Utility of CD56 Immunohistochemical Studies in Follow-up of Plasma Cell Myeloma

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Abstract

Although 70% to 80% of plasma cell myelomas (PCMs) express CD56, few data are available on the usefulness of CD56 immunohistochemical analysis in assessing residual disease. We retrospectively reviewed 127 PCM posttreatment bone marrow (BM) specimens, classifying them as positive or negative for residual disease (independent of CD56 immunohistochemical studies) based on abnormal plasma cell (PC) morphologic features or flow cytometry (FC) and/or light chain restriction by immunohistochemical studies (conventional criteria). CD56 immunohistochemical analysis was performed on these and 20 negative lymphoma staging BM specimens. Of 127 BM specimens, 74 were positive and 53 were negative for residual PCM by conventional criteria. Of 74 BM specimens positive by conventional criteria, 59 (80%) demonstrated CD56 (strong+) PCs in clusters and/or with cytologic atypia. Of the 53 BM specimens negative by conventional criteria, 3 showed CD56 (strong+) morphologically atypical PCs in clusters or scattered. CD56 immunohistochemical analysis is useful for detecting residual PCM, particularly in morphologically equivocal cases in which light chain restriction cannot be demonstrated, and may serve as a potential response criterion.

CD56 is a membrane glycoprotein that mediates adhesion between neural cells and, as such, is also known as neural cell adhesion molecule.1 In the bone marrow (BM), CD56 is normally expressed on natural killer (NK) cells, a subset of T cells, and osteoblasts rimming bone trabeculae.1-3 In 1990, Van Camp et al4 first described strong CD56 positivity in the plasma cells (PCs) of patients with PC myeloma (PCM) by using immunohistochemical analysis and flow cytometry (FC). In subsequent studies, aberrant CD56 expression has been demonstrated in 70% to 80% of PCMs at diagnosis.2,3,5

The International Myeloma Working Group (IMWG) proposed new diagnostic criteria in 2003 for the diagnosis of PC dyscrasias, including symptomatic myeloma, asymptomatic myeloma, and monoclonal gammopathy of undetermined significance (MGUS). Criteria for symptomatic myeloma include serum and/or urine monoclonal protein, clonal PCs in the BM or plasmacytoma, and myeloma-related end-organ damage, such as hypercalcemia, renal failure, anemia, and/or lytic bone lesions.6 Assessment of residual disease in the BM relies on PC percentage, morphologic features, immunoglobulin light chain expression, and PC immunophenotype. In 2006, the IMWG published new response criteria for the evaluation of PCM therapy. In this proposal, response categories of complete response (CR), stringent complete response (sCR), very good partial response (VGPR), and partial response were defined by varying criteria, including serum free light chain ratios, serum and/or urine protein electrophoresis/immunofixation results, and BM examination.7 The response categories of CR and sCR require negative serum and urine immunofixation results and BM findings of 5% or fewer PCs and 5% or fewer PCs plus absence of clonal PCs by immunohistochemical analysis or FC, respectively.
clonality defined by an abnormal light chain ratio. However, when PCs are present in low numbers in follow-up BM specimens, demonstration of light chain restriction and/or aberrant immunophenotypes by immunohistochemical analysis or FC may be extremely difficult. Nevertheless, categorization into appropriate uniform response groups is vital for the evaluation of new treatment modalities.

We observed a cluster of CD56+ PCs on immunohistochemical staining of a clot section in a myeloma follow-up BM with few PCs and no demonstrable light chain restriction by immunohistochemical analysis or FC, and this finding was interpreted as low-level residual disease. However, few data are available on the usefulness of CD56 immunohistochemical analysis on BM sections in assessing residual disease. We sought to retrospectively review follow-up PCM BM specimens for expression of CD56 by immunohistochemical analysis and characterize its potential role in residual disease monitoring.

Materials and Methods

Study Group

We retrospectively reviewed 127 follow-up BM specimens that had been obtained between January 2007 and February 2008 at Froedtert Hospital/Medical College of Wisconsin, Milwaukee, from 111 patients diagnosed with PCM who had undergone various treatment protocols. The original diagnosis of PCM was established according to the 2001 World Health Organization criteria or the IMWG 2003 criteria.

Cases were initially categorized by a hematopathologist as positive or negative for residual PCM, independent of the CD56 immunohistochemical results. Bouin-fixed, H&E-stained core biopsy specimens and/or clot sections and Wright-Giemsa–stained aspirate smears were reviewed quantitatively and qualitatively for PCs. When possible, 500-cell aspirate smear differential counts were performed. Immunoglobulin light chain expression was assessed by immunohistochemical analysis and/or FC for PCs. When present, ≥2 PCs. CD56 staining intensity was assessed relative to that of osteoblasts when present, so that strong positivity equaled the osteoblast intensity and weak positivity was dimmer than that of these internal controls. Cytologically normal PCs were identified as small to medium-sized cells with eccentric nuclei and coarsely clumped chromatin Image 1A and Image 1B, morphologically distinguishable from large granular lymphocytes (LGLs) based on chromatin pattern and a lower nuclear/cytoplasmic ratio Image 1C. Atypical PCs were identified as described previously. κ and λ stains, when performed, were evaluated for monoclonality within the PCs, defined as κ/λ ratios of less than 0.5 or more than 4.0. § 9, 10

Flow Cytometry

Heparinized bone marrow aspirate specimens were lysed with an ammonium chloride solution followed by 3 rounds of centrifugation and resuspension with an RPMI/penicillin-streptomycin solution. Cell suspensions (approximately 2 × serum and/or urine protein electrophoresis data were collected, when available. The study was approved by the Medical College of Wisconsin Institutional Review Board.

Immunohistochemical Studies

Immunohistochemical analysis for CD56 was performed on core biopsy specimens or clot sections of all follow-up BM specimens and 20 age-matched, negative lymphoma staging BM specimens, which served as control samples. An indirect immunoperoxidase staining method was performed on Bouin-fixed, paraffin-embedded, 3-μm-thick tissue sections, using mouse anti-CD56 antibodies (Novocastra, Newcastle upon Tyne, England), antimouse EnVision+ System–HRP labeled polymer (DAKO, Glostrup, Denmark), and diaminobenzidine (DAB) as the chromogen. Endogenous peroxidase was blocked with 3% hydrogen peroxide for 10 minutes, followed by application of a DAKO pH 6.0 Target Retrieval Solution incubated for 35 minutes at 99°C. A 20-minute room temperature incubation followed. The CD56 antibody was applied for 35 minutes and followed by application of the EnVision+ detection polymer, using the DAKO Autostainer. Staining was concluded with application of DAB for 7 minutes and counterstaining with hematoxylin. Immunohistochemical analysis for immunoglobulins was performed as above, using rabbit polyclonal anti-κ and anti-λ antibodies (DAKO), with substitution of an antirabbit EnVision+ System–HRP-labeled polymer (DAKO) for detection.

Interpretation of Immunohistochemical Studies

CD56 stains were reviewed blindly, without reviewer knowledge of the diagnosis. The distribution, staining intensity, and morphologic features of CD56+ PCs were recorded and compared between the control and study groups. Clusters were defined as more than 2 PCs. CD56 staining intensity was assessed relative to that of osteoblasts when present, so that strong positivity equaled the osteoblast intensity and weak positivity was dimmer than that of these internal controls. Cytologically normal PCs were identified as small to medium-sized cells with eccentric nuclei and coarsely clumped chromatin Image 1A and Image 1B, morphologically distinguishable from large granular lymphocytes (LGLs) based on chromatin pattern and a lower nuclear/cytoplasmic ratio Image 1C. Atypical PCs were identified as described previously. κ and λ stains, when performed, were evaluated for monoclonality within the PCs, defined as κ/λ ratios of less than 0.5 or more than 4.0. § 9, 10

Flow Cytometry

Heparinized bone marrow aspirate specimens were lysed with an ammonium chloride solution followed by 3 rounds of centrifugation and resuspension with an RPMI/penicillin-streptomycin solution. Cell suspensions (approximately 2 ×
10^6 cells/mL) were stained using CD14–fluorescein isothiocyanate, CD56–phycoerythrin, CD45–peridinin chlorophyll protein, and CD38–allophycocyanin. After incubation for 30 minutes at 1°C to 9°C in the dark, the cells were washed with phosphate-buffered saline containing azide, followed by fixation in 1% formaldehyde. Intracellular immunoglobulin expression was evaluated by using a monoclonal κ–fluorescein isothiocyanate, monoclonal λ–phycoerythrin, CD45–peridinin chlorophyll protein, and CD38–allophycocyanin tube. Following addition to the cell suspension, the CD38 and CD45 antibodies were incubated in the dark for 15 minutes at 20°C to 25°C, and then the immunoglobulin antibodies were incubated similarly for 20 minutes, following the addition of the Caltag Fix and Perm Reagents (Caltag, Burlingame, CA). All antibodies were products of Becton Dickinson, Franklin Lakes, NJ. Data were analyzed by using cluster analysis and Becton Dickinson Paint-A-Gate software. CD38 (bright+) events were regarded as PCs.\textsuperscript{11} Monoclonality was defined as κ/λ ratios less than 0.5 or more than 4.0.\textsuperscript{7,9,10}

Results

The control group (n = 20) was composed of 11 men and 9 women, ranging in age from 37 to 79 years (median, 58 years), and the myeloma follow-up group (n = 127 BM specimens from 111 patients) consisted of 65 men and 46 women, ranging in age from 35 to 78 years (median, 57.8 years). Of 20 control samples, 11 (55%) contained few, weakly CD56+ PCs. In all cases, the CD56+ PCs were cytologically normal. Two of the cases with CD56+ PCs (2/11) showed pairs of positive PCs (Image 1B), with the remaining majority demonstrating single, scattered positive PCs (Image 1A). Of the 11 patients with CD56+ PCs, 3 had previously undergone

![Image 1](image1.png)

**Image 1** Weak CD56+ plasma cells singly (A, ×1,000) and in pairs (B, ×1,000) and large granular lymphocytes (C, ×1,000) in control marrow specimens.
various treatment regimens and these BM examinations represented restaging procedures, compared with 6 of 9 cases without CD56+ PCs. PCs accounted for an average of 1.2% (range, 0%-4%) of cells in the aspirates from this group.

Of the 127 PCM follow-up BM specimens, 74 were positive and 53 were negative for residual myeloma by conventional criteria Table 1 and Figure 1. PCs accounted for an average of 17.9% of aspirate smear cellularity (range, 0%-100%; median, 7%) in the positive cases and 0.70% (range, 0%-3%; median, 0.9%) in the negative follow-up cases. Of 74 positive follow-up cases, 63 (85%) had serum and/or urine monoclonal proteins identified and/or cytogenetic evidence of residual disease. Of 53 negative cases, 21 (40%) had monoclonal proteins detected in the serum and/or urine.

Of 74 positive BM specimens, 59 (80%) demonstrated CD56 (strong+) PCs (Table 1; Figure 1). In all of these cases, CD56+ PCs were present in clusters and/or showed overt cytologic atypia. FC results were available in 46 of these 59 immunohistochemically positive cases, with 37 (80%) of 46 demonstrating CD56+ PCs by FC (average, 1.8% PCs by aspirate morphologic examination; range, 0.02%-38%). FC results were available in 13 of 15 positive cases without CD56 (strong+) PCs on immunohistochemical analysis, showing 2 cases with a CD56+ PC subset. The prior CD56 status was known in 8 of the 15 negative immunohistochemical cases, with 3 of 8 previously having CD56+ PCs identified on the diagnostic marrow or positive follow-up examinations.

Of the 53 negative BM specimens, 3 (6%) showed CD56 (strong+) PCs (Table 1; Figure 1). In all 3 cases, PCs lacked CD56 expression by FC and were polytypic by FC and immunohistochemical analysis. The aspirate smears demonstrated 0%, 0.2%, and 3% PCs in these cases. In 2 of the 3 cases, clusters of CD56 (strong+), cytologically atypical PCs were identified Image 2A and Image 2B. One of these cases had no biochemical evidence of disease and was, therefore, clinically considered in sCR at the time of BM examination, while the other case had a normalized free light chain ratio and a small free κ band on urine immunofixation and was, therefore, clinically in VGPR (Figure 1). In the third case, immunohistochemical analysis highlighted rare, scattered CD56 (strong+) PCs that were enlarged and had dispersed chromatin and prominent nucleoli Image 2C. This patient had a greater than 90% reduction in M protein on serum protein electrophoresis, which represented a VGPR (Figure 1).

The prior CD56 status was known in only 30 of 53 cases negative for residual disease cases, with 24 of 30 cases having

**Table 1**

<table>
<thead>
<tr>
<th></th>
<th>Positive (n = 74)</th>
<th>Negative (n = 53)</th>
</tr>
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<tbody>
<tr>
<td>Mean (range) PCs (%)</td>
<td>17.9 (0-100)</td>
<td>0.70 (0-3)</td>
</tr>
<tr>
<td>Median PCs (%)</td>
<td>7</td>
<td>0.90</td>
</tr>
<tr>
<td>No. (%) of cases with serum, urine, or cytogenetic evidence</td>
<td>63 (85)</td>
<td>21 (40)</td>
</tr>
<tr>
<td>No. (%) of cases with CD56 (strong+) PCs by immunohistochemical analysis</td>
<td>59 (80)</td>
<td>3 (6)</td>
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PCs, plasma cells.
previously documented CD56+ PCs by FC in diagnostic marrow specimens or positive follow-up examinations.

Discussion

Based on the fact that the majority of PCMs aberrantly express CD56, a number of studies have demonstrated the diagnostic usefulness of CD56 in discriminating between PCM and reactive plasmacytoses of the BM (majority are CD56–) and B-cell lymphomas with plasmacytic differentiation (majority are CD56–). Several studies addressing the discriminatory power of CD56 in differentiating PCMs from MGUS cases have been performed; however, results are contradictory, with some studies identifying few to no cases of MGUS with CD56+ PCs and others identifying CD56 positivity in the majority of these PC dyscrasias. In contrast, the literature contains very few data specifically addressing the utility of CD56 immunohistochemical analysis as a tool for monitoring residual disease in PCM, despite the published role of CD56 in measuring minimal residual disease by FC. We therefore sought to investigate CD56 expression by immunohistochemical analysis in follow-up myeloma BM specimens.

We used negative lymphoma staging BM specimens as control samples, one quarter of which had mild plasmacytoses (2%-4% PCs). Interestingly, approximately half of these control samples demonstrated weak CD56 staining (less intense than osteoblasts) in a small subset of PCs, which were scattered singly or in pairs. Although Ely and Knowles and Van Camp et al reported weak CD56 expression in few PCs in small numbers of BM specimens with polyclonal plasmacytoses and essentially normal BM specimens, respectively, ours is the first study to detail the

**Image 2** Strong CD56+ plasma cells in clusters (**A**, ×1,000; **B**, ×1,000) and singly (**C**, ×1,000) in the 3 negative myeloma follow-up bone marrow specimens.
distribution of this PC subset. Awareness of this subset in normal BM specimens and specimens containing reactive plasmacytoses will lessen the risk of misinterpretation.

Interpretation of CD56 staining in the BM is also dependent on recognition of other cells that express CD56, namely NK cells and a subset of T cells. In our study, this observation was especially important because we were assessing follow-up cases that may have low-level involvement. NK cells and CD56+ T cells are usually LGLs at the morphologic level. While LGLs may share the feature of an eccentric nucleus with PCs, their chromatin pattern is usually quite distinct, having a dense, more even, and uniform quality. This contrasts with PCs, which have a distinctly, irregularly condensed chromatin pattern. In addition, LGLs generally have a higher nuclear/cytoplasmic ratio in tissue sections than do PCs, and the combination of these features will enable ready distinction from PCs. Again, recognition of LGLs in the BM lessens the risk of potential false-positive interpretation.

Consistent with the literature for diagnostic marrow specimens, we observed strong CD56 expression by immunohistochemical analysis in 80% of our positive follow-up cohort. In all, CD56+ PCs were present in clusters (defined as >2 PCs) and/or possessed overt cytologic atypia. The majority of cases that had corresponding FC also demonstrated CD56 positivity by this method. In a minority of cases, CD56+ PCs were detected by immunohistochemical analysis and not by FC. This phenomenon may have resulted from hemodilution or loss of PCs during FC processing, the latter being a well-recognized consequence of specimen processing.\(^\text{18}\)

Our results suggest that strong CD56 expression on clusters of PCs and/or on overtly atypical PCs represents evidence of residual PCM. Of 53 follow-up BM specimens that were negative for residual PCM by morphologic and immunophenotypic criteria, 3 contained small numbers of cytologically atypical PCs that were highlighted by their strong expression of CD56. By the IMWG criteria for response categories, 1 of these cases was considered an example of sCR. This response category is defined by having negative biochemical studies and an absence of clonal PCs by immunohistochemical analysis or FC, with clonality defined as an abnormal light chain ratio by those methods. In our case, the cluster of CD56 (strong+) PCs was interpreted as evidence of clonality and, therefore, low-level residual disease; however, as CD56 positivity is not a criterion for response categorization, this case would still be clinically categorized as sCR instead of, perhaps more appropriately, CR. Given this example, it is reasonable to consider whether CD56 immunohistochemical analysis should be incorporated into the response criteria for sCR. The other 2 cases that were negative for residual PCM by conventional criteria but contained atypical CD56 (strong+) PCs were clinically categorized as VGPRs. While this category is not dependent on bone marrow findings but rather M protein levels, CD56 positivity in these 2 cases represented crucial evidence of residual disease in the marrow. These 3 cases also illustrate that light chain assessment is insensitive in marrow specimens with small numbers of residual neoplastic PCs in a background of normal polytypic PCs, similar, in our opinion, to CD138 immunohistochemical analysis. CD56 immunohistochemical analysis, by highlighting a distinct immunophenotypic aberrancy in neoplastic PCs, appears capable of detecting small numbers of residual myeloma cells when light chain restriction is not demonstrable, thus serving as a useful way of monitoring disease status.

The applicability of CD56 immunohistochemical analysis to PCF follow-up is limited by the fact that PCs do not uniformly express CD56. It is expected that 20% to 30% of the PCs in our cohort would be CD56–; therefore, there would seem to be no role for residual disease monitoring by CD56 immunohistochemical analysis in approximately one quarter of our cases. However, we did not know the CD56 status at diagnosis or in positive follow-ups in slightly fewer than half of our cases that were negative for residual disease, so we cannot confirm this prediction. Furthermore, Cao et al\(^\text{19}\) recently reported instability of the immunophenotype of myeloma cells, describing examples in which CD56 expression by FC was both gained and lost following therapy, suggesting that CD56 immunohistochemical analysis in disease monitoring may still be applicable in some PCMs lacking CD56+ PCs at diagnosis.

Recently, Joshi et al\(^\text{20}\) described the superiority of BM core biopsies over aspirates for the evaluation of residual disease in their cohort of 106 myeloma follow-ups, largely because of the added benefit of immunohistochemical analysis (\(\kappa, \lambda, \text{CD56},\) and cyclin D1) for core biopsies. The authors used aberrant CD56 expression as a criterion for diagnosing residual disease, which contrasts with our study in which we interpreted follow-up BM specimens independent of CD56 immunohistochemical analysis. It is unclear from the data presented whether CD56 reactivity was ever the sole criterion for a diagnosis of residual disease, as would be the case in the 3 BM samples in our study. In another follow-up PCM study using CD56 immunohistochemical analysis, Chang et al\(^\text{21}\) correlated CD56 status with prognosis in a cohort of patients treated with high-dose melphalan-based therapy and autologous stem cell transplantation. In this study, CD56 positivity at follow-up was defined when more than 50% of the CD138+ cells were CD56-reactive, differing from our study, which used strength of staining pattern and distribution as evidence of positivity. To the best of our knowledge, our study represents the first addressing the utility of CD56 immunohistochemical analysis in disease monitoring.

CD56 immunohistochemical analysis may have a role in the evaluation of residual disease in PCM, particularly in assessing low-level involvement. We identified 3 cases in
which CD56+ PCs were clearly identified in otherwise negative follow-up BM specimens (normal morphologic features; polytypic/equivocal light chain expression). CD56 positivity by immunohistochemical analysis in follow-up cases is suggestive of clonality, and, therefore, its incorporation into PCM response criteria should be considered.

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