www.jmscr.igmpublication.org
Impact Factor (SJIF): 6.379
Index Copernicus Value: 79.54
ISSN (e)-2347-176x ISSN (p) 2455-0450
crossrefDOI: https://dx.doi.org/10.18535/jmscr/v7i1.62

IGM Publication
Journal Of Medical Science And Clinical Research
An Official Publication Of IGM Publication

Microvessel Density, Ki 67 Index and Haematological Profile in Multiple Myeloma

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Abstract
Background: *Multiple myeloma is a haematological malignancy with varying clinical presentations. Microvessel density (MVD) and proliferation markers are used in predicting prognosis of multiple myeloma patients. Microvessel density was assessed using CD 34 immunostaining and proliferation marker by Ki 67 index. These parameters are correlated with various prognostic markers in multiple myeloma like stage of the disease.*
Material & Method: *A cross sectional study was done in the Department of Pathology, in a Tertiary care centre in central part of Kerala during a period of 18 months (1/ 2/2014 - 30/7/2015). 30 newly diagnosed cases of multiple myeloma were included in the study. Clinical, haematological, radiological and biochemical investigations collected. Immunohistochemisrty for microvessel density and Ki 67 index were done on bone marrow trephine biopsy. Computerized image analysis was done for MVD assessment. Study was approved by institutional ethical committee.*
Results: *MVD was higher in patients with higher stage disease. MVD and Ki 67 index showed positive correlation which was statistically significant.*
Conclusion: *MVD increases with stage of the disease. Patients with high MVD have high proliferation index like Ki 67.*
Keywords: *Multiple myeloma, MVD, Ki 67 index.*

Introduction

Multiple myeloma is a plasma cell neoplasm which comprises about 1% of malignant tumours & 10 – 15% of haematopoietic neoplasms. 20% deaths from haematological malignancies are due to multiple myeloma. Plasma cell myeloma results from expansion of a clone of immunoglobulin secreting terminally differentiated B cells. These cells will secrete a single homologous or monoclonal immunoglobulin. Plasma cell neoplasms include a spectrum of disorders ranging from Monoclonal gammopathy of undetermined significance (MGUS) to plasma cell leukemia[(1)].
Direct & indirect interactions between myeloma cells, nonmyeloma cells & extracellular matrix in the bone marrow are essential for growth, survival

& drug resistance in myeloma. Myeloma causes clinical symptoms by way of tumour mass effects, cytokine production (e.g., anaemia), bone destruction (e.g., pain), protein deposition in visceral organs (e.g., kidney and heart), and immunosuppression (e.g., infection)[(2)].

Most malignant neoplasms depend on angiogenesis for tumour progression. Recently, in multiple myeloma, bone marrow angiogenesis is correlated with plasma cell labelling index & disease activity[(3)]. Microvessel density (MVD) is a measure of tumour angiogenesis. Ki 67 is a proliferation marker. Its expression is correlated with survival rate of myeloma patients[(4)]. Microvessel density & Ki 67 index can be used as prognostic markers in newly diagnosed cases of multiple myeloma.

Aims of the Study

1. To assess microvessel density in bone marrow trephine biopsy in multiple myeloma using CD34 immunohistochemistry.
2. To assess proliferative activity in bone marrow trephine biopsy in multiple myeloma, using Ki 67 immunohistochemistry.
3. To correlate microvessel density & Ki 67 index along with haematological and biochemical parameters.

Materials & Method

It was a cross sectional study. 30 newly diagnosed cases of multiple myeloma in the department of pathology, in a tertiary care centre, in central part of Kerala during the period 1/2/2014 to 31/7/2015 were studied. Newly diagnosed & not treated cases of Multiple myeloma, during the study period were included in this study. Treated cases, relapse or recurrence of Multiple myeloma were excluded.

Patient data collected include name, age, sex, clinical diagnosis, haemoglobin, ESR, Peripheral smear, Bone marrow aspirate & trephine biopsy, biochemical investigations & radiological investigations. Biochemical investigations included renal function test, serum Ca^{2+} level, serum total protein, albumin, serum protein electrophoresis, urine Bence Jones protein (BJP) & immunoglobulin assay in few patients.

Radiological investigation included plain X ray, CT scan & MRI. Lytic lesions in radiological investigations were recorded

Bone marrow aspiration & trephine biopsy were done under strict aseptic precautions. Bone marrow aspirate & imprint smears were fixed in methanol. Later staining done with Romanowsky stain. Both Leishman & Wright's staining were done.

Bone marrow trephine biopsy specimens were fixed in bouins fixative & decalcified in 5% nitric acid. Biopsy was processed & sections were taken from paraffin embedded tissues. 5 mm sections were cut with microtome. The sections were stained with hematoxylin & eosin. Stained sections were studied under light microscopy. In the trephine biopsy, distribution of plasma cells were noted as diffuse, interstitial, nodular/ focal, paratrabecular & combined.

Diagnosis of multiple myeloma was done based on International Myeloma Foundation Criteria. Staging of the disease was done based upon Durie & Salmon criteria.(Table 1)

Table 1

Stage 1	Stage 2	Stage 3
Low M protein level (Ig G < 50g/l, Ig A < 30g/l, Urine BJP < 4g/24hr) Absent/solitary **bone lesion** **Hb, S. Ca** & non M protein Ig - Normal	Values between stage 1 & 3	Any one /more required **High M protein** (IgG >70g/l , Ig A >50g/l, urine light chain > 12g/24hr) **Advanced** multiple lytic lesion Hb < 8.5g/dl , S.Ca > 12 mg/dl

Assessment of Microvessel density and Ki 67 index

Immunohistochemisrty staining was performed on trephine biopsy. CD34 antibody was used for staining endothelial cells. Ki 67 staining done for assessing proliferative activity of neoplastic cells. Stained slides were studied under light microscope.

CD34 immunostained slides were examined under scanner, x100 & x400 magnifications.. Microvesseldensity was assessed using image analysis software VMS 3.6. Software was calibrated using Neubauer chamber. Microvessels within an area of 0.04 mm^2(corresponding to Neubauer chamber RBC square) was counted. 3 hot spots were selected where microvessel density was maximum. Using eye piece camera, photomicrographs were taken at x100 magnification. Number of blood vessels were counted in each 0.04mm^2 area. Blood vessels near the trabeculae were not counted. Average value of 3 hotspots were taken as microvessel density & expressed as number of blood vessels per 0.04mm^2 area.

Microvessel density of normal controls were also calculated.

H & E stained slide of each case was examined under x100 & x400 magnification. Areas with increased plasma cells were noted. Corresponding areas were examined in Ki 67 immunostained slides. 1000 plasma cells were counted & out of 1000 cells, cells with nuclear positivity for Ki 67 were counted & expressed in percentage. 3 such areas were counted & average value of 3 hotspots were taken as Ki 67 index.

Data Analysis

Data collected and entered in Microsoft office excel 2007 sheet. This was then analyzed using software SPSS version 16.Correlation of MVD and Ki 67 index with other parameters were calculated using Pearson correlation, ANOVA, t test and Kendall's tau_b correlation

Observations & Results

The age of the patients range from 42 – 89 years (mean age – 59.1 yrs). Most frequent age group in this study was 41 – 60 yrs (73.3%). In the 30 cases studied, 18 cases (60%) were females & 12 cases (40%) were males. Out of 30 cases, 10 cases (33.3%) had haemoglobin > 10 gm/dl, 10 case (33.3%) had haemoglobin between 9.9 & 8.5 gm/dl & 10 cases (33.3%) had haemoglobin < 8.5 gm/dl. Out of 30 cases, 6 cases (20%) had hypercalcemia. 15 cases (50%) had normocytic normochromic anaemia with increased rouleaux formation in peripheral smear examination.

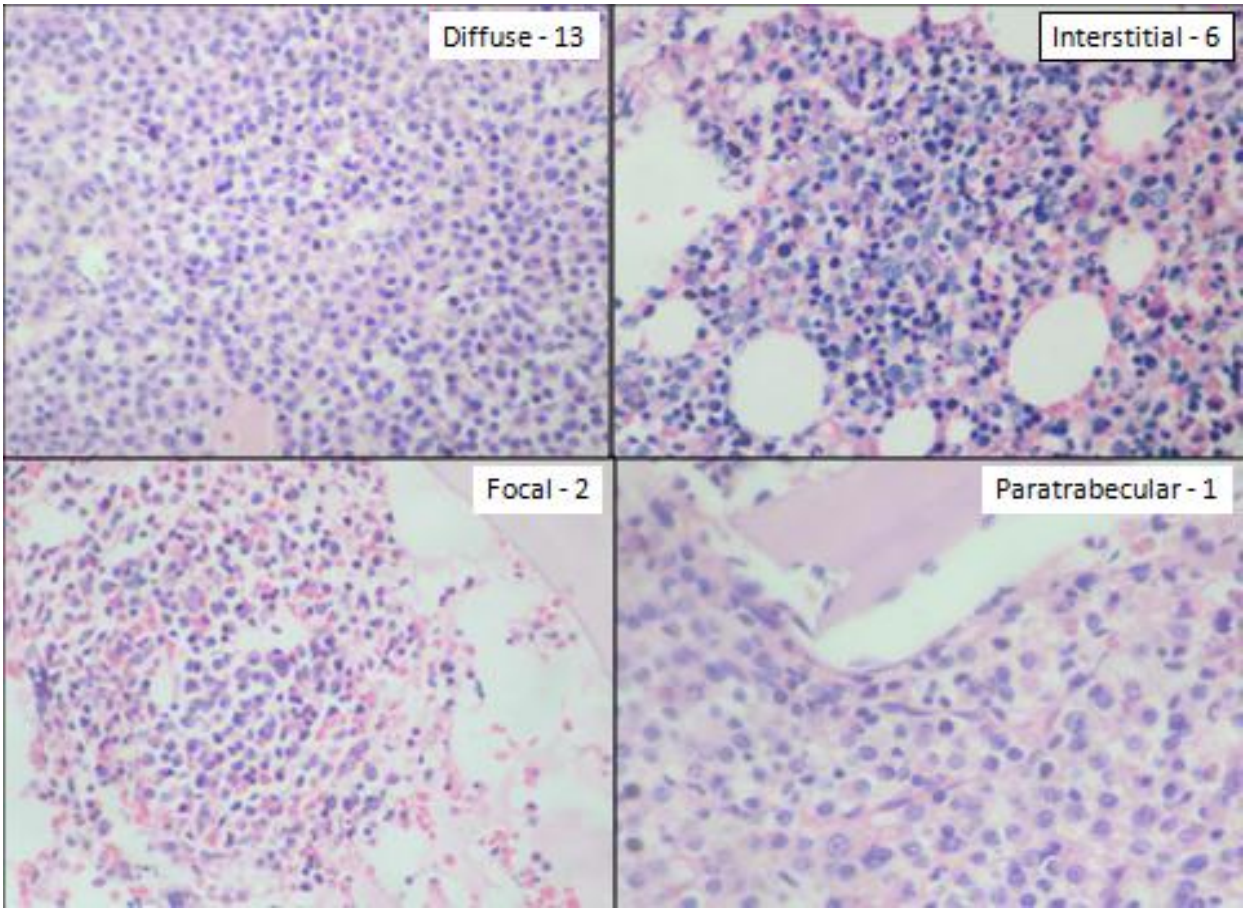
Table 2 : Peripheral smear report

Peripheral Smear Report	Frequency	Percentage
NCNCA + RF	15	50%
NCNCA	4	13.3%
LEBP	3	10%
NCNCA+RF+Thrombocytopenia	3	10%
NCNCA+RF+Lymphocytosis	2	6.7%
NCNCA+RF+Eosinophilia	1	3.3%
NCNC blood picture	1	3.3%
Pancytopenia	1	3.3%
Total	30	100%

Note: NCNCA - Normocytic normochromic anaemia, RF - Rouleaux formation, LEBP- Leukoerythroblastic blood picture.

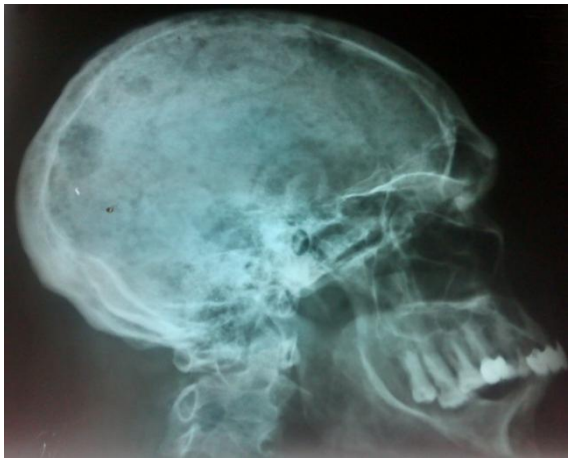
The pattern of distribution of plasma cells in bone marrow trephine biopsy was diffuse in 13 cases(43.3%). 6 cases(20%) had interstitial pattern of involvement. 8 cases (26.7%) had combined diffuse & interstitial involvement. Focal or nodular plasma cell distribution noted in 2 cases (6.7%).One case (3.3%) had paratrabecular involvement.

Picture No.1: Pattern of Bone marrow involvement



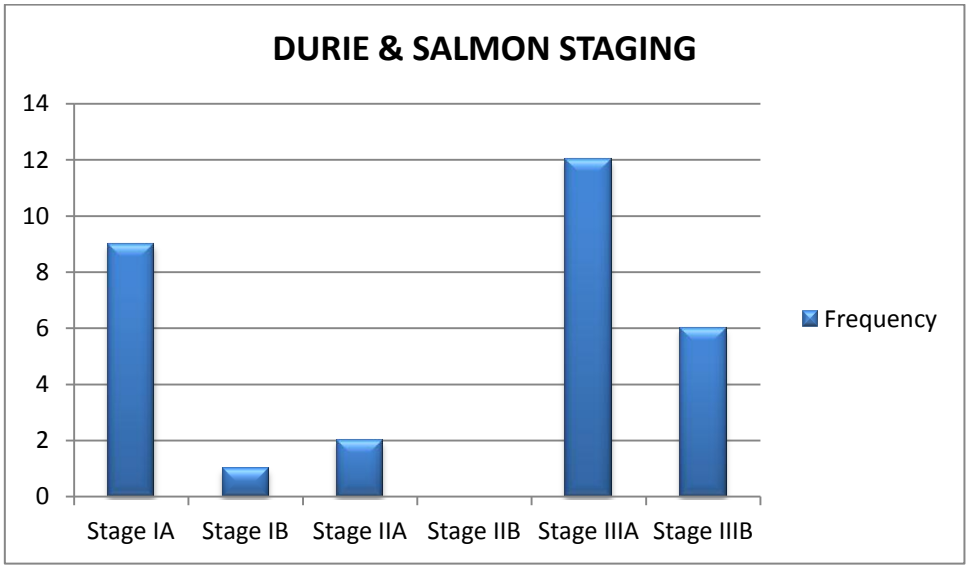
Of the 30 cases, 7 cases (23.3%) had renal impairment with S.creatinine >2mg/dl. 23 cases (76.7%) had normal S.creatinine level. Out of 30 cases, 29 cases (96.7%) had monoclonal protein (M band) on serum protein electrophoresis. Bone lytic lesions were present in 22 cases (73.3%). 8 cases (26.7%) had no bone lytic lesions.

Picture No. 2: Bone lytic lesion – Skull xray



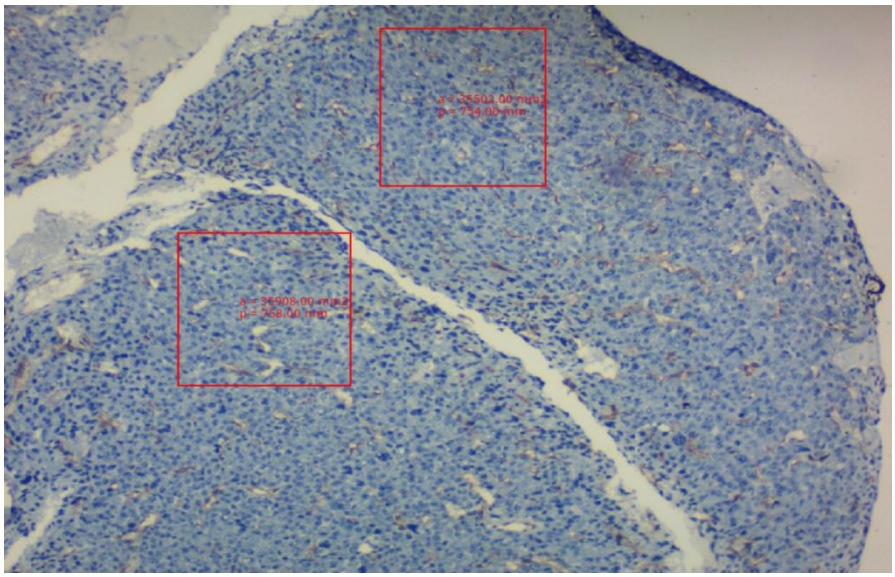
Out of the 30 cases studied, 10 cases (33.3%) were in stage I. 2 cases(6.6%) were in stage II. 18 cases (60%) were in stage III. Each stage was again divided into A & B depending upon the level of S.Creatinine.

Picture No.3: Staging of Multiple myeloma

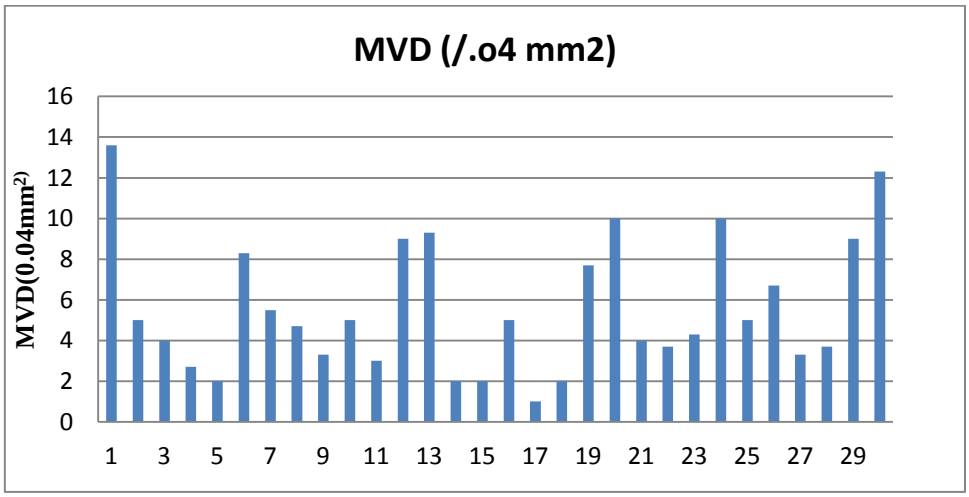


Microvessel density assessed using CD 34 staining of endothelial cells. Microvessel density range from 1 - 13.6 / o.o4mm^2 area (Mean - 5.57/0.04mm^2 & standard deviation – 3.281)

Picture No.4: Microvessel density



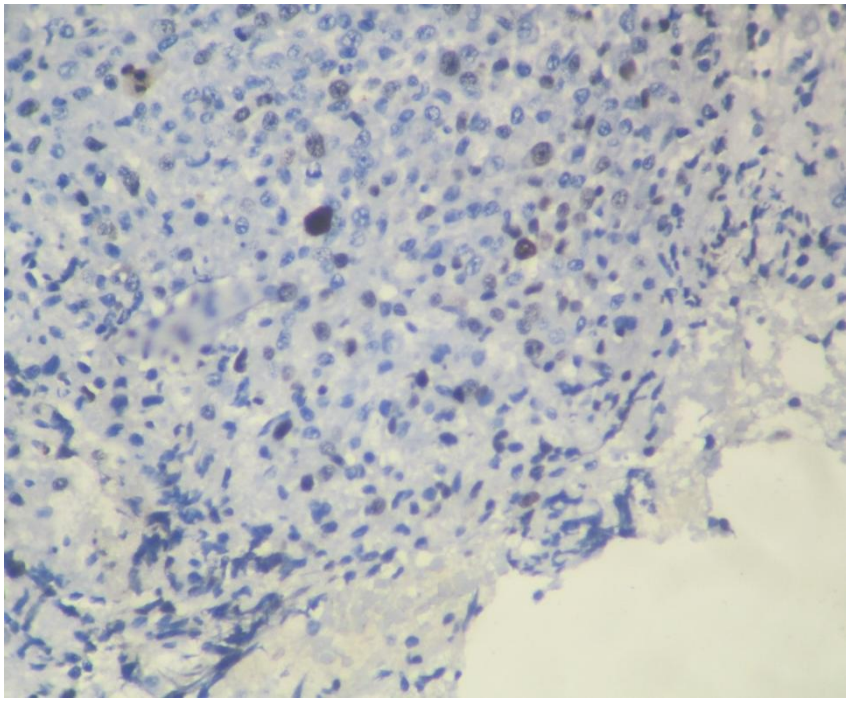
Picture No.5: Microvessel density



Ki 67 INDEX

Ki 67 positivity ranges from 0 – 4.8 % with mean being 0.457 (Standard deviation – 0.878)

Picture No.6: Ki 67 Index



Picture No.7: Ki 67 Index

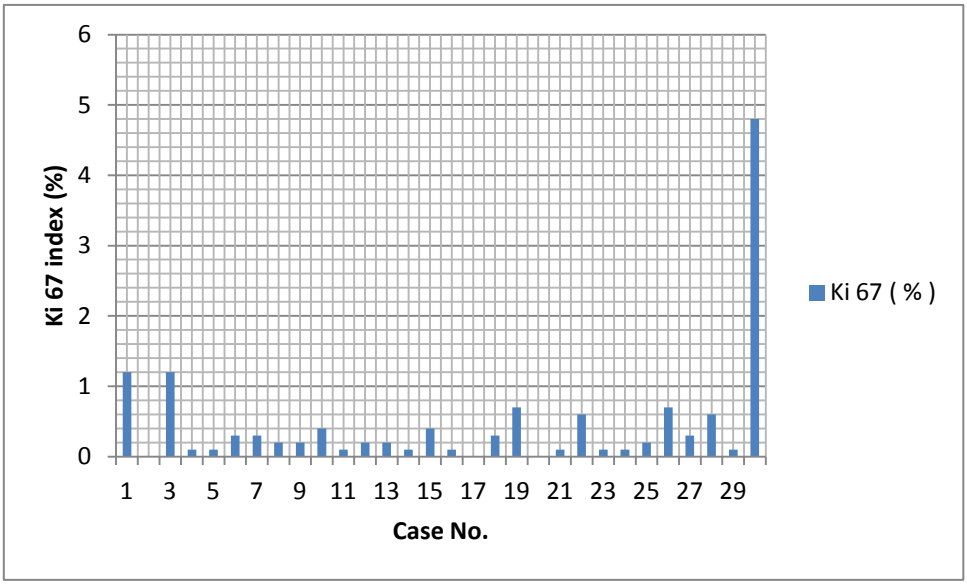


Table No.3 : MVD & Ki 67 Index

Case No.	Microvessel density (/0.04mm^2)	Ki 67 index (%)
1	13.4	1.2
2	5	0
3	4	1.2
4	2.7	0.1
5	2	0.1
6	8.3	0.3
7	5.5	0.3
8	4.7	0.2
9	3.3	0.2
10	5	0.4

11	3	0.1
12	9	0.2
13	9.3	0.2
14	2	0.1
15	2	0.4
16	5	0.1
17	1	0
18	2	0.3
19	7.7	0.7
20	10	0
21	4	0.1
22	3.7	0.6
23	4.3	0.1
24	10	0.1
25	5	0.2
26	6.7	0.7
27	3.3	0.3
28	3.7	0.6
29	9	0.1
30	12.3	4.8

Discussion

In this study age of the patients range from 42 – 89 yrs with female predominance which was comparable with a study by Stifer et al. Their study also showed age ranging from 42 - 90 yrs[(5)]. Clinical presentation included anaemia, pancytopenia, bone lesions and chronic kidney disease. In a study by Seema Singhal et al most of the patients presented with anaemia, bone lesion, chronic kidney disease & infection[(6)].

Another feature of multiple myeloma is hypercalcemia which was present in only 20% cases in this study similar to a study by Kyle RA et al where only 25% cases had this finding[(7)]. Anaemia was present in all 30 cases. Normocytic normochromic anaemia with rouleaux formation was present in 50% cases. Kyle RA et al also found that normocytic normochromic anaemia with rouleaux formation was the common blood picture in multiple myeloma accounting for 75% of cases[(7)].

Pattern of plasma cell distribution in bone marrow biopsy is categorized into diffuse, interstitial, focal/nodular, paratrabecular & combined diffuse interstitial pattern. In the present study 43.3% cases had diffuse pattern of distribution of plasma cells. In 20% interstitial, 26.7% both interstitial & diffuse pattern, 6.7% nodular & 3.3% paratrabecular distribution were seen. According to a study by Yang L et al the plasma cell distribution patterns were categorized into interstitial, microaggregates, diffuse & large nodules. 52% had interstitial pattern. 23% had microaggregates, 17% large nodule & diffuse pattern was seen only in 8% cases[(8)].

In the present study, staging was done based on Durie & Salmon criteria for staging.10 patients (33.3%) were in stage I, 2 patients (6.7%) in stage II & 18 (60%) were in stage III. According to study done by Conté L G et al, 11% were in stage I, 12% in stage II & 73% in stage III[(9)]. In a study by Myung – Ju Ahn et al, 86.6% cases were in stage III. Stage I & II had 6.7% cases in each group[(10)].

Angiogenesis plays important role in proliferation and survival of neoplastic cells in any neoplasm. Angiogenesis is increased in many haematological malignancies like leukemia and multiple myeloma [(11,12,13)]. Angiogenesis is assessed by microvessel density of the neoplasm. Increased MVD in multiple myeloma is associated with reduced progression free survival. It is also significantly associated with other prognostic markers in multiple myeloma like stage[(14)].

Various markers are available to demonstrate microvessel density. Few such markers are VEGF, Factor VIII and CD 34. All these markers will stain the endothelial cells of these newly formed

vessels[14,15]. In our study we used CD 34 immunohistochemical marker to demonstrate endothelial cells.

Microvessel density in our study ranges from 1 - 13.6 / o.o4mm^2 area (Mean - 5.57/0.04mm^2 & standard deviation – 3.281). Microvessel density was calculated using computerized image analysis. Babarović E et al used computerized image analysis to find out microvessel density[16]. There was a significant positive correlation with stage of the disease & microvesssel density (P value < 0.05). Microvessel density was more in higher stage diseases. Alexandrakis MG et al studied about microvessel density in multiple myeloma & found that MVD was higher in stage III cases (P value <0.05) when compared with stage I cases[17]. On comparing with normal bone marrow microvessel density (2.4/0.04mm^2), in this study higher stage myeloma cases had more microvessel density. According to Alexandrakis MG et al, there was significant difference between untreated myeloma patients & controls (P value < 0.0001)[17].

In the present study correlation between Haemoglobin level & microvessel density was assessed using Pearson correlation. There was significant correlation between haemoglonbin level & microvessel density (Pearson coefficient:-0.433, P value 0.017).

Microvessel density had no correlation with peripheral smear report, serum calcium level, bone marrow involvement pattern & bone lytic lesion in this study (Assessed by t test). According to Alexandrakis MG et al study, there was significant correlation (P value <0.05) between plasma cell infiltration & microvessel density[17]. Study by Ahn MJ showed that there was only a weak correlation with plasma cell infiltration in bone marrow. There was no significant correlation with stage of disease & microvessel density[18].

In the present study, Ki 67 positivity range from 0 - 4.8% (mean:0.457%,SD:0.8776). Ki67 had no significant correlation with haemoglobin level, serum calcium level, peripheral smear report, bone marrow involvement pattern, Durie & Salmon staging & bone lytic lesion. In the study by Alexandrakis MG et al, Ki 67 positivity was significantly high (P value < 0.01) in stage III myeloma cases when compared to stage I & II. In their study, there was a significant correlation between Ki67 index, plasma cell infiltration in bone marrow & microvessel density (P value < 0.05) (17). In this study correlation between MVD & Ki 67 index was studied using Pearson correlation. Positive significant correlation was found between these two parameters (Pearson coefficient: 0.447, P value: 0.013).

Conclusion

MVD and Ki 67 index at the time of diagnosis can be considered as prognostic markers. MVD increases with stage of the disease. As MVD and Ki 67 index has positive correlation, it indicates that cases with high MVD have high proliferation index.

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