RESEARCH ARTICLE

Ki-67 Immunostaining and its Correlation with Microvessel Density in Patients with Mutiple Myeloma

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Abstract

Purpose: To compare Ki-67 index and microvessel density MVD) in multiple myeloma and non-myeloma patients and their correlation with each other and other prognostic markers. Materials and Methods: Forty patients were enrolled in this study between 2011-2013, 30 with multiple myelomas and 10 with non-malignant disease as controls. Proliferative activity was analyzed by Ki-67 and microvessel density (MVC) was assessed by CD34 and compared between two groups. In myeloma patients, correlation between Ki-67, MVD and other prognostic factors was assessed by Pearson correlation coefficient. Results: According to Durie Salmon staging criteria, 13 patients were of stage 1, 5 of stage II and 12 of stage III. Ki-67 expression showed a positive correlation with MVD (r=0.729, p<0.001) and was significantly higher (p<0.0001) in myeloma patients (range 35-80%, mean 60.1%) as compared to controls (range 8-25%, mean 18.1%). MVD/mm² was also significantly (p<0.0001) higher in myeloma patients (range 62-237/mm², mean 178.0/mm²) than controls (range 5.2-50/mm², mean 18.3/mm²). Ki-67 and MVD, both increased progressively with increasing stage of myeloma. Ki-67 showed significant positive correlation with blood urea and lactate dehydrogenase and a significant negative correlation with serum albumin. MVD showed a significant positive correlation with blood urea, lactate dehydrogenase, serum creatinine, β2 microglobulin and skeletal lesions. Conclusions: Ki-67 and MVD are indicators of aggressiveness and poor prognosis having significant correlation with each other and other prognostic markers of multiple myeloma. Routine assessment of these markers may help to identify high risk patients, who may benefit from with more aggressive therapy.

Key words: Multiple myeloma - microvessel density - Ki-67 index - prognostic markers - angiogenesis

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Introduction

Multiple myeloma (MM) is a neoplastic plasma cell disorder characterized by the clonal proliferation of plasma cells in the bone marrow and production of monoclonal proteins in the blood or urine (Sridevi et al., 2015). It accounts for approximately 10-13% of all hematological malignancies and 1% of all malignancies (Fernando RC et al., 2015). Worldwide, incidence of multiple myeloma is 6.3 per 1, 00,000 population per year, with mortality rate of 0.003%. In India the reported incidence is 0.3 to 1.9 per 1, 00,000 for men and 0.4 to 1.3 per 1, 00,000 for women with the highest incidence being in Delhi (Kumar et al., 2006). In 2015, it is estimated that there will be 26,850 new cases of myeloma and an estimated 11,240 people will die of this disease (Fernando RC et al., 2015). Durie-Salmon staging system (DSS) and International Staging system (ISS) are used for staging and prognostication in MM. DSS uses tumor burden and renal function to stage these patients while ISS categorizes patients on the basis of $\beta 2$ microglobulin and albumin. Five-year survival rate of patients with MM is 46.6%, as it is characterized by inevitable relapse after standard or high dose chemotherapy. Prognostic factors are required to distinguish low risk disease from aggressive forms. But MM has a heterogeneous spectrum of clinical entities and outcome. Due to wide variation in outcome there is need to define other prognostic variables.

Previous studies in literature have shown that proliferation and angiogenesis are important prognostic indicators. Biological markers for proliferation and angiogenesis and their correlation with other prognostic factors can play an important role in risk stratification of these patients.

Ki-67 is a marker of proliferative activity in several human tumors (Alexandrakis et al., 2004). Though Ki-67 has been studied in hematological malignancies including multiple myeloma, it is not determined routinely due to scant information regarding its relevance and association with other prognostic factors.

Angiogenesis, characterized by the formation of new blood vessels from pre-existing vasculature, plays an important role in invasion and metastasis of tumors (Tichy et al., 2010). Microvessel density (MVD) is a marker for

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angiogenesis and reported to be related to disease activity and poor survival in MM patients. (Rajkumar et al., 2000; Lee et al., 2015).

This study was aimed to compare Ki-67 expression and MVD in myeloma and non-myeloma patients and their correlation with each other and other prognostic factors including stage of disease, serum calcium, serum creatinine, blood urea, serum LDH and total proteins including albumin.

Materials and Methods

Patients and controls

Between September 2011 and February 2013, 30 patients of MM, diagnosed as per standard criteria (International Myeloma Working Group 2003) and 10 patients of non-malignant diseases taken as controls were enrolled in the study. Staging was done according to Durie-Salmon staging system. Patients receiving any therapy were excluded from the study. All relevant clinical details were noted. Informed written consent was obtained before enrolment into study. The study was approved by the Institutional Ethical Committee.

Investigations

Bone marrow aspiration (Wright stain) and biopsy were performed on all 40 patients. Bone marrow biopsies were fixed in 10% formalin and decalcified in 10% EDTA (ethylenediaminetetracetic acid) for 48 hours. After routine processing, 4μ m sections were cut and stained with hematoxylin and eosin. The sections obtained from patients of myeloma were examined for morphological changes with special reference to pattern of plasma cell infiltration. Complete hemogram (Automated hematology analyzer LH 500), serum calcium, serum creatinine, total proteins including albumin, LDH and β 2 microglobulin were measured in all myeloma patients using standard biochemical procedures. In controls only Ki-67 and MVD was assessed and compared with myeloma patients.

Immunohistochemistry

Ki-67 immunostaining (Rabbit monoclonal Ki-67 antibody SP 6, prediluted, Cell Marque) was used to assess proliferation index and CD34 immunostaining (Q Bend/10, prediluted, Cell Marque) to assess MVD.

Lysinated slides were prepared from the bone marrow biopsy for immunostaining. After deparaffinization in xylene rehydration through graded alcohol and distilled water, sections were subjected to antigen retrieving microwave (Citrate buffer 2.1g/1000 ml, pH 6) for ten minutes. The slides were cooled to room temperature and washed twice (Tris buffer 6.05g/1000 ml, pH 7.2-7.4). Peroxidase block 4% was given for 30 minutes and the slides were washed again thrice with Tris buffer. Sections were then incubated with primary monoclonal antibody (Ki-67 & CD34) and placed overnight in a refrigerator. After bringing the sections to room temperature and washing in Tris buffer, secondary antibody (Polymer HRP Label) was added for 30 minutes. Before addition of DAB buffer, slides were washed thrice in Tris buffer and the reaction was stopped with distilled water on appearance of brown color. The sections were counterstained by hematoxylin for 30 seconds, air dried and mounted in DPX. Positive and negative controls were run with each batch of immunostaining.

Measurement of Ki-67 immunostaining

A minimum of 500 plasma cells were counted within a positive area. The nuclei showing dark staining (Ki-67 positive) were counted and percentage of the total plasma cell population expressing Ki-67 antigen was calculated as Ki-67 index.

Measurement of CD34 immunostaining

MVD was assessed independently by two observers. The mean of the two counts was taken as the final count. The sections were initially scanned at 100X and three areas containing the highest number of microvessels were identified. Individual microvessels were then counted at 400X and result was expressed as mean of the three values as MVD/ high power field (MVD/hpf). Any red staining cells seen singly or in nests and clearly separated from one another including any cluster of endothelial cells with or without a lumen were considered as microvessels and counted. Using an ocular micrometer, the field diameter of 40X objective lens of the microscope (Nikon eclipse 80i) was determined to be 0.55 mm. Dividing the diameter by half, to give a radius of 0.275mm and using the formula πr^2 , the field area of each high power field was determined to be 0.237mm² and expressed as MVD/mm² of tissue.

Statistical analysis

Data analysis was done using SPSS software (version 16.0) and interpretation of result was done by applying chi square test and Pearson correlation coefficient test. Level of significance and confidence were 5% and 95% respectively.

Results

Patients characteristics

Median age of myeloma patients was 55 years (range 35 - 70 years) and 56 years (range 49 - 69 years) for controls. Eighteen of 30 (60%) myeloma patients were

Table 1. Range and Mean ± SD of Ki-67 and MVD in Patients and Controls

Parameters	Patients (n=30)		Control	Dyvalue	
	Range	Mean±SD	Range	Mean±SD	P value
Ki-67 (%)	35-80	60.1±12.4	8-25	18.1±6.9 %.	< 0.0001
MVD/mm2	62-237	178.0±72.3	5.2-50	18.3±13.4	< 0.0001
MVD/hpf	14.7-79.9	42.2±17.1	1.2-6	3.3±1.9	< 0.0001

MVD= Microvessel density

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males and 12 (40%) were females while 5 (50%) were males and 5 (50%) were females in control group. According to Durie Salmon staging criteria, 13 patients were of stage I, 5 of stage II and 12 of stage III. The most frequent clinical manifestations in myeloma patients were weakness and fatigue (73.3%). Other complaints included backache, fever, bone pain and pathological fractures. Radiological examination revealed skeletal lesions in 23(77%) patients, which consisted of lytic lesions in 19 (82.6%), and extensive destruction of skeleton with pathological fractures in 4 (17.4%).

Hematological parameters

Twenty-nine (96.6%) myeloma patients had anemia which was normocytic and normochromic in majority (80%) of cases. Peripheral blood smear revealed rouleaux formation in 24 (80%) patients (Figure 1A). A monoclonal band was detected in 26 (86%) patients (serum electrophoresis agarose gel, pH 8.6). Bone marrow aspirate was hypercellular in most (n = 26,86%) myeloma patients. Mature and immature forms of plasma cells in the bone marrow aspirate ranged from 13-86 % (Figure 1B). Bone Marrow biopsy was hypercellular and showed different patterns of infiltration of plasma cells. Interstitial pattern was seen in 10 (33.3%), nodular in 10 (33.3%) and 10 (33.3%) patients had diffuse pattern (Figure 1C-D).

Biochemical parameters

Hypercalcemia and elevated serum creatinine were present in 33.3% and 40% myeloma patients, respectively. Hypoalbuminemia was observed in 53.3%, while serum lactate dehydrogenase and blood urea was elevated in 13.3% and 46.6% patients, respectively. β 2-microglobulin was elevated in all MM patients.

Ki-67 index and MVD

Ki-67 index was significantly (p<0.0001) higher in myeloma patients as compared to controls (Figure

 Table 2. Ki-67 and MVD Positivity in Different Stages

 of Myeloma

Stage	N (%)	MVD/mm ² (Mean ±SD)	Ki-67 (Mean ±SD)
Ι	13 (43)	118.6±28.9	49.8±9.8
II	05 (17)	148.0±21.7	60.0±5.2
III	12 (40)	255.0±41.0	71.2±5.5

MVD= Microvessel Density; Ki-67-I vs. II, p=0.046, I vs. III, p<0.001, II vs. III, p=0.028; MVD-I vs. II, p=0.237, I vs. III, p<0.001, II vs. III, p<0.001

2A-2B) (Table 1). MVD/mm² in myeloma patients was significantly (p<0.0001) higher than controls. MVD/hpf followed a similar trend (Figure 2C-2D) (Table 1).

Ki-67 showed a significant positive correlation with MVD/hpf and MVD /mm²(r=0.729, p<0.001) in myeloma patients (Figure 3)



Figure 1. (A) Peripheral Blood Film Showing Rouleaux Formation (Wright stain) (100X) (B) Bone Marrow Aspirate Showing an Increase in Numbers of Plasma Cells (Wright stain 400X) (C-D) Hypercellular Bone Marrow Biopsy from a Patient with Myeloma (H & E 100X & 400X)



Figure 2. (A) Nuclear Positivity of Ki-67 in Multiple Myeloma (400X) (B) Occasional Cells Showing Nuclear Positivity (↑) of Ki-67 in Controls (400X) (C) CD34 Immunostaining in Multiple Myeloma (100X & 400X) (D) Occasional Vessels Seen on CD34 Immunostaining in Controls (↑) (400X)

Table 3. Correlation of Ki-67 & MVD with Biochemical Parameter
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Ki-67%	Serum	Total Protein	Serum Albumin	Serum Creatinine	Blood Urea	Serum LDH	β2 microglobulin
Calcium							
r value	0.352	-0.145	-0.476**	0.286	0.525**	0.396*	0.364
P value	0.057	0.443	0.008	0.126	0.003	0.03	0.254
MVD							
r value	0.339	-0.358	-0.341	0.395*	0.562**	0.522**	0.653*
P value	0.067	0.052	0.065	0.031	0.001	0.003	0.021

MVD= Microvessel Density;* correlation is significant at the level 0.05, ** correlation is significant at the level 0.0



Figure 3. Correlation of Ki-67 and MVD

Correlation of Ki-67 and MVD with other parameters

Significant positive correlation was observed between Ki-67 proliferation index and percentage of bone marrow plasma cells (r=0.383, p<0.005). Ki-67 positivity was significantly higher in patients with diffuse infiltration of plasma cells as compared to those with nodular (p=0.11) and interstitial (p=0.005) patterns of infiltration. Ki-67 positivity increased progressively with increasing stage of myeloma (Table 2).

In myeloma patients Ki-67 showed a statistically significant negative correlation with hemoglobin (Hb) (p=0.004), RBC count (p=0.019), MCV (p=0.746), platelet count (p=0.032) and also serum albumin (p=0.008). A statistically significant (p=0.003) positive correlation was observed between Ki-67 and blood urea and LDH (p=0.030). Ki-67 did not show any significant correlation with serum calcium, serum creatinine and β_2 microglobulin (Table 3). An increasing trend was observed in mean Ki-67 values with increasing severity of skeletal lesions.

MVD showed a statistically significant negative correlation with Hb (p<0.001) and RBC count (p=0.005) and statistically significant (p=0.001) positive correlation with blood urea (p<0.001), serum LDH (p=0.003), creatinine (p=0.031), and β 2 microglobulin (p=0.021). Though there was a negative correlation with total proteins and serum albumin this was not statistically significant (Table 3).

A significant positive correlation was observed between MVD and plasma cell number (r= 0.464, p= 0.010). A statistically significant higher MVD was observed in myeloma patients with diffuse infiltration of plasma cells as compared to those with nodular (p=0.003) and interstitial (p<0.001) pattern. MVD increased significantly (p<0.001) with increasing stage of myeloma (Table 2). A significantly (p=0.022) higher MVD was seen in patients with extensive skeletal destruction as compared to those with lytic lesions only.

Discussion

We have evaluated proliferative activity (Ki-67 index) and angiogenesis (MVD) in 30 newly diagnosed patients of multiple myeloma and compared with 10 non-myeloma patients. Prognostic factors are required to stratify patients into low risk and aggressive disease. Previous studies in literature have described various prognostic markers in MM, important one includes proliferation and angiogenesis (Rajkumar et al., 2000; Alexandrakis et al., 2004). In our study we found statistically significant correlation between Ki-67 and MVD (r=0.729, p<0.001). Similar correlation was also found by other authors (Xu et al., 2002; Tichy et al., 2010). However, in some of the previous studies a different methodology has been used for assessment of these parameters (Vacca et al., 1994; Rajkumar et al., 2000).

We have used Ki-67 as proliferation marker. It recognizes nuclear antigen present during G1, S, G2 and M phases of cell cycle but not in G0 phase. Plasma cell labelling index (PCLI) is another method used by other authors to determine proliferation. Increased PCLI predicts short remission and survival but it does not predict response to therapy (Dispenzieri et al., 2009). Few authors studied Ki-67 expression in myeloma and concluded that Ki-67 labeling was simple and rapid and can replace PCLI and flow cytometry for detection of proliferating cells in hematological malignancies (Mark et al., 2014). Therefore, we assessed role and feasibility of Ki-67 to determine proliferative activity in myeloma. As proliferating cells are more sensitive to chemotherapeutic agents therefore the study of proliferating fractions in myeloma may give valuable information about response to therapy and prognosis.

Similarly, angiogenesis was assessed by using immunohistochemical stain for von Willebrand factor (vWF), CD31and Factor VIII by many authors (Vacca et al., 1994; Rajkumar et al., 2000). These stains are nonspecific as they can be expressed in various bone marrow cells including megakaryocytes and myeloid cells thus making an accurate enumeration of MVD less practical. Immunohistochemical staining for CD34 gives the best results and is now used to assess angiogenesis widely (Pruneri et al.,1999; Alexandrakis et al., 2004). We also used CD34 as marker for MVD.

Ki-67 and MVD showed positive correlation with each other in previous studies (Xu et al., 2002; Alexandrakis MG et al., 2004; Tichy M et al., 2010). Alexandris MG et al in their study had correlated both these markers with C-Reactive Protein and bone marrow infiltration only. Similarly, Xu JL et al had correlated Ki-67 and MVD with clinical stage and cytological grade. In our study we correlated Ki-67 and MVD with various known prognostic factors like clinical stage, bone marrow infiltration, serum calcium, serum creatinine, serum LDH, total proteins, blood urea and β 2 microglobulin. We have also found that patients having high Ki67 index also possess higher degree of angiogenesis.

Furthermore, ours is the first study to compare Ki-67 and MVD in myeloma and non-myeloma patients and further assessing its correlation with other known prognostic markers that may help in classifying patients for various treatment options (intensive chemotherapy and autologous or allogenic bone marrow transplantation versus standard chemotherapy). Till date most studies have observed correlation of various prognostic factors with either Ki-67 or CD34. Studies which combine Ki-67 and MVD and evaluate their correlation with all known prognostic factors are few, especially in Indian patients. Moreover, in our study blood urea emerged as an important prognostic marker which have shown positive correlation with both Ki-67 and CD34.

Regarding role of Ki-67 in multiple myeloma, a study done by Drach et al on 42 patients of myeloma, Ki-67 was observed to be significantly higher in patients who relapsed than in those at diagnosis or plateau phase. Also it correlated with clinical stage and β 2 microglobulin as is observed in our study. The authors concluded, Ki-67 a useful marker in evaluation of risk profile of MM patients and recommend its use routinely in these patients (Drach et al.,1992).

Other authors have reported similar results (Lokhorst H et al., 1988; Lai R et al., 1998;). However, the percentage positivity of Ki-67 seen in our study is higher (range, 35-80%) than that reported by other authors (Alexandris MG et al., 2004; Markovic O et al., 2005).

In another study by Kyle R et al on 1027 patients' anemia, thrombocytopenia, hypoalbuminemia, PCLI and raised serum creatinine were reported to be adverse prognostic factors. In our study Ki-67 correlated with all these factors except serum creatinine though a positive correlation was observed with blood urea (Kyle et al., 2003).

The findings observed in our study suggests that proliferation rate of plasma cells assessed by Ki-67 indicates more aggressive disease as suggested by its correlation with increased percentage of plasma cells, diffuse pattern of infiltration in bone marrow biopsy and stage of disease. Measurement of proliferation rate is essential for opting treatment strategies. As patients who relapse after multiple therapies have weaker immune system and use of anti-proliferative agents with high potency can become a good therapeutic option (Richardson PG et al., 2013). Newer immunomodulatory drugs with potent anti-proliferative mechanism like Pomalidomide are on clinical trials to benefit patients and improving overall survival.

Role of CD34 in assessing angiogenesis has been described in previous studies (Rajkumar SV et al., 2000; Ahn MJ et al., 2001). It is reported to be an adverse prognostic factor in several solid tumors and has been recognized as an important prognostic marker in hematological malignancies including myeloma (Medinger M and Passweg J, 2014). Increased angiogenesis with advancing stage has been reported in myeloma (Vacca A et al., 1994; Lee N et al., 2015). The results of our study are in concordance with previous studies as we observed increased angiogenesis with advancing stage of myeloma.

In one study increased survival was observed in patients with low and intermediate grade of angiogenesis as compared to high grade angiogenesis (Rajkumar SV et al., 2000). In our study grading of angiogenesis is not done. However, significantly (p<0.0001) higher MVD is observed in patients as compared to controls. Moreover, a statistically significant correlation is observed with plasma cell number and MVD in our study which was

not observed in the above said study.

Previous studies in literature have shown correlation of MVD with other prognostic factors including $\beta 2$ microglobulin, LDH, serum creatinine, diffuse infiltration in bone marrow biopsy (Sezer O et al., 2000; Ahn MJ et al., 2001; Rajkumar SV et al., 2002), however in one study no correlation was found between MVD grade and $\beta 2$ microglobulin (Rajkumar SV et al., 2000). In our study a positive correlation is observed with the above said prognostic factors and also blood urea. Demonstration of increased angiogenesis in patients of myeloma has led authors to explore its use in therapeutic application of anti-angiogenic agents. Ongoing clinical trials are also evaluating anti-angiogenic agents like antibodies to VEGF.

Alexandris MG et al found that after treatment in myeloma patients, proliferative activity was normalized, however MVD decreased but didn't normalized to base level, providing evidence of persistent disease and can lead to relapse, thus realizing the rationale of use of antiangiogenic and antineoplastic drugs in maintenance therapy in myeloma (Alexandris MG et al.,2004).

Multiple myeloma is a heterogeneous disease with variable disease course and outcome. Variables such as clinical and laboratory features, β 2- microglobulin and LDH are conventionally used as prognostic markers (Dispenzieri A et al., 2009). With the availability of newer drugs for treatment of these patients, there is a need for tumor biology related factors such as angiogenesis and proliferation markers for risk stratification of the patients. Antiangiogenic and antiproliferative immunomodulatory drugs may be able to overcome some of the poor prognostic factors and achieve clinical benefit in relapsed patients also.

In conclusion, our study thus concludes that Ki-67 and MVD are indicators of aggressiveness in multiple myeloma. They have significant correlation with each other and other prognostic markers and thus can be used for risk stratification of patients. The routine determination of these parameters in multiple myeloma may help to identify patients with aggressive disease and poor prognosis, who may benefit from more intense therapy and helps in achieving optimal clinical goals.

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