von Willebrand Factor Is the Most Reliable Immunohistochemical Marker for Megakaryocytes of Myelodysplastic Syndrome and Chronic Myeloproliferative Disorders

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Key Words: Bone marrow; Chronic myeloproliferative disorder; Diagnosis; von Willebrand factor (factor VIII–related antigen); Immunohistochemistry; Myelodysplastic syndrome

Abs t r a c t

To find the best immunohistochemical marker for megakaryocytes in normal marrow, myelodysplastic syndrome (MDS), and chronic myeloproliferative disorders (CMPD), 57 marrow biopsy specimens were studied semiquantitatively with immunohistochemical methods using a panel of 7 antibodies. The staining intensity was graded 0 to 3 for scoring 100 consecutive megakaryocytes in each stained section. The final score for each stain was the sum of these 100 megakaryocytes individually multiplied by their corresponding grade. In normal marrow (11 cases), the average scores for anti-von Willebrand factor (vWF) and Ulex europaeus agglutinin-1 (UEA-1) were 177.1 and 195.1, respectively. The scores for the other 5 markers, including anti–platelet-derived growth factor-BB, 2 anti–transforming growth factor-beta 3, anti-CD61, and anti-CD79a ranged from 96.1 to 124.1. In MDS (27 cases), the scores were 200.8 (vWF), 152.6 (UEA-1), and 28.7 to 98.5 (others). In CMPD (19 cases), the scores were 220.5 (vWF), 179.2 (UEA-1), and 64.8 to 101.2 (others). These results show that vWF and UEA-1 are good immunohistochemical markers for megakaryocytes in normal marrow, and vWF is the best marker in MDS and CMPD. For routine practice, vWF is the most reliable marker for identifying atypical megakaryocytes, especially in the cases of 5q– syndrome and agnogenic myeloid metaplasia.

Normal megakaryocytes are easy to identify because of their multilobulated nuclei and large amount of cytoplasm. However, in various hematologic disorders, abnormal megakaryocytes may be difficult to identify because of their atypical morphologic features.

The myelodysplastic syndromes (MDSs) are a group of clonal hematopoietic neoplasms with ineffective trilineage hematopoiesis that results in peripheral cytopenia and, in about one third of cases, progression to acute leukemia. They may occur as de novo diseases or as secondary or therapy-related disorders. In marrow biopsy sections from patients with MDS, morphologic abnormalities of the dysplastic megakaryocytes are most pronounced and easily detectable. Some dysplastic megakaryocytes, especially in the patients with chromosome 5q deletion,¹ are monolobulated micromegakaryocytes (“dwarf forms”) with centrally located round nuclei; thus, they simulate histiocytes, erythroblasts, or myeloid precursors. They are not identified easily in routine H&E-stained sections.

The chronic myeloproliferative disorders (CMPDs) are a group of clonal hematopoietic neoplasms with uncontrolled proliferation of 1 or more myeloid cell lines. There are 4 well-recognized entities: chronic myeloid leukemia (CML), polycythemia vera, essential thrombocythemia, and agnogenic myeloid metaplasia (AMM) (or chronic idiopathic myelofibrosis). In AMM, the number of atypical megakaryocytes is increased, with abnormal clustering. They are particularly prominent in areas with marked myelofibrosis, and morphologic identification is difficult, especially when accompanied by crush artifact.

The results of bone marrow trephine biopsy rarely are used alone to make the initial diagnosis of a hematologic disorder. However, in certain situations, smears of peripheral
blood or marrow aspirate are inadequate or not available for study, for example, a dry tap because of bone marrow fibrosis, hypocellular marrow aspirate, technical problems, or the unexpected finding of acute leukemia. Thus, pathologists may need to rely on paraffin blocks of bone marrow specimens to diagnose, and even to classify, hematologic disorders.

In a previous study, Chuang and Li\(^2\) showed that von Willebrand factor (vWF; factor VIII–related antigen) is a relatively reliable marker for identifying megakaryoblasts in acute megakaryoblastic leukemia (M7 in the French-American-British classification). The aim of the present study was to evaluate the usefulness of various megakaryocytic markers in identifying atypical megakaryocytes in MDS and CMPD. If a reliable marker can be found for megakaryocytes, it will be of practical value for hematopathologists.

**Materials and Methods**

Fifty-seven bone marrow biopsy specimens were retrieved from the files of Mayo Clinic Rochester, Rochester, MN. Eleven specimens were normal marrow from patients with negative lymphoma staging. Twenty-seven specimens were from cases of MDS, including 5 refractory anemia, 3 refractory anemia with ringed sideroblasts, 3 refractory anemia with excess of blasts, 2 refractory anemia with excess of blasts in transformation, 4 unclassified MDS, 3 therapy-related MDS, and 7 5q– syndrome. Nineteen specimens were from cases of CMPD, including 1 5q– atypical CMPD, 4 essential thrombocythemia, 4 AMM, 3 CML-chronic phase, 2 CML-accelerated phase, 1 CML-blastic transformation, and 4 unspecified CMPD.

Bone marrow biopsy specimens were fixed in B-5 for 2 hours, transferred to neutral buffered formalin, and processed routinely, beginning with decalcification in a formic acid solution for 1.5 hours.

The paraffin sections were cut at a thickness of 4 µm, placed on silanized glass slides, and air dried in an oven at 60 C for 60 minutes. After deparaffinization, removal of mercury, and rehydration, endogenous peroxidase activity was blocked with 3% hydrogen peroxide and methanol. Immunohistochemical staining was performed with the horseradish peroxidase–labeled streptavidin-biotin method using the antibodies listed in Table 1, except for *Ulex europaeus* agglutinin-1 (UEA-1) and anti-CD79a (HM57).

**Table 1**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>Pretreatment</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>von Willebrand factor</td>
<td>1/750</td>
<td>Autoprotease 2*</td>
<td>Dako, Carpinteria, CA</td>
</tr>
<tr>
<td><em>Ulex europaeus</em> agglutinin-1</td>
<td>1/200</td>
<td>None</td>
<td>Dako</td>
</tr>
<tr>
<td>Y2/5 (CD61)</td>
<td>1/10</td>
<td>Autoprotease 2*</td>
<td>Dako</td>
</tr>
<tr>
<td>Transforming growth factor-beta 3</td>
<td>1/100</td>
<td>None</td>
<td>Oncogene Science, Uniondale, NY</td>
</tr>
<tr>
<td></td>
<td>1/100</td>
<td>None</td>
<td>Research Diagnostics, Flanders, NJ</td>
</tr>
<tr>
<td>Platelet-derived growth factor-BB</td>
<td>1/150</td>
<td>None</td>
<td>Genzyme, Cambridge, MA</td>
</tr>
<tr>
<td>HM57 (CD79a)</td>
<td>1/400</td>
<td>None</td>
<td>Dako Catalyzed Signal Amplification System, Carpinteria, CA</td>
</tr>
</tbody>
</table>

\(^*\)Autoprotease 2, Ventana Medical System, Tucson, AZ.

Staining for anti-CD79a (HM57) was done manually. The Dako Catalyzed Signal Amplification System (Dako) was used for immunostaining with anti-CD79a (HM57).\(^3\) The only modification we made was the 40% dilution of the amplification agent (biotinyl tyramine) with a 0.05-mol/L concentration of tris(hydroxymethyl)aminomethane buffer (pH 8.0), 0.1% polysorbate (Tween) 20, and 0.03% hydrogen peroxide. After deparaffinization and blocking of endogenous peroxidase activity, the sections were treated sequentially with a protein block solution for 5 minutes, primary antibody for 20 minutes, link antibody solution for 15 minutes, streptavidin-biotin complex for 15 minutes, 40% diluted amplification reagent for 15 minutes, streptavidin-peroxidase for 15 minutes, and 3,3’-diaminobenzidine tetrahydrochloride solution for 5 minutes, after which the sections were counterstained with hematoxylin for 5 minutes. After gradual dehydration in absolute ethanol, the sections were immersed in xylene, and coverglasses were applied with a synthetic xylene-based mounting medium.

Staining with the other monoclonal antibodies was performed in the Ventana ES 320 Autostainer (Ventana Medical System, Tucson, AZ) at 37 C. After the endogenous
peroxidase activity was blocked, the sections were treated with protease solution (Autoprotease 2, Ventana Medical System) in the autostainer or with a 10-mmol/L concentration of citrate buffer for 7 minutes or 0.4% pepsin in 0.01N hydrochloric acid for 48 seconds by microwave treatment or not treated at all, as listed in Table 1. In the autostainer, the primary antibodies were incubated for 30 minutes and the biotinylated secondary antibodies and streptavidin–horseradish peroxidase for 8 minutes each. 3-Amino-9-ethylcarbazole and hydrogen peroxide solution were applied simultaneously for 8 minutes. The slides then were counterstained with hematoxylin, and coverglasses were applied with an aqueous mounting medium.

For each stained section, the staining intensity of 100 consecutive megakaryocytes was graded as 0, 1, 2, or 3. The final score for each staining was the sum of these 100 megakaryocytes individually graded.

Results

In the 11 specimens of normal marrow, megakaryocytes showed strong and intense cytoplasmic staining with vWF and UEA-1, and cells of the other lineages except endothelial cells stained negatively. The majority of the megakaryocytes were graded as 2 or 3 Image 1A. The average scores were 177.1 and 195.1, respectively Table 2. The staining with anti-CD61 was much weaker than with vWF and UEA-1, with the majority of megakaryocytes being grade 1 and occasional cells being grade 2 Image 1B. The staining with the 2 monoclonal antibodies against transforming growth factor (TGF)-beta 3 revealed weak cytoplasmic staining of most megakaryocytes and was graded as 1. Granulocytes also were stained. The staining with anti–platelet-derived growth factor (PDGF)-BB showed grade 1 weak cytoplasmic staining in almost every megakaryocyte. The cells of the other lineages stained negatively. With anti-CD79a, megakaryocytes showed more heterogeneous staining, and the grading ranged from 0 to 2. Small lymphocytes stained intensely.

In the 27 specimens of MDS, the megakaryocytes were intensely positive for vWF except for 5 specimens, which had lower scores of 107, 129, 103, 124, and 124. The specimens with scores of 107 and 129 were cases of refractory anemia, and the other 3 were cases of 5q– syndrome. The average score for these 27 cases of MDS was even higher than that for normal marrow (200.8 vs 177.1). Staining results with UEA-1 were highly variable, with scores ranging from 13 to 258 (average, 152.6). In the majority of the cases, staining was weak with anti-CD61. The results of staining with 2 anti-TGF-beta 3 monoclonal antibodies and anti-PDGF-BB were similar to the results obtained in normal marrow. Compared with normal marrow, the megakaryocytes in the MDS group stained more weakly and more heterogeneously with anti-CD79a; however, small lymphocytes stained intensely.

In the 19 CMPD cases, the megakaryocytes stained intensely with vWF in every case, with scores ranging from 193 to 256 (average, 220.5, which was the highest average score in the present study) Image 2A. In the 12 CMPD cases, the results of staining with UEA-1 were similar to those of vWF. However, the other 7 CMPD cases had scores
ranging from 59 to 114 (1 AMM, 1 CML-chronic phase, 2 CML-accelerated phase, 1 CML-blastic transformation, and 2 unspecified CMPD). In 1 case, staining with anti-CD61 was negative, and it was very weak in the other 8 cases (scores, 3 to 25) Image 2B. The average score for the whole group was 64.8. Staining results for the 2 anti-TGF-beta 3 monoclonal antibodies, anti-PDGF-BB, and anti-CD79a were similar for all the biopsy specimens of the normal, MDS, and CMPD groups.

For testing the interobserver variation of this study, a second pathologist (Y.-C.Y.) rescored the 3 most important immunostains (anti-vWF, UEA-1, and CD61) on 28 sequential cases included in the present study (8 normal, 9 MDS, and 11 CMPD) using the same method as the initial scoring pathologist (S.-S.C.). The results for each of the 3 immunostains from the 2 pathologists were analyzed with the Pearson correlation method for interobserver reliability. The r values for vWF, UEA-1, and CD61 were 0.714, 0.936, and 0.956, respectively (P < .01), indicating fairly good interobserver reproducibility.

### Table 2

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Normal (n = 11)</th>
<th>MDS (n = 27)</th>
<th>CMPD (n = 19)</th>
<th>Total (n = 57)</th>
</tr>
</thead>
<tbody>
<tr>
<td>von Willebrand factor</td>
<td>177.1</td>
<td>200.8</td>
<td>220.5</td>
<td>202.8</td>
</tr>
<tr>
<td>Ulex europaeus agglutinin-1</td>
<td>195.1</td>
<td>152.6</td>
<td>179.2</td>
<td>169.6</td>
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<tr>
<td>Y2/5 (CD61)</td>
<td>124.0</td>
<td>28.7</td>
<td>64.8</td>
<td>59.1</td>
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<tr>
<td>Transforming growth factor-beta 3</td>
<td>102.5</td>
<td>95.4</td>
<td>95.2</td>
<td>96.7</td>
</tr>
<tr>
<td>Platelet-derived growth factor-BB</td>
<td>96.1</td>
<td>89.8</td>
<td>91.7</td>
<td>91.6</td>
</tr>
<tr>
<td>HM57 (CD79a)</td>
<td>100.8</td>
<td>98.5</td>
<td>101.2</td>
<td>99.8</td>
</tr>
</tbody>
</table>

CMPD, chronic myeloproliferative disorder; MDS, myelodysplastic syndrome.

*Results are given as the average score of all cases in each category.

### Discussion

Immunohistochemical staining for vWF using rabbit polyclonal anti-vWF antibody is a good method for detecting normal megakaryocytes, immature and atypical megakaryocytes in various myeloproliferative disorders, and megakaryoblasts in M7. Monoclonal antibody Y2/5 against platelet glycoprotein IIia (CD61) also has been applied to bone marrow biopsy specimens to detect normal megakaryocytes and abnormal megakaryocytes in MDS and CMPD. Fox et al found considerable dysmegakaryocytopoiesis and an increased proportion of CD61-positive micromegakaryocytes with immunohistochemical methods. In a previous study, anti-vWF and anti-CD61 (Y/5) had similar cell-lineage specificity for detecting leukemic megakaryoblasts. However, the proportion of positive-staining cells and the intensity of staining were much higher with anti-vWF than with anti-CD61 (Y2/5). In the present study, anti-vWF was much more sensitive than anti-CD61 for detecting megakaryocytes, especially in cases of MDS and CMPD.

UEA-1, a lectin from gorse seed of *Ulex europaeus*, has a selective binding affinity with vascular endothelial cells and megakaryocytes. In our previous study, we showed that (1) the heterogeneity in the staining pattern of megakaryocytes by UEA-1 reflected different stages of megakaryocytic differentiation and (2) UEA-1 staining is a practical method for studying megakaryocytopoiesis in routinely processed marrow biopsy specimens. In the present study, we found that UEA-1 staining is most sensitive for detecting megakaryocytes in normal marrow and is second only to anti-vWF in cases of MDS and CMPD.

TGF-beta belongs to a superfamily of structurally related regulatory proteins and has 3 mammalian isoforms: TGF-beta 1, TGF-beta 2, and TGF-beta 3. TGF-beta 3 is a potent inhibitory cytokine that inhibits the stimulatory cytokine-induced growth of hematopoietic progenitor cells. The 3 TGF-beta isoforms may have a lineage-specific inhibitory feedback role in hematopoietic regulation. In the previous study, positive staining results using anti-TGF-beta 3 monoclonal antibodies from 2 different companies (Oncogene Science, Uniondale, NY and Research Diagnostics, Flanders, NJ) indicate its lineage specificity for megakaryocytes and granulocytes. In the present study, the staining pattern and low intensity were the same in all the cases of normal marrow, MDS, and CMPD, using both anti-TGF-beta 3 monoclonal antibodies.

As is well known, PDGF and TGF-beta are present in the alpha granules of human megakaryocytes and platelets and are probably the cytokines important in the pathogenesis of myelofibrosis in CMPD. In the present study, anti-PDGF-BB staining was restricted to megakaryocytes, thus confirming its distribution in the marrow hematopoietic cells. The staining intensity with this monoclonal antibody was too low for it to be used routinely as a megakaryocytic marker.

The CD79 molecule consists of 2 polypeptide chains (CD79a and CD79b) and is associated with a B-cell membrane immunoglobulin for signal transduction after antigen binding. It has been considered the B-cell equivalent of CD3 on T cells. Monoclonal antibody against CD79a reportedly reacts with normal and neoplastic B cells in paraffin sections, including the majority of B-cell neoplasms and acute lymphoblastic leukemia of precursor B-cell type. A previous immunohistochemical study of acute leukemia demonstrated immunoreactivity of megakaryocytes and B-lineage acute lymphoblastic leukemia by using anti-CD79a. That phenomenon was the reason for including anti-CD79a in the present study. Again, we found that megakaryocytes along with small B lymphocytes could be highlighted by anti-CD79a. However, the staining intensity with this monoclonal antibody was too variable for its practical use as a megakaryocytic marker.

Our results demonstrate that vWF and UEA-1 are good immunohistochemical markers for megakaryocytes in normal marrow, and vWF is the better of the 2 in MDS and CMPD. For routine practice, we recommend vWF as the marker to identify normal and atypical megakaryocytes, especially in the cases of 5q– syndrome and AMM.

References


