

Role of CD138, CD56, and light chain immunohistochemistry in suspected and diagnosed plasma cell myeloma: A prospective study

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Abstract

Introduction: Plasma cells (PCs) have conventionally been counted on the bone marrow aspirate, and small focal involvement may be missed even on bone marrow biopsy sections. **Material and Methods:** We aimed to study the role of CD138, CD56, anti- κ , and anti- λ immunohistochemistry (IHC) to separate PC myeloma from reactive plasmacytosis and to study the utility of these in cases suspected as myelomas and lacking >10% PCs on bone marrow aspirate. The study comprised 35 diagnosed myelomas, 20 reactive plasmacytosis, and 19 M-band positive suspected myelomas. CD138 IHC was performed on all cases along with CD56, anti- κ , and anti- λ IHC. PCs were counted on CD138-immunostained sections by manual count and by image analysis. In addition, CD56 expression was correlated with clinical features in diagnosed myeloma group. **Results:** In all cases, both manual counts and image analysis, PC counts were significantly higher on the CD138 stained sections than bone marrow aspirates. It was seen that the manual PC counts and image analysis counts were equivalent in diagnosed myeloma cases. CD56 expression was seen in ~62.85% diagnosed myeloma cases while it was negative in cases of reactive plasmacytosis. CD56 expression was significantly higher in patients with lytic lesions (78.26% vs. 21.74%). CD138, anti- κ , and anti- λ IHC also helped classify 11/19 (57.8%) cases correctly. **Conclusion:** The use of CD138 along with the light chain and CD56 IHC adds a high diagnostic value in myeloma patients and suspected myeloma cases. The PCs can be counted manually on the CD138-immunostained sections and correlate well with the counts obtained by image analysis.

Key words: CD138, CD56, lytic lesions, myeloma, plasma cell percentage, reactive plasmacytosis, suspected myeloma

Introduction

Plasma cells (PCs) are mature antibody secreting B-cell which are increased in the bone marrow in both reactive and neoplastic states. There are a multitude of malignant PC disorders which are further subclassified into plasmacytoma, monoclonal gammopathy of undetermined significance (MGUS), asymptomatic PC myeloma (PCM), symptomatic myeloma, and primary amyloidosis.^[1] PC enumeration is critical to the diagnosis of these disorders and to differentiate them from each other. Classically, a cut-off of 10% clonal PCs is used to differentiate MGUS from symptomatic and asymptomatic PCM.^[2] The international myeloma working group in 2014 myeloma classification has identified >60% PCs in the bone marrow as a biomarker of disease progression and has been included as myeloma defining the event. These patients, even when they lack the related organ tissue injury are still classified as active myeloma and are candidates for therapy.^[2]

The method employed for counting PCs on well-stained bone marrow aspirate smears may not give an accurate count and may show lesser number of PCs compared to bone marrow biopsy as: (1) Marrow involvement may be focal and may not be at the site of aspirate, (2) PCs express many adhesion molecules such as CD138, CD56 which makes them stick to one another and the marrow stroma, (3) due to the lipophilic nature of PCs, they may get stuck in the marrow particles, and (4) bone marrow aspirate samples suffer from hemodilution.^[3-6] For these reasons, evaluation of bone marrow biopsy is absolutely essential in myeloma.

In Hematoxylin and Eosin (H and E) stained marrow biopsy sections, small foci of PCs may be missed and therefore, underestimate PC percentage. Immunohistochemistry (IHC) on the bone marrow biopsy would be superior in delineating PCs. Syndecan-1/CD138 expression in the bone marrow is specific for polyclonal and clonal PCs,^[7] and counting has been done on

CD138-stained section by either visual estimation,^[8,9] manual count on the section^[8] or image analysis.^[4,7-9] CD138 in conjunction with κ and λ light chains helps in differentiating reactive from clonal PCs.^[10] Clonal PCs express certain markers of which CD56 is useful as it has a positive predictive value. It is aberrantly expressed in 67%–79% of PCM cases^[11] CD56 expression also has been found to correlate with lytic lesions in PCM.^[12]

The present study was designed to assess the utility of CD138 IHC along with anti- κ and anti- λ chain IHC to count PCs in bone marrow biopsy and compare it with bone marrow aspirates in both reactive plasmacytosis and PCM. In addition, we also tried to evaluate its utility in cases suspected to be PCM showing <10% PCs on bone marrow aspirate. In addition, CD56 immuno-staining was done in all cases and its expression was correlated with features of CRAB (hypercalcemia, renal failure, anemia, and bone lesions) in diagnosed PCM cases.

Materials and Methods

Patient selection

This was an observational, prospective study conducted in the Department of Hematology, All India Institute of medical sciences, New Delhi from January 2010 to March 2011 and consisted of three groups. The first group included a total of 35 cases of PCM (including 31 symptomatic PCM, 4 myeloma patients on therapy, and 1 case of POEMS syndrome [Polyneuropathy organomegaly endocrinopathy monoclonal gammopathy and skin changes]). The second group comprised 20 cases of reactive plasmacytosis and the third group of 19 cases of suspected PC dyscrasia. The patient's relevant clinical details were recorded. Ethical clearance was obtained from the Institutional Ethics Committee. Baseline complete blood counts, serum creatinine, and skeletal survey were included for all patients.

The WHO 2008 criteria were used for the diagnosis of PC dyscrasias including MGUS, symptomatic and asymptomatic

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PCM, and primary amyloidosis. Reactive plasmacytosis cases were selected if bone marrow aspirate showed >5% PCs and these cases were: Visceral leishmaniasis in 7 cases, tuberculosis in 1 case, immune thrombocytopenia in 3 cases, suspected Non-Hodgkin lymphoma in 2 cases, aplastic anemia in 5 cases, Myelodysplastic syndrome and hypersplenism in 1 case each.

For the group of suspected PC dyscrasias, cases included were those with <10% PCs on the aspirate and the marrow biopsy was not diagnostic of myeloma with at least one of the following features:

1. Presence of M-band on serum or urine protein electrophoresis
2. Lytic lesions in the bone/osteoporosis
3. Evidence of primary amyloidosis with a tissue biopsy positive for amyloid by the Congo red stain
4. Evidence of renal failure.

Patients were chosen without any selection bias with respect to the PC percentage, stage or any specific clinical feature except only on the availability of adequate bone marrow aspirate and bone marrow biopsy.

Evaluation of bone marrow aspirates, biopsies, and immunohistochemistry

In all bone marrow aspirates stained with Jenner-Giemsa stain, a 500 cell myelogram was done. Bone marrow biopsy was processed using the Hammersmith protocol.^[13] H and E stained section was evaluated in all cases.

Formalin-fixed paraffin sections of 3 μ m thick were used for IHC staining. IHC was performed for CD138, CD56, anti- κ , and anti- λ antibodies. CD138 (Clone MI15) was used in 1:100 dilution, anti- κ (Clone L1C1) was used in 1:10,000 dilution, anti- λ (Clone HP6054) was used in 1:10,000 dilution and CD56 (Clone 123C3.D5) was used in 1:25 dilution. All antibodies were obtained from vision diagnostics. Microwave-based antigen retrieval using a citrate buffer at pH 6.0–6.2 was utilized for CD138, anti- κ , and anti- λ antibodies. For CD56, antigen retrieval was performed using pressure cooker with TRIS-EDTA buffer at pH 9.0.

A κ : λ ratio of >8 or <0.2 was used to determine clonality.^[14] The determination of the PC percentage on CD138 stained bone marrow biopsy sections was performed by both manual counts and by computer image analysis by an observer blinded to the clinic-pathological data. Normally, the a low magnification visual assessment of the CD138 stained immunosection is done, but for refined analysis, we employed a high magnification manual count of PCs as described by Smith *et al.*^[9] The section was examined first at low magnification ($\times 100$) to assess distribution and PC concentration. Following representative areas were counted under high magnification ($\times 400$). PCs were counted as a percentage of all nucleated cells after delimiting the field diaphragm. We counted at least 500 nucleated cells instead of 200 as used by Smith *et al.*^[9] Computer-assisted image analysis was performed using a calibrated Olympus (BX51, Olympus™, Japan) microscope. A representative $\times 40$ field was photographed taking care to exclude areas of hemorrhage, necrosis, periosteum, bony trabeculae, and dense fibrosis and crush artifacts. Acquired images were imported into the Image ProPlus™ software. The total area was marked, and the fat spaces were excluded. Then, the PC areas were marked and the all the PC areas were added. The cellular area was calculated by subtracting fat space area from

total area, and PC area percentage was calculated as a fraction of the total cellular area. Wherever there were markedly different areas of infiltration, two or more fields were calculated.^[9]

Statistical analysis

For the categorical variables, Fisher's exact test and for continuous variables, the Mann–Whitney test was used. To calculate correlations between PC percentages by various methods, Pearson's correlation coefficient was used. Wilcoxon rank sum test was used to determine the differences between the PC percentages obtained by different methods. $P < 0.05$ was considered as statistically significant.

Results

The study was conducted on a total of 74 cases comprising 35 cases of myelomas (Group 1), 20 reactive plasmacytosis (Group 2) and 19 cases where a diagnosis of myeloma was suspected but not proven by the bone marrow aspirate and biopsy (Group 3). In Group 1, 34 cases had an M band either in urine or serum protein electrophoresis with the single negative case being POEMS syndrome that was positive by serum immunofixation electrophoresis (IFE). None of the patients in Group 2 were evaluated for a paraprotein. In the Group 3, 9 patients had an M band in either serum or urine. However, none of the patients underwent IFE. The demographic profile is given in Table 1.

Plasma cell counts on bone marrow aspirate and CD138 immunostained sections

PCs were counted on the bone marrow aspirate [Figure 1], and on CD138 immunostained section by both manual count [Figure 2] and image analysis performed by a blinded observer [Figure 3]. The median PC% by these three methods and statistical analysis (by Wilcoxon signed rank test) are provided in Table 2.

When the myeloma cases (Group 1) were grouped into categories of $\leq 10\%$, 11–30%, 31–50%, and >50% PCs, PC percentage was found to be higher on manual counts on CD138 stained biopsy sections when compared with that seen in marrow aspirate in 22 of the cases (65.82%), i.e., the PC percentage was discordant between the two methods. Split between groups, the discordance in the $\leq 10\%$ group was 75% (3/4 cases), in 11–30% group, it was 88.8% (8/9 cases), and in the 31–50% group, it was 100% (11/11 cases). However, all cases in the >50% PC group were concordant when PC percentage on marrow aspirate with manual counts on CD138 stained immunosection. The detailed distribution is provided in Table 3.

It was also seen that the patterns of involvement on the H and E stained sections and the CD138 stained sections also showed discordance in 13 of the cases (37.14%). Among the six cases thought to have an interstitial pattern on H and E, four showed nodules and two showed microaggregates, five cases judged to have nodular aggregates showed diffuse replacement of one or more marrow spaces and two cases with small aggregates demonstrated nodules on CD138 stained sections.

Correlation between “CRAB” and CD56 expression

CD56 was positive in 22/35 of the myeloma cases (62.85%) while in reactive plasmacytosis it was negative in all cases 0/20 (0%). Among the cases of suspected myelomas, it was positive in 3/28 cases (10.71%). All the three cases were proven to be myelomas by the use of CD138 IHC and clonality analysis with kappa and lambda.

Table 1: Demographic profile of the three groups of bone marrow plasmacytosis

	PCM (n=35)	Reactive plasmacytosis (n=20)	Suspected MM (n=19)
Male:female	22:13	12:8	20:8
Age, median (range) (years)	55 (25-75)	35 (7-67)	56 (30-78)
M- band in serum/urine	Both positive in 31 (1 negative case had POEMS syndrome, showed IFE positive) 4 in urine only	Not evaluated	Positive in all cases either in serum or urine
Hemoglobin, median (range) (g/L)	87 (51-140)	64 (31-80)	110 (49-130)
TLC, median (range) ($\times 10^9/L$)	6.7 (3.2-23.0)	2.6 (0.58-22.42)	6.6 (3-19.0)
Platelet count, median (range) ($\times 10^9/L$)	170 (21-401)	64 (2-535)	200 (7-533)

PCM=Plasma cell myeloma, TLC=Total lymphocyte count, IFE=Immunofixation electrophoresis, POEMS=Polyneuropathy, organomegaly, endocrinopathy, monoclonal gammopathy, skin changes, MM=Multiple myeloma

Table 2: Plasma cell% in all three groups by application of three plasma cell counting techniques

	Mean (range) (%)			P value between PC% BMA and CD138 manual count	P value between CD138 manual count and image analysis	P value between PC% BMA and CD138 image analysis
	PC% BM aspirate	PC% in CD138 IHC by manual count	PC% in CD138 IHC by image analysis			
Myeloma (n=35)	35 (2-88)	80 (5-100)	69.9 (5.6-100)	0.0001	0.791	0.0001
Reactive plasmacytosis (n=20)	6 (5-10)	13 (5-70)	10.55 (4-31)	0.0001	0.007	0.001
Suspected myeloma (n=19)	4 (0-10)	13 (5-60)	9.6 (3-56.6)	0.0001	0.007	0.0001

IHC=Immunohistochemistry, BMA=Bone marrow aspirate, BM=Bone marrow

Table 3: Comparison between plasma cell % on bone marrow aspirate and manual count on CD138 stained biopsy section in myeloma patients (n=35)

Plasma cell% in BMA	Manual plasma cell count % on CD138 IHC on marrow biopsy (BMBx)			
	$\leq 10\%$	11%-30%	31%-50%	$> 50\%$
$\leq 10\%$	1	1	1	1
11%-30%		1	3	5
31%-50%				11
$> 50\%$				11

BMA=Bone marrow aspirate, IHC=Immunohistochemistry, BMBx=Bone marrow biopsy

Twenty-three of the 35 myeloma cases in Group 1 had lytic lesions, fractures or osteopenia. CD56 expression in these cases significantly higher than in cases without lytic lesions using the Fisher's exact test [Table 4, $P = 0.024$].

However, there was no correlation between CD56 expression and the presence of renal failure, hypercalcemia, and anemia.

Use of CD138 IHC in suspected myeloma cases

Among the 19 M-band positive cases of suspected myeloma group, it was seen that 6 cases could be reclassified as myeloma based on the clonal PCs. Two cases demonstrated $> 50\%$ clonal PCs on the bone marrow biopsy, and the aspirate had shown a small lymphocyte-like morphology which led to initial misclassification as lymphoma infiltration. In addition, the diagnosis of primary amyloidosis was confirmed in 2 cases, MGUS, plasmacytoma and Waldenstrom macroglobulinemia in 1 case each. Hence after the application of IHC, we were able to classify 11/19 cases in distinct categories. The details of these 11 cases are given in Table 5. Rest of the cases were not myelomas.

Discussion

The diagnosis of PCM essentially is based on the detection of a monoclonal M protein and clonal PCs on the bone marrow. Although for diagnosis, any number of PCs is sufficient in cases with presence of a CRAB criteria and M band, a PC number $> 10\%$ clonal PCs as used by the Mayo criteria is very useful.^[15] Moreover, the new IMWG criteria for the diagnosis of myeloma have defined $> 60\%$ clonal PCs on the bone marrow biopsy/aspirate as a biomarker for diagnosing active myeloma.

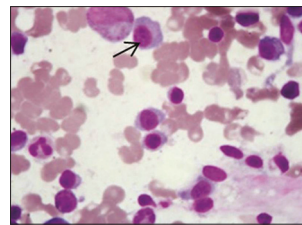


Figure 1: Oil immersion view (x1000) of a Jenner-Giemsa stained bone marrow aspirate shows increased plasma cells in a patient of myeloma

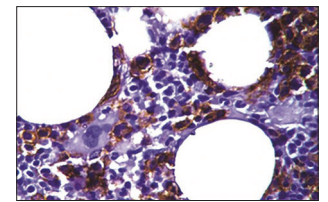


Figure 2: A high power field selected for manual count on a CD138 immunostained section (x400)

Thus, even cases otherwise lacking CRAB will be classified as myeloma if they have $> 60\%$ clonal PCs.^[2] A false negative report of low PC% in marrow aspirate and H and E stained marrow biopsy delays in reaching a clear diagnosis in many suspected cases of PC dyscrasia.

We performed this study to see if the use of CD138 immunostaining alone could enhance the identification of PCs and help in diagnosis. Cases where a diagnosis of myeloma was suspected but not confirmed were analyzed using CD138 and clonality analysis to determine if this could establish a conclusive diagnosis in these cases where a false-negative report may be sent from the laboratory due to a low number of PCs on the aspirate smears.

As in other studies,^[4,5,8] the present study also shows that CD138 is a sensitive marker to identify PCs and is positive in both normal and monoclonal PCs. The single case of Waldenstrom macroglobulinemia expressed CD138 only in the PCs and was completely negative in the lymphocytes. Our finding is consistent with O'Connell *et al.* who showed CD138 activity restricted to PCs in all four cases of Waldenstrom macroglobulinemia.^[7]

We performed a manual PC percentage count on the CD138 immunostained bone marrow biopsy section as described by Smith *et al.*^[9] followed by computer image analysis to determine the immunopositive area fraction.^[8,9,16] We noted that the PC percentages on the Giemsa stained aspirate smears were always significantly lower than those obtained on the CD138 stained sections by either manual counts or image analysis in myeloma, reactive plasmacytosis and suspected myeloma cases. A similar result has been reported by Ng *et al.*^[4] in myeloma cases taken

both at diagnosis and follow up, Smith *et al.*^[9] and Stifter *et al.*^[16] in myeloma cases. The $\times 400$ magnification was used to count the PCs from an area selected under the low power field on the CD138 stained biopsy section as per Smith *et al.*^[9] This was a manual count on at least 500 nucleated cells which was a modification from the 200 cell count performed by Smith *et al.*^[9] This was compared with the PC percentage obtained on the image analysis. We found a highly significant correlation between manual counts and image analysis performed on the CD138 immunostained sections like Smith *et al.*^[9] In their study, the manual PC counts on CD138 immunostained sections had shown the highest correlation with the count obtained on image analysis while marrow aspirate PC count and visual estimation of CD138 immunostained sections were less correlated with the image analysis count.

There is no published study which compared the PC percentages on CD138 sections in reactive plasmacytosis while in the single study^[17] which included borderline cases does not clearly mention the CD138 PC percentage in this group of patients. Of the 19 M-band positive suspected myeloma cases, 11 were correctly classified based on the IHC into 6 cases of myeloma, of which two cases had a small lymphocytic morphology, 2 cases as

primary amyloidosis, 1 case each of MGUS, plasmacytoma, and Waldenstrom macroglobulinemia. For the rest of the 8 cases, PC% was higher on the CD138 stained section, but clonality could not be established. These cases also require follow-up as they did have at least one positive “CRAB” criteria and they might have small numbers of clonal PC in the background of normal polyclonal PCs or they might have any other lymphoproliferative disorder which could account for the M band.

We were able to demonstrate CD56 expression in 62.85% of the myeloma cases, 0% of the reactive plasmacytosis and 15.78% in the suspected myeloma group with a positive control in osteoblasts.^[18] This is comparable to the 71% positivity found by Ely *et al.* in myeloma cases and 0% in the reactive plasmacytosis group.^[12] However, Martin *et al.* reported a 92%CD56 positivity in myeloma and 1/5 cases of reactive plasmacytosis were CD56 positive. Incidentally, the positivity for CD56 was found to be higher on bone marrow biopsy samples when compared with bone marrow smears in the same myeloma cases.^[18] The higher reported incidence in myeloma may be due to the small sample size of

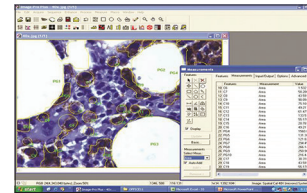


Figure 3: The same high power field when analysed by image analysis using ProPlus software

Table 4: CD56 expression and bone lesions in plasma cell myeloma patients (n=35)

	Bone lesions (n=23), n (%)	No bone lesions (n=12), n (%)
CD56 positive (n=22)	18 (78.26)	4 (33.33)
CD56 negative (n=13)	5 (21.74)	8 (66.67)

Table 5: Details of the 11 cases resolved by IHC

Clinical features	Diagnosis based on marrow aspirate and biopsy	CD138 IHC PC%	Final diagnosis
63 year old male with FNAC proven plasmacytoma of the soft tissue	Aspirate-4% plasma cells Scattered interstitial plasma cells on biopsy	25%, kappa restricted	Myeloma
66 year old female with symptomatic anemia	Aspirate-7% plasma cells. Biopsy- diffuse lymphocytic infiltrate	60% plasma cells, kappa restricted	Myeloma (with small lymphocytic morphology)
40 year male with low backache, lytic lesions and M-band	Aspirate- 6% plasma cells biopsy- Scattered interstitial plasma cells	30% plasma cells, lambda restricted	Myeloma
40 year male with low backache, lytic lesions and M band	Aspirate- 3% plasma cells Biopsy- clusters of plasma cells	56% plasma cells, kappa restricted	Myeloma
54 year male with swelling in the sternum	Aspirate- 10% plasma cells Biopsy- diffuse lymphocytic infiltrate	52% plasma cells, kappa restricted	Myeloma (with small lymphocytic morphology)
50 year male with renal amyloidosis	Aspirate- 4% plasma cells Biopsy- Scattered interstitial plasma cells	30% plasma cells, lambda restricted	Myeloma with amyloidosis
30 year male with soft tissue plasmacytoma	Aspirate- 5% plasma cells Biopsy- no increase in plasma cells	10% plasma cells, no clonality	Plasmacytoma, follow up required
69 year male with MGUS diagnosed 2 years back, now with anemia	Aspirate- 2% plasma cells, no increase on biopsy	8% plasma cells, no clonality	MGUS, cause of anemia to be investigated
40 year male with pedal edema, renal amyloidosis	Aspirate-8% plasma cells Biopsy-no increase	15% plasma cells, lambda restricted	Primary amyloidosis
42 year old male with restrictive cardiomyopathy, cardiac biopsy amyloidosis positive	Aspirate- 6% plasma cells, no increase on biopsy	5% plasma cells, lambda restricted	Primary amyloidosis
62 year male with anemia requiring transfusions	Aspirate- 7% plasma cells, biopsy- increase in lymphocytes	25% plasma cells, kappa restricted Rest of the cells negative for CD138	Waldenstrom macroglobulinemia

FNAC=Fine-needle aspiration cytology, MGUS=Monoclonal gammopathy of undetermined significance, IHC=Immunohistochemistry

15 cases. In their study, the single CD56 positive case of reactive plasmacytosis might have had a small clonal population. We also found a significant association between bone lesions and CD56 expression in myeloma but not with anemia and renal failure. This finding is similar to Ely *et al.*^[12] and Chang *et al.*^[19] who showed a significant association with lytic lesions, but they have not reported their data for an association with anemia and renal failure. In the present study, we found that heat-induced epitope retrieval (HIER) performed with pressure cooker gave superior results with CD56 than the microwave-based technique which gave good results for all other antibodies used. For CD56 immunostaining, in our experience, TRIS-EDTA buffer combined with a pressure cooker for HIER gave much better results than the microwave method with citrate as none of the cases using the latter technique showed positive staining in osteoblasts which act as an internal control.^[18]

The present study shows that CD138 IHC in conjunction with κ/λ should be performed in all cases suspected of PCM in which the marrow aspirate shows <10% PCs. Since the manual PC counts on CD138 immunostained sections show a very good correlation with the image analysis, manual PC count method is adequate. This would reduce the false negative reports of lower PCs in marrow aspirate and H and E stained marrow biopsy due to focal distribution on marrow biopsy or in cases with PCs of a small lymphocytic morphology. In addition, CD56 has a good positive predictive value for diagnosis of myeloma and correlates well with bone lesions and may be correlated with the pathogenesis of lytic lesions.

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Conflicts of interest

There are no conflicts of interest.

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