Original Research Article

Immunohistochemical Study of Tumor Associated-Macrophages and Microvessel Density in Multiple Myeloma

Abstract

Background: Multiple myeloma (MM) is described as an increase in malignant plasma cells in the bone marrow (BM), which is linked with end-organ damage including kidney failure, lytic bone lesion, anemia, and immunosuppression. This study evaluated the degree of tumor-associated macrophages infiltration and its diagnostic value in newly identified multiple myeloma cases. **Methods:** This retrospective study included 100 patients who had multiple myeloma. Patients were classified as follow: Patients with low Tumor associated macrophages, patients with high tumor associated macrophages, cases with low grade micro vessel density and patients with high grade micro vessel density. All cases underwent to detailed history taking, Clinical assessment, bone scan and laboratory tests including Immunohistochemistry of BM biopsy samples to confirm diagnosis of abnormal plasma cells (CD138 and monoclonal light chain). **Results:** There was a strong positive correlation between CD163 and CD68. There was a strong positive correlation between MVD and CD68. There was a strong positive correlation between MVD and CD163. There was decreased overall survival with increased CD 68 TAMs content with median OS (50 months) in low CD 68 TAMs vs. (16.5 months) in high CD 68 TAMs. There was decreased overall survival with increased CD163 TAMs content with median OS (49.3 months) in low CD 163 TAMs vs. (25.6 months) in high CD 163 TAMs. There was decreased overall survival with increased MVD with median OS (54.6 months) in low MVD vs. (25.6 months) in high MVD. **Conclusions:** Important role of TAMs expression and MVD in the prognosis of the multiple myeloma, as the elevated TAMs expression and high grade MVD associated with poor prognosis.

Keywords: Immunohistochemical, Tumor Associated-Macrophages, Microvessel Density, Multiple Myeloma

Introduction:

Multiple myeloma (MM) is described as an increase in malignant plasma cells in the bone marrow (BM), which is related to end-organ damage including renal failure, lytic bone lesion, anemia, and immunosuppression [(1)].

MM patients are concerned about relapse due to drug resistance as one of their top treatment concerns. The communication between MM cells and the stromal cells and extracellular matrix proteins of the BM microenvironment leads in their production, survival, angiogenesis, and resistance to therapy [(2)].

Tumor-associated macrophages (TAMs) are M2-type macrophages with low interleukin (IL)-2 and high IL-10 production; they are one of the most important components of cancer-related inflammation in tumor tissues. In contrast to macrophages with an M1-like phenotype, TAMs play a crucial role in angiogenesis and tissue remodeling, although their tumoricidal activity is restricted [(2, 3)].

Micro vascular density (MVD) is an indication of angiogenesis, and it is higher in patients with effective MM than in those with MGUS and smoldering MM [(4)].

Examination of tumor-associated macrophages using anti-CD163 and anti-CD68 monoclonal antibodies, as well as factor VIII-labeled evaluation of MVD [(5)].

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Some studies proved that increased TAM and MVD is associated with poor prognosis [6]. This study evaluated tumor-associated macrophages infiltration degree and its prognostic value in newly diagnosed MM cases, and establish the relationship between tumors related macrophages and microvascular density.

Methods:

This retrospective study included 100 consecutive cases with multiple myeloma in the Research Building, Mayo Clinic, Scottsdale, Arizona, USA and Clinical Pathology Department in Tanta University hospital. After informed written consent from patients or their guardians.

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How did you get informed :[4h]Comment consent in a retrospective study?

Exclusion criteria were patients with any other malignancies except Multiple Myeloma

Cases underwent detailed history (age, sex and symptoms of bone pain as back pain, weakness, numbness, symptoms of anemia as fatigue, dizziness and headache and symptoms of hypercalcemia as confusion, somnolence, bone pain, constipation, nausea, and thirst). **Clinical examination:** Pallor, purpuric eruptions, pathological fracture, inability to move, recurrent infection and renal impairment. **Bone scan**: To determine the status of the bone lesions. **Laboratory Investigations**: 1-Complete blood count done by Sinduri Biotech SB 6610 Fully Automated Hematology Analyzer with thorough examination of peripheral blood smears stained with giemsa stain. 2-Serum calcium, creatinine, LDH, B2 microglobulin, albumin (Clinic Automatic Chemistry Biochemistry Analyzer (poweam A8). 3- BM biopsy samples immunohistochemistry to confirm diagnosis of abnormal plasma cells (CD138 and monoclonal light chain).

All previous data obtained from the database of the Hematology department.

4- Staining technique: One hundred individuals with readily accessible samples of abnormal bone marrow were chosen. Examination of tumor-associated macrophages utilizing anti-CD68 and anti-CD161 monoclonal antibodies, in addition to evaluation of MVD with Factor VIII staining. In xylene that had been hydrated with increasing quantities of ethanol, sections were deparaffinized. The slides were heated with citrate buffer to extract antigens. 3 percent quantity of hydrogen peroxide was employed to inhibit endogenous peroxidase. For protein blocking, non-immune protein blocking was conducted for one minute. Separate slides were coated with anti-CD68, anti-CD163, and factor VIII monoclonal antibodies and incubated at room temperature for two hours (primary antibodies). The secondary antibodies were applied and kept for 30 minutes at room temperature. The mixture of Streptovidin and biotin was administered for 30 minutes.

The sections were dyed by incubating them for 10 minutes in chromogen solution and then washing them with distilled water. After each step, the slides were rinsed with phosphate-buffered saline (PBS). The median extent of infiltration (40/HPF field) in each histologic site was utilized to differentiate between individuals with low and high TAM density [7]. And according to MVD, MVD less than 20 /HPF was designated as low grade and equal or greater than 20 /HPF as high grade [8].

Statistical analysis: IBM's SPSS v27 (Chicago, Illinois, United States) was utilized for statistical analysis. Using the Shapiro-Wilks test and histograms, the distribution of the data was judged to be normal. Using an unpaired student t-test, the mean and standard deviation (SD) of parametric quantitative data were investigated. Using the Mann-Whitney test, the median and interquartile range (IQR) of nonparametric quantitative data were determined. If appropriate, qualitative variables were presented as frequency and percentage and analyzed using Chi-square or Fisher's exact test. The

prognostic significance of the analyzed parameters was established using Kaplan-Meier product limit estimates of survival. A two-tailed P value < 0.05 was considered statistically significant.

Results:

Clinical characters and laboratory records of studied cases were illustrated in Table 1.

Table (1): Clinical characters and laboratory data of studied cases

Patients (n = 100)			
Parameter		**N**	**%**
Durie-Salmon staging	I	12	12
	II	22	22
	III	66	66
ISS	I	15	15
	II	19	19
	III	66	66
Bone Lytic lesion	≤ 2 lesions	41	41
	> 2lesions	59	59
Creatinine (mg/dl)	≤ 2 mg/dl	63	63
	> 2 mg/dl	37	37
LDH	Normal	62	62
	High > 480 IU/L	38	38
Ca++, mg/dl	≥12 mg/dl	60	60
	<12 mg/dl	40	40
Electrophoresis for M component	"M" component in serum	90	90
	"M" component in urine	40	40
M protein subtype	IgG kappa	37	37
	IgG lambda	39	39
	IgM lambda	2	2
	IgA kappa	12	12
	IgA lambda	5	5
	Light chain	5	5
Plasma cells percentage	>50%	64	64
	<50%	36	36
Plasma cell morphology	Mature (low grade)	32	32
	Immature (intermediate grade)	56	56
	Plasmablastic (high grade)	12	12
Pattern of infiltration	Interstitial	6	6
	Nodular	6	6
	Mixed	32	32
	Diffuse (packed)	56	56
Peripheral blood findings	Hb (<10 g/dl)	85	85
	Normal platelet count	72	72
	Platelet count < 100000/cmm	28	28
	Raised ESR	80	80

Data were expressed as mean ± SD, number (%), *: significant as p value < 0.05.

There was insignificantly different between the two groups regarding age, sex, DS staging, ISS, bone lytic lesions. LDH had considerably elevated in high TAMs compared to low TAMS group (p=0.001). Bad outcome was significant in high TAMs compared to low TAMS group (P =0.001). **Table 2**

Table 2: Comparison between low TAMs and high TAMs according to the age, sex, DS staging, ISS, bone lytic lesions, LDH and Response after treatment

Prepare a more reader-friendly :[5h]Comment table

	CD68+ TAMs				T test	P value	CD163+ TAMs				T test	P value
	Low (n= 66)		High (n=34)				Low (n= 62)		High (n=38)			
Age	63.18 ± 9.53		61.84 ± 8.19		0.689	0.487	62.46 ± 9.41		62.17 ± 9.13		0.152	0.880
Gender	CD68+ TAMs						CD163+ TAMs					
	Low		High				Low		High			
	N	%	N	%	0.405		N	%	N	%		0.384
Male	35	53	21	61.8			32	51.6	23	60.5		
Female	31	47	13	38.2			30	48.4	15	39.5		
DS staging system												
Stage I	10	15.2	2	5.9	0.351		9	14.5	3	7.9		0.414
Stage II	13	19.7	9	26.5			15	24.2	7	18.4		
Stage III	43	65.2	23	67.6			38	61.3	28	73.7		
ISS												
Stage I	10	15.2	5	14.7	0.959		11	17.7	4	10.5		0.428
Stage II	12	18.2	7	20.6			13	21	6	15.8		
Stage III	44	66.7	22	64.7			38	61.3	28	73.7		
Bone lytic lesion												
≤ 2 lesions	28	42.4	13	38.2	0.687		25	40.3	16	42.1		0.860
> 2 lesions	38	57.6	21	61.8			37	59.7	22	57.9		
LDH												
Normal	50	75.8	12	35.3	0.001*		47	75.8	15	39.5		0.001*
High	16	24.2	22	64.7			15	24.2	23	60.5		
Response after treatment												
Complete remission	23	34.8	5	14.7	0.001*		24	38.7	4	10.5		0.001*
very good partial response	20	30.3	4	11.8			18	29.1	6	15.8		
Partial response	23	34.8	20	58.8			19	30.6	24	63.2		
Progressive disease	0	0.0	5	14.7			1	1.6	4	10.5		

Data were expressed as mean ± SD, number (%), *: significant as p value < 0.05.

There was insignificantly different between the two groups regarding age, gender, DS staging, and ISS. There was statistically significant increase of plasma cell percentage, plasma cell morphology and plasma cell infiltration in high MVD than low MVD group (p=0.001). There was statistically significant poor outcome in high MVD than low MVD group (p=0.001). LDH had increased significantly in high MVD than low MVD (P =0.001). **Table 3**

Table 3: Comparison between low MVD and high MVD according to age, gender, DS staging, ISS, plasma cell percentage, plasma cell morphology, pattern of infiltration, response to treatment and LDH

	MVD				P value
	Low (n= 40)		High (n=60)		
Age	62.97 ± 8.75		61.24 ± 8.74		0.335
Gender	N	%	N	%	
Male	21	52.5	38	63.3	0.281
Female	19	47.5	22	36.7	
DS staging					
Stage I	4	10	8	13.3	
Stage II	13	32.5	9	15	0.117
Stage III	23	57.5	43	71.7	
ISS					
Stage I	6	15	9	15	
Stage II	8	20	11	18.3	0.978
Stage III	26	65	40	66.7	
Plasma cell %					
< 50 %	30	75	6	10	0.001*
> 50 %	10	25	54	90	
Plasma cell morphology					
Mature (low grade)	28	70	4	6.7	
Immature (intermediate grade)	10	25	46	76.7	0.001*
Plasmablastic (high grade)	2	5	10	16.7	
Pattern of infiltration					
Interstitial	5	37.5	1	1.7	
Nodular	4	10	2	3.3	
Mixed	20	50	12	20	0.001*
Diffuse (packed)	11	27.5	45	75	
Response to treatment					
Complete remission	18	45	10	16.7	
very good partial response	16	40	8	13.3	0.001*
Partial response	5	5	38	63.3	
Progressive disease	0	0	5	8.3	
LDH					
Normal	50	80.6	12	31.6	0.001*
High	12	19.4	26	68.4	

A substantial positive connection existed between CD163 and CD68. Strong positive association existed between MVD and CD68. A high positive connection was observed between MVD and CD163. **Table 4**

Table 4: Correlation between CD163 and CD68 and MVD and correlation between CD68 and MVD

	CD 68	CD 163

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	r.	p	r.	p
CD 163	0.945	0.001*		
MVD	0.684	0.001*	0.754	0.001*

There was decreased overall survival with increased CD 68 TAMs content with median OS (50 months) in low CD 68 TAMs vs. (16.5 months) in high CD 68 TAMs. **Figure 1**

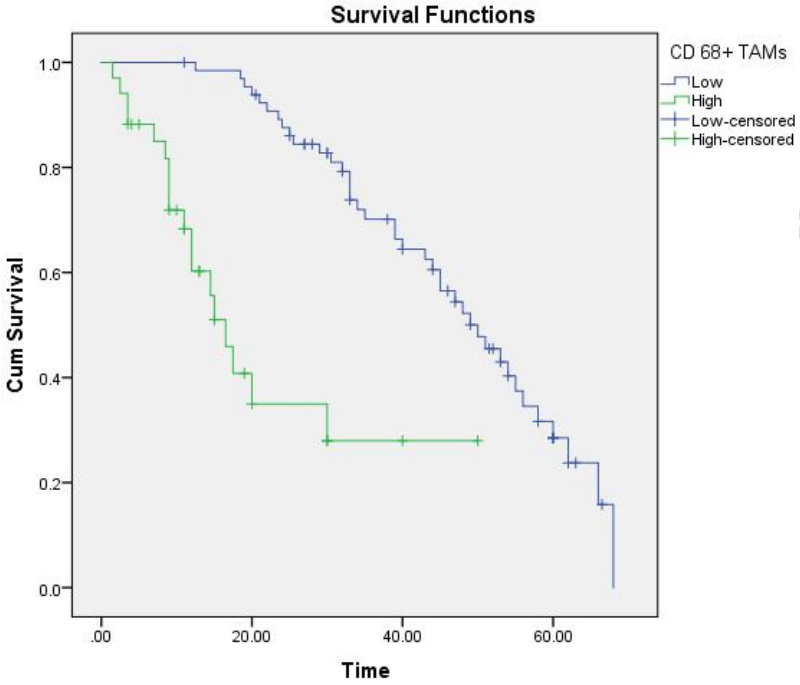

Figure 1: Survival outcome of patient based on CD 68 TAMs content

There was decreased overall survival with increased CD163 TAMs content with median OS (49.3 months) in low CD 163 TAMs vs. (25.6 months) in high CD 163 TAMs. **Figure 2**

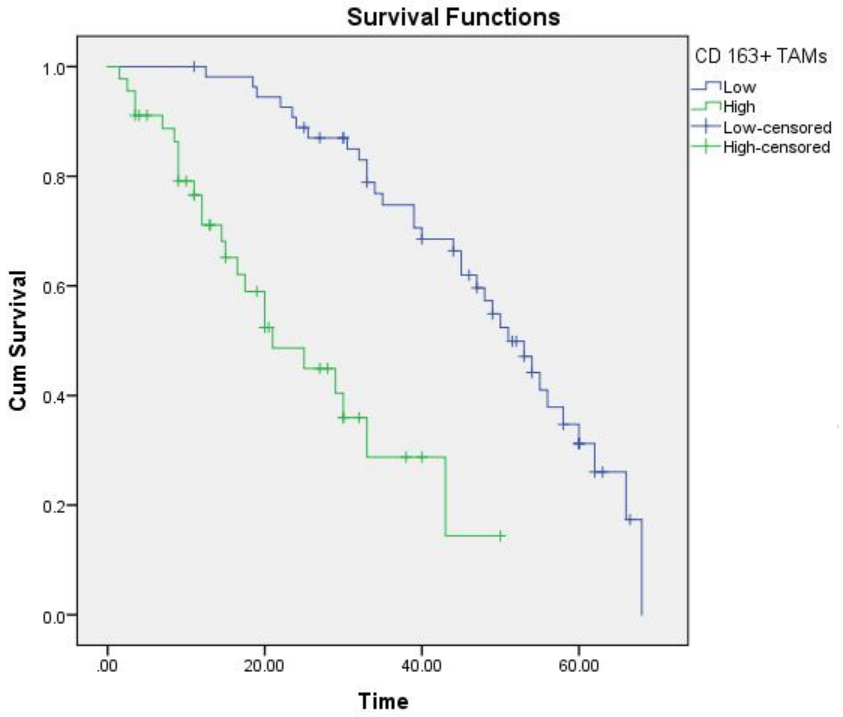

Figure 2: Survival outcome of patient based on CD 163 TAMs content:

There was decreased overall survival with increased MVD with median OS (54.6 months) in low MVD vs. (25.6 months) in high MVD. **Figure 3**

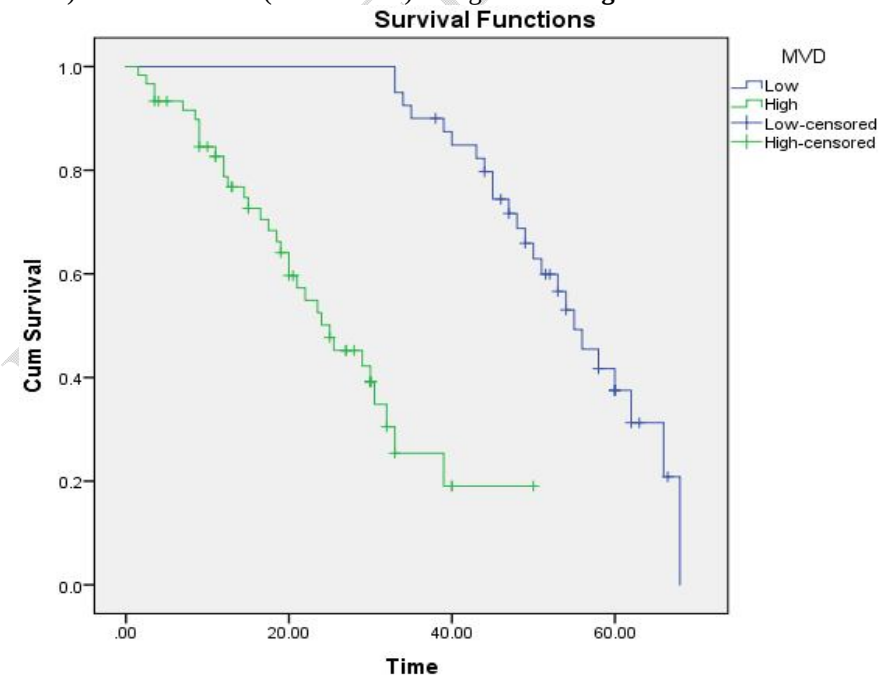

Figure 3: Survival outcome of patient based on MVD content:

Discussion

MM is a plasma cell neoplasm that is branded by malignant plasma cell clonal growth in the bone marrow, monoclonal protein synthesis in the blood or urine, and end-organ failure [9].

Regarding age, sex, and staging, the current investigation indicated no statistically significant differences between the groups with low and high TAMs. **Suyanı *et al.*** [5] came in line with this result.

According to bone lytic lesions, there was insignificantly different in TAMs expression between both groups. **Chen *et al.*** [7] reported the same result**.**

The current work's finding revealed a statistically significant increase of LDH in high TAMs group in comparison to those with low TAMS group. Also, there was statistically significant poor prognosis in high TAMs compared to low TAMs group. In addition, an increase in TAMs was associated with a statistically significant decrease in overall survival, showing that TAMs may promote the development and progress of myeloma. These results were in agreement with same findings reported by **Wang *et al.*** [10] and **Chen *et al.*** [7].

Due to the presence of TAMs, **Montovani and Allavena,** [11] observed that poor prognosis and shorter overall survival are associated with cancer treatment resistance. M2 macrophages may develop chemoresistance in tumour cells, therefore shielding tumour cells from chemotherapy [12].

Despite the fact that CD68+ and CD163+ TAMs are known to be detrimental to OS, they do not differentiate between M1 and M2 subsets, as shown by clinical investigations concentrating on M2 macrophages in a variety of malignancies [12].

CD163 assessed by IHC, or serum is inversely linked with MM case survival, according to **Panchabhai *et al.*** [13].

Chen *et al.* [7] examined the content of TAMs in 240 individuals of MM by immunohistochemistry and established that a high level of diametrically polarised TAMs is a unique independent poor prognostic factor; the widespread adoption of novel drugs has led to significant advances in the treatment of MM.

In the age of new medications, few research have examined the prognostic importance of TAMs in the tumour microenvironment of MM.

A high MVD in multiple myeloma patients has been shown to be a predictor, particularly for disease progression. Therefore, a high MVD implies a poor prognosis for individuals with MM.

At the time of first diagnosis, MVD must be examined with standard BM biopsy. Increased angiogenesis in the microenvironment of bone marrow (BM) promotes myeloma cell metastasis [14].

These results, together with those of research analysing micro-vessels in multiple myeloma patients, highlight the value of angiogenesis and imply that antiangiogenic medicines may be a viable treatment for MM [14].

Additional evidence indicates that angiogenesis in bone marrow corresponds with the production of neoplastic plasma cells and has a critical role in the course of disease [8].

Our study revealed insignificantly different between low and high MVD group as regard age, sex and staging. This was consistent with **Rana *et al.*** [15].

This study reported that increased plasma percentage in B.M associated with increase in MVD, and this result was in agreement with **Rana *et al.*** [15].

In the present study, it was revealed that MVD increased with immaturity of plasma cells. It was supported by **Palta *et al.*** [8].

Our study revealed a statistically significant increase of plasma cell infiltration in high MVD compared to low MVD group, **Rana *et al.*** [15] reported the same result.

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Also, in the present study, there was statistically significant poor outcome in high MVD compared to low MVD group. This result was in agreement with those reported by **Palta *et al.*** [8].

Moreover, in the present study, there was strong positive correlation between CD163 and CD 68 expression. This result was in accordance with those revealed by **Suyanı *et al.*** [5].

Moreover, in the present study, there was strong positive correlation between TAMs and MVD expression. This result was in accordance with those revealed by **Suyanı *et al.*** [5].

Scavelli *et al.* [16] confirmed that BM macrophages in active MM cases help to build neovessel in vasculogenic mimicry, parallel to the growth of plasma cell tumours and macrophages in patients with MM who were exposed to VEGF and FGF-2, which are important angiogenic cytokines released by plasma cells and present in the BM milieu, changed into cells functionally and phenotypically comparable to paired MMECs, replicating MMECs' capillary-like networks.

In the present study, it was revealed that overall survival is considerably longer in patients with low-grade MVD than cases with high-grade MVD. This agrees with **Lee *et al.*** [14] who reported that decreased overall survival with increased microvessel density as angiogenesis aid in proliferation and metastasis of multiple myeloma and also increased resistance to chemotherapy.

Targeting tumour angiogenesis with innovative antiangiogenic drugs is an exciting and promising therapy strategy that is the topic of current research [8].

Conclusions:

From the result of this study, we can conclude that important role of TAMs expression and MVD in the prognosis of the multiple myeloma, as the elevated TAMs expression and high grade MVD associated with poor prognosis. TAMs and MVD were found as a unique independent marker for both monitoring and predicting MM patient outcomes.

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