal antibody to CD138 (clone MI15, m7228, DAKO, Glostrup, Denmark). Microwave antigen retrieval was done in the presence of 1mmol/L EDTA (pH 8.0) buffer. Slides were then incubated with anti-CD138 monoclonal antibody at 1:25 dilutions for 30 minutes at room temperature. Immunoglobulin k (No. 40191, DAKO) and immunoglobulin λ (No. 40193, DAKO) antibodies to detect the restriction of immunoglobulin light chains and to confirm monoclonality were done.

Monoclonal antibody anti-CD34 class II (m7165 clone QBEnd10, DAKO) was used to highlight endothelial cells. Epitope retrieval was achieved by immersing slides in Tris-EDTA (Merck, Damstadt, Germany) buffer (pH 9.0) and boiling for 15 minutes in a water bath at 97°C. Slides were then incubated with CD34 monoclonal antibody at 1:100 dilutions for 30 minutes at room temperature.

Computerized image analysis (CIA) of angiogenesis:

All slides stained with anti-CD34 were scanned and analyzed with Alphelys Spot Browser 2 integrated system (Alphelys, France), using a software-controlled (Alphelys Spot Browser 2.4.4, Alphelys), stage-positioning Nikon Eclipse 50i microscope (Nikon, Japan) mounted on a 1360x1024 resolution Microvision CFW-1310C digital camera (Microvision Instruments, France). Slides were scanned at x 20 magnifications to identify the section area of slide and then scanned at x 200 magnifications to create images for quantification.

Computer-assisted image analysis was used to determine the total count of microvessels per square millimeter and total area occupied by microvessels (as a percentage of the total section

area). During digital image analysis, the software detected objects of interest based on pixel color properties (wavelength, intensity, and saturation) and morphometry (size and shape). The analysis software used created color segmentation algorithms on all slides which were designed for the detection and elimination of empty space, determination and count of positive areas, and measurement of mean vessel diameter. These measurements were used to calculate average MVD (quantity of microvessels per square millimeter) and TVA (percentage of microvessel area in total section area).

Analysis of data:

Statistical analysis was performed using SPSS version 18 software. The measures of central tendency for continuous data such as plasma cell infiltrates, MVD and TVA were compared by using the Student-*t*-test or Mann-Whitney test, depending on data distribution. The Fisher exact test and the Chi-square test were used to compare differences in nominal variables. Correlation between microvessel density, total vascular areas and other laboratory and clinical data was done by w2 test (univariate analysis).

RESULTS

A total number of 38 newly diagnosed multiple myeloma patients were enrolled in the present study. They were 29 males and 9 females with a male to female ratio of 3.2:1. 52.6% of the patients had monoclonal IgG, while 26.3% of patients had monoclonal IgA. The age of the patients ranged from 47 to 63 with a mean of 54.8±6.3 years. B_2 microglobulin level ranged from 2400 to 13,500 with a mean of 5800±2900 ng/dl. Bone marrow aspirate plasma cell percentage ranged from 13% to 76% with a mean of 45±12%. Table (1) shows the different clinical and laboratory parameters of the patient's group and Figs. (1-4) demonstrate morphologic patterns of marrow infiltration, immunohistochemistry and angiogenesis.

Table (1): Clinico-pathologic and laboratory parameters of the multiple myeloma patient group.

	Number (38)	Percent (100%)
Histological pattern:		
Interstitial	13	34.2
Sheets	9	23.7
Nodular	2	5.3
Diffuse	14	36.8
Degree of marrow fibrosis:		
No fibrosis	17	44.7
Grade I	10	26.3
Grade II	11	29
M protein level:		
<2gm%*	6	15.8
2-4gm%	20	52.6
>4gm%	12	31.6
Durie and salmon staging:		
Stage I	8	21.1
Stage II	8	21.1
Stage III	22	57.8
Plasma cell % in bone marrow aspirate:		
<20%	9	23.7
20%-50%	18	47.4
>50%	11	28.9
B_2 microglobulin level:		
<3400ng/dl	16	42.1
3400-10000ng/dl	19	50
>10000ng/dl	3	7.9

*The level of 2gm% was considered due to the percentage of IgA.

MVD (the quantity of microvessels/square mm) and TVA (percentage of microvessel area in the total section area) were measured for all patients and controls. MVD ranged from 12.3-430.5 with a median of 163.6 for the control group and ranged from 13.7-1079.1 with a median of 174.4 for the multiple myeloma group. TVA ranged from 0.1-16.4 with a mean of 1.9±1.3% for the control group and ranged from 18.2-36.1 with a mean of 6.4±6.5% for the multiple myeloma group. There was a significant difference between MVD and TVA in the myeloma group versus the control group (p<0.001 for each). Patients with higher angiogenesis had significantly higher B_2 microglobulin levels (p=0.012 for MVD and p=0.029 for TVA) and higher levels of M protein (p=0.039 for MVD). MVD and TVA were significantly higher in diffuse pattern of marrow infiltration compared to other patterns of infiltration (p=0.008 and p=0.023 respectively).

Angiogenesis was significantly correlated with the percentage of plasma cells, for MVD r=0.48 and p=0.001 and for TVA r=0.45 and p=0.003. It was also significantly correlated with the level of M protein (r=0.44 and p=0.004 for MVD), with no significant correlation with TVA (p=0.066). No significant correlation could be detected between angiogenesis and either the clinical staging of the disease (p=0.089 for MVD and p=0.113 for TVA) or the degree of marrow fibrosis (p=0.069 for MVD and p=0.093 for TVA).

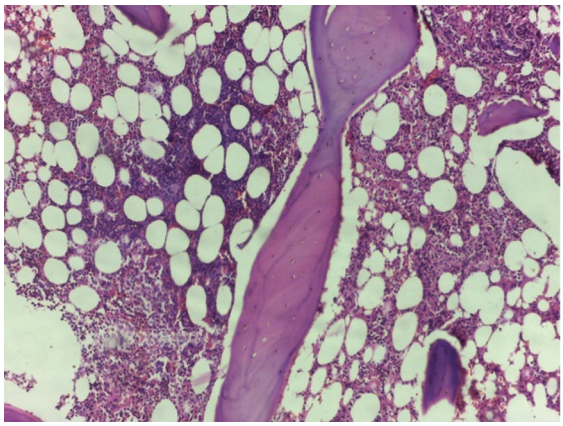

Fig. (1): Sheet of mature plasma cells (x20).

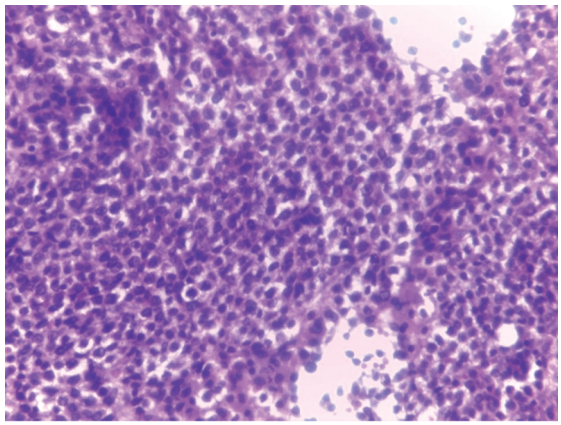

Fig (2): Diffuse infiltration with plasma cells (x40).

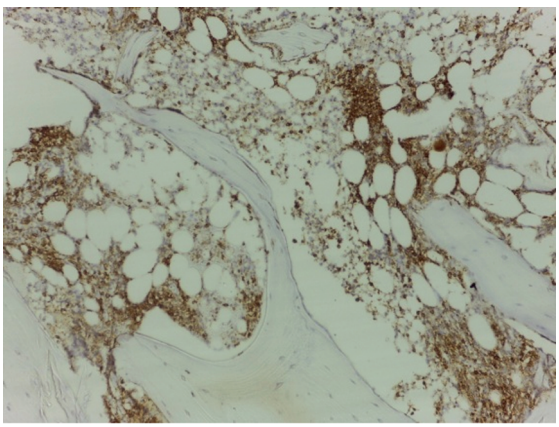

Fig. (3): CD138 positive plasma cells (x20).

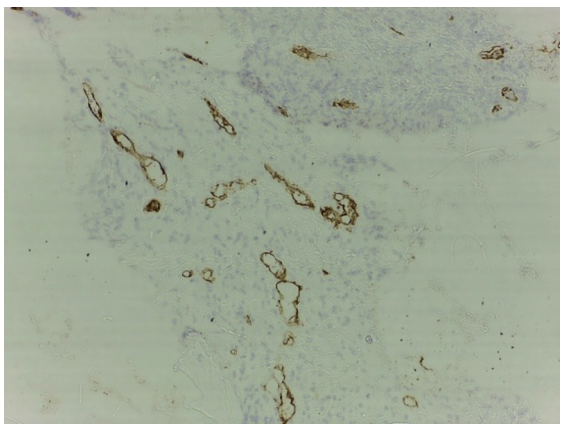

Fig. (4): Abundant CD34 lined newly formed vessels (x40).

DISCUSSION

In a malignancy such as MM, in which multiple therapeutic approaches with various mechanisms of action are available, accurate and standardized techniques for monitoring patients is critical to confirm disease responsiveness, to enable prompt detection of ineffective therapy, and to detect relapse before the occurrence of organ damage **[12]**. Not much attention is given to the morphologic characteristics of the bone

marrow, although it has been shown in several studies that the use of BMB is a more accurate method for the evaluation of plasma cell infiltration **[13]**. Therefore, the aim of the present study was to analyze accurately one of the most important components of the bone marrow microenvironment in BMB samples of patients with MM; namely angiogenesis. Most of the previous studies included only simple quantitative evaluation of MVD and used methods developed primarily for the characterization of angiogenesis in solid tumors. Such studies measured MVD in the hot spots, which are areas of the bone marrow biopsy carrying the highest number of microvessels, based on conventional light microscopy. Our study measured angiogenesis: MVD, and TVA on the whole area of the slides. Therefore, the usually applied, so-called hot-spot technique should be amended by an appropriate and more elaborate computer-assisted morphometric analysis of the microvessel structures. Such a computer standardized spectrum of information regarding quantity and quality of angiogenesis enables further understanding of the morphologic changes in the course of the disease and accordingly on the effect of various therapies on bone marrow vascularization.

The results of our study linked plasma cell infiltration with angiogenic activity in MM because both angiogenic parameters, MVD and TVA, correlated with plasma cell percentage "Tumor burden" and the diffuse pattern of marrow infiltration. This association is in accordance with other studies **[14-17]**. We also correlated MVD and TVA with increased levels of B_2 microglobulin, above 3400ng/dl as reported by Bhati et al., **[15]** and with increased level of M protein as reported by Babarović et al., **[17]**. A limitation of the present study is the small number of patients, which might have impacted our failure to detect an association between angiogenesis parameters and either of the extent of marrow fibrosis and the clinical staging of the disease.

Our study underlines the role of BMB not only in establishing the diagnosis of MM but also in patient monitoring and providing important prognostic information, as well as highlighting the importance of angiogenesis being correlated to other prognostic parameters, which qualify it as a potential prognostic one.

REFERENCES

1- Landis SH, Murray T, Bolden S, Wingo PA. Cancer statistics, CA Cancer J. Clin. 1999; 49: 8-31, 1999.

2- Bataille R, Harousseau JLS. Multiple myeloma. N Engl J Med. 1997; 336: 1657-1664.

3- Folkman J. Seminars in medicine of the Beth Israel Hospital, Boston. Clinical applications of research on angiogenesis. N Engl J Med. 1995; 333: 1557-1563.

4- Rajkumar SV, Leong T, Roche PC, et al. Prognostic value of bone marrow angiogenesis in multiple myeloma. Clin Cancer Res. 2006; 3111.

5- Ribatti D, Vacca A. The role of microenvironment in tumor angiogenesis. Genes Nutr. 2008; 3: 29-34.

6- Fowler JA, Edwards CM. Croucher PI. Tumor-host cell interactions in the bone disease of myeloma. Bone. 2011; 48: 121-128.

7- Ito A, Hirota S, Mizuno H, Kawasaki Y, Takemura T, Nishiura T, Kanakura Y, Katayama Y, Nomura S, Kitamura Y. Expression of vascular permeability factor (VPF/VEGF) messenger RNA by plasma cells: Possible involvement in the development of edema in chronic inflammation. Pathol. Int. 1995; 45: 715-720.

8- Bellamy WT, Richter L, Frutiger Y, Grogan TM. Expression of vascular endothelial growth factor and its receptors in hematopoietic malignancies. Cancer Res. 1999; 59: 728-733.

9- Vacca A, Ribatti D, Roncali L, Dammacco F. Angiogenesis in B cell lymphoproliferative diseases. Biological and clinical studies. Leuk. Lymphoma. 1995; 20: 27-38.

10- Mckenna RW, Kyle RA, Kuehi WM, et al. Plasma cell neoplasms. In: Swirlow S, Campo E, Harris N, et al. eds. WHO classification of tumors of hematopoietic and lymphoid tissues. International Agency for research on cancer, Lyon. 2008; pp. 200-213.

11- Durie BG, Salmon SE. A clinical staging system for multiple myeloma: Correlation of measured myeloma cell mass with presenting clinical features, response to treatment and survival. Cancer. 1975; 36: 842.

12- Chee CE, Kumar S, Larson DR, et al. The importance of bone marrow examination in determining complete response to therapy in patients with multiple myeloma. Blood. 2009; 114: 2617-2618.

13- Štifter S, Babarović E, Valković T, et al. Combined evaluation of bone marrow aspirate and biopsy is superior in the prognosis of multiple myeloma. Diagn Pathol. 2010; 5: 30. Doi: 10.1186/1746-1596-5-30.

14- Rajkumar SV, Mesa RA, Fonseca R, et al. Bone marrow angiogenesis in 400 patients with monoclonal gammopathy of undetermined significance, multiple myeloma, and primary amyloidosis. Clin Cancer Res. 2002; 8: 2210-2216.

15- Bhatti SS, Kumar L, Dinda AK, et al. Prognostic value of bone marrow angiogenesis in multiple myeloma: Use of light microscopy as well as computerized image analyzer in the assessment of microvessel

density and total vascular area in multiple myeloma and its correlation with various clinical, histological, and laboratory parameters. Am J Hematol. 2006; 81: 649-656.

16- Rana C, Sharma S, Agrawal V, et al. Bone marrow angiogenesis in multiple myeloma and its correlation with clinicopathological factors. Ann Hematol. 2010; 89: 789-794.

17- Babarović, Valković T, Štifter S, et al. Assessment of bone marrow fibrosis and angiogenesis in monitoring patients with multiple myeloma. Am J Clin Pathol. 2012; 137: 870-878.