

Review Article

Histological evaluation of myeloproliferative neoplasms

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In 2017, the revised World Health Organization was published. Regarding myeloproliferative neoplasms, histological findings of bone marrow biopsy is becoming more important for diagnosis. This article highlights particularly the morphology of megakaryocytes and evaluation of myelofibrosis for pathological diagnosis, and immunohistochemistry which can detect somatic mutation.

Keywords: myeloproliferative neoplasms; megakaryocyte; myelofibrosis

INTRODUCTION

Myeloproliferative neoplasms (MPNs) are clonal hematopoietic stem cell disorders characterized by the proliferation of cells of one or more of the myeloid lineages i.e. granulocytic, erythroid, and megakaryocytic. In the fourth edition of the World Health Organization (WHO) classification, MPNs were classified as follows: *BCR-ABL1*-positive chronic myelogenous leukemia, chronic neutrophilic leukemia, polycythemia vera (PV), primary myelofibrosis (PMF), essential thrombocythemia (ET), chronic eosinophilic leukemia, chronic eosinophilic leukemia-not otherwise specified, mastocytosis, or unclassifiable myeloproliferative neoplasm.¹

In May 2016, an outline of the revisions to the fourth WHO classification was announced,² and in 2017, the revised WHO classification was published.³ A major modification regarding MPNs included a description of PMF according to each stage (prefibrotic/early stage and overt fibrotic stage). Furthermore, mastocytosis was listed as an independent disease category.

Moreover, regarding PV, PMF, and ET, which are *BCR-ABL1*-negative MPNs, greater emphasis was placed on bone marrow biopsy for making a diagnosis and predicting the prognosis in the present revision. Histological characteristics broadly include the proliferation of cells of one or more myeloid lineage, and varying degrees of age-matched hypercellularity of the bone marrow. Megakaryocyte morphology is particularly characteristic, with characteristic malformation and maldistribution observed in ET and PMF. In addition, myelofibrosis (MF) is often seen, and in such instances, a dry tap occurs during the smear examination. Therefore, histological evaluation using biopsy specimens is indispensable. MF is generally a factor of poor prognosis. Therefore, the

WHO classification recommends noting the level of fibrosis (grading). Furthermore, the differentiation of other hematopoietic diseases that cause MF can be a problem. In the present revision, the evaluation methods for reticulin and collagen fibrosis underwent minor revisions.³

Moreover, for the pathological diagnosis of MPNs, in addition to HE-stained samples, the grading of reticulin and collagen fibrosis is evaluated using silver impregnation, such as using Gömöri staining, for MF.^{4,5} However, to verify the increase in the number of collagen fibers, additional trichrome staining is recommended. To evaluate abnormalities in the distribution and level of maturity of hematopoietic cells, immunostaining with lineage-specific markers is performed. In recent years, immunohistochemical methods capable of detecting some chromosomal and genetic abnormalities of hematopoietic tumors have been used. Such methods will also be presented in this study.

MORPHOLOGICAL ABNORMALITIES AND MALDISTRIBUTION OF MEGAKARYOCYTES

In the diagnosis of ET and PMF, the morphology and distribution of megakaryocytes among hematopoietic cells play a particularly important role in pathological diagnosis.

Characteristic morphology of megakaryocytes for ET and prefibrotic PMF

The description by Koopmans *et al.* is useful for performing histological evaluations,⁶ although the current WHO classification also describes cluster formation, size, and nuclear lobulation of megakaryocytes, as well as characteristic morphological features.³ In normal bone marrow, intertrabecular megakaryocytes are found in an isolated manner. However,

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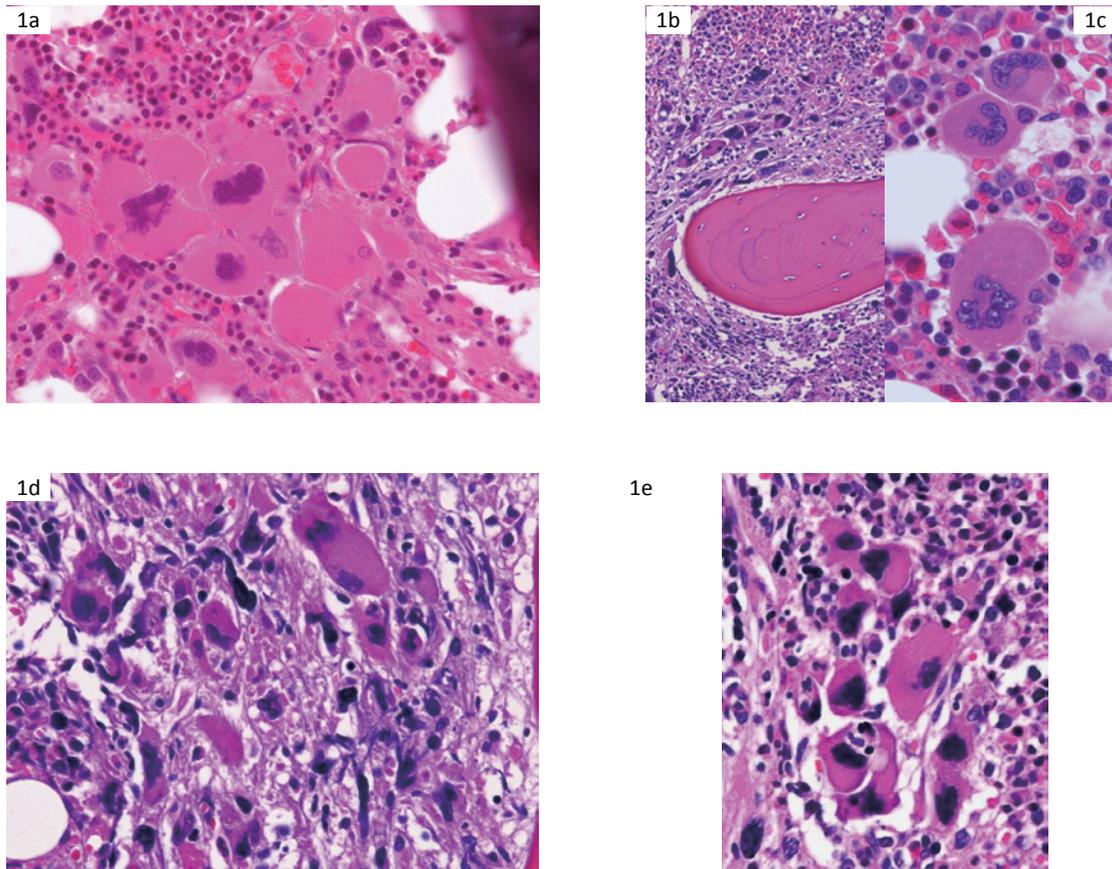


Fig. 1. Nuclear morphology of megakaryocytes in ET and PMF. *a.* Megakaryocytes forming tight clusters (PMF patient). *b.* Megakaryocytes are distributed around the trabeculae (PMF patient). *c.* Staghorn-like nuclei (ET patient). *d.* Megakaryocytes exhibiting dysplastic morphology (PMF patient). *e.* Megakaryocyte with darkly stained nuclear chromatin (PMF patient)

in MPNs, megakaryocytes exhibit an uneven distribution, whereas PMF and ET, in particular, are characterized by megakaryocyte clustering. In PMF, a dense arrangement is observed (referred to as “tight clusters”); distribution adjacent to the trabeculae (“paratrabeular”) and sinuses in the bone marrow are characteristic (Figs. 1a, 1b). On the other hand, in ET, a sparse arrangement is often observed, wherein hematopoietic cells are intermixed. Furthermore, the morphology of nuclei is characterized by large megakaryocytes with lobulated nuclei (“staghorn-like”) in ET (Fig. 1c), whereas in prefibrotic/early stage PMF, severe nuclear atypia (“dysplastic”), nuclear hypo-lobulation, abnormal nuclear lobulation, such as cloud-like, chromatin abnormalities, such as hyperchromatic nuclei, abnormal nucleus/cytoplasm ratio, and bare nucleus with pachychromatic chromatin are characteristic, differing from other MPNs (Figs. 1d, 1e).

How to differentiate prePMF from ET?

However, it is difficult to state that these morphological characteristics of megakaryocytes are disease-specific; for example, nuclear hypolobulation and/or megakaryocytes with dysplastic nuclei in the fibrotic stage in ET, and hyper-lobulated nuclei in PMF are sometimes detected, and morphological overlapping is often noted in both. Koopmans and colleagues reported that the frequency of dysmorphic nuclei,

dysmorphic megakaryocytes, or dense clustering was lower (<25%) in ET cases compared with in prePMF, and noted that these morphological findings are more important,⁶ whereas the WHO 2016 states that “no single morphological feature is pathognomonic of a specific subtype”.² Barbui *et al.* conducted a review of 1,104 patients diagnosed with ET and reported that 180 patients (16%) were diagnosed with prefibrotic/early stage PMF.⁷ Thiele *et al.* also found that among 295 patients diagnosed with ET or prefibrotic/early stage PMF, the consistency between two groups of pathologists was 78%.⁸ Thus, to differentiate the two, it is recommended that the diagnosis is made in a comprehensive manner, combining the morphological features of megakaryocytes with histological findings such as cell density and fibrosis. Recently, it has also been reported that PMF can be predicted from the hemoglobin concentration, the white blood cell count, and the lactate dehydrogenase value in the peripheral blood in cases where an ET-like clinical course has been followed.^{9,10}

MYELOFIBROSIS

MF develops from several causes, including inflammatory disease, metabolic disease, and neoplastic disorders.¹¹ It is broadly divided into hyperplasia with only increased reticulin

Table 1. Semiquantitative bone marrow fibrosis (MF) grading system proposed by Thiele J et al, with minor modifications concerning collagen and osteosclerosis

Grade	Definition
MF-0	Scattered linear reticulin with no intersections (cross-overs), corresponding to normal bone marrow
MF-1	Loose network of reticulin with many intersections, especially in perivascular areas
MF-2	Diffuse and dense increase in reticulin with extensive intersections, occasionally with focal bundles of thick fibers mostly consistent with collagen and/or associated with focal osteosclerosis
MF-3	Diffuse and dense increase in reticulin with extensive intersections and course bundles of thick fibers consistent with collagen, usually associated with osteosclerosis

Table 2. Diagnostic criteria for PV

Major criteria
1. Elevated hemoglobin concentration (> 16.5 g/dL in men; > 16.0 g/dL in women) or elevated hematocrit (>49 % in men; >48% in women) or increased red blood mass (> 25% above mean normal predicted value)
2. Bone marrow biopsy showing age-adjusted hypercellularity with trilineage growth (panmyelosis), including prominent erythroid. Granulocytic, and megakaryocytic proliferation with pleomorphic, mature megakaryocytes (differences in size)
3. Presence of <i>JAK2</i> V617F or <i>JAK2</i> exon 12 mutation
Minor criteria
Subnormal serum erythropoietin level

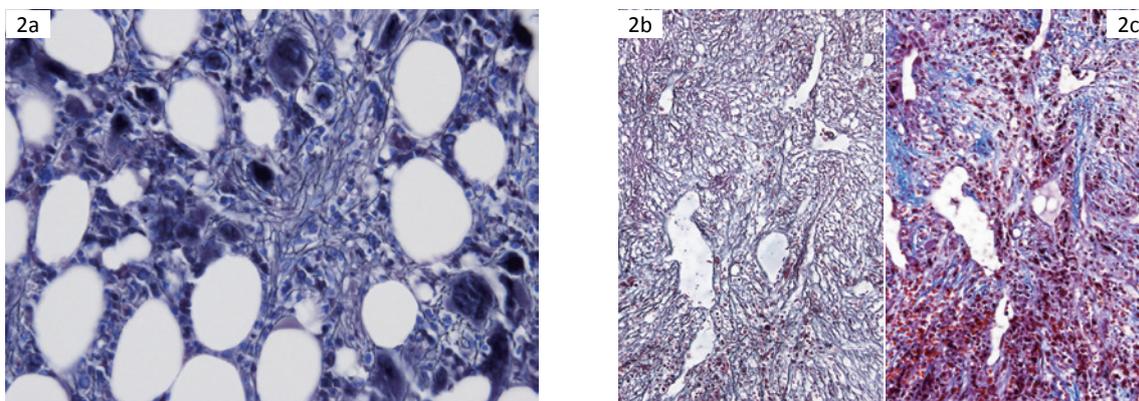


Fig. 2. Evaluation of myelofibrosis. *a.* Silver impregnation (pre-PMF patient). Isolated hyperplasia of reticular fibers can be seen, and crossing-over of the fiber network was confirmed. Equivalent to MF-1. *b.* Silver impregnation (overt PMF patient). Diffuse-dense hyperplasia of reticular fibers is seen, and crossing-over of the fiber network was confirmed. *c.* Masson trichrome staining (overt PMF patient). Blue collagen fiber bundles can be seen.

fibrosis and that with increased reticulin and collagen fibrosis. However, in MF following PMF, PV, or ET (post-PV or post-ET MF), the level of reticulin fibrosis is the main subject; this is followed by an increase in the number of collagen fibers. Furthermore, trabecular thickening and osteosclerosis caused by fibrosis are observed. There are two MF classifications: the European classification⁵ and the classification by Manoharan *et al.*¹² The WHO classification utilizes the former, and in the 2017 revisions, slight changes to the definition of MF-2 and MF-3 were added regarding collagen fibers and osteosclerosis (Table 1).^{2,3} In bone marrow biopsy specimens of patients who develop fibrosis, microscopic examination of HE-stained samples, as well as silver impregnation and trichrome staining are recommended. After silver impregnation, reticulin fibers are stained black and collagen fibers are stained reddish; thus, the two types of fibers can be distinguished (Figs. 2a, 2b). Collagen fibers appear eosinophilic on HE staining and are blue on Masson's trichrome staining (Fig. 2c).

MAJOR MODIFICATIONS TO THE DIAGNOSTIC CRITERIA OF EACH DISEASE TYPE

This section outlines the major modifications to the diagnostic criteria noted in the revised WHO classification for

PV, PMF, and ET (*BCR-ABL1*-negative MPNs). Importance has been placed on histological findings.

Polycythemia vera

Table 2 lists the diagnostic criteria. One modification among the major criteria was a reduction in the hemoglobin (Hb) value. In addition, histological findings, a minor criterion in the fourth edition,¹ were listed under major criteria, which made histological examination by bone marrow biopsy necessary for establishing a diagnosis.^{2,3} Underlying these changes is the necessity of red cell mass (RCM) for the definite diagnosis of PV in the diagnostic criteria to date. However, the number of institutions that can quantify the RCM is limited; therefore, as an alternative, the Hb or hematocrit (Hct) value of peripheral blood has been used. The presence of “masked PV,” whereby thrombocytosis and moderate erythrocytosis develop, and PV progresses despite not satisfying the diagnostic criteria, has been reported.¹³ Recent reports have indicated that elevated Hb and Hct value do not necessarily reflect elevated RCM.^{14,15} On the other hand, the morphological features of bone marrow biopsy specimens were found to reflect the RCM.¹⁴ Therefore, we believe that this has led to a greater emphasis being placed on histological findings.

Regarding histological findings, “panmyelosis” (listed under the major criteria), is defined as “age-adjusted bone

Table 3. Diagnostic criteria for primary myelofibrosis, prefibrotic/early stage

Major criteria
1. Megakaryocytic proliferation and atypia, without reticulin fibrosis grade > 1, accompanied by increased age-adjusted bone marrow cellularity, granulocytic proliferation, and (often) decreased erythropoiesis
2. WHO criteria for <i>BCR-ABL1</i> -positive chronic myeloid leukemia, polycythemia vera, essential thrombocythemia, myelodysplastic syndromes, or other myeloid neoplasms are not met
3. <i>JAK2</i> , <i>CALR</i> , or <i>MPL</i> mutation or presence of another clonal marker or absence of minor reactive bone marrow reticulin fibrosis
Minor criteria
Presence of at least one of the following, confirmed in 2 consecutive determinations:
- Anemia not attributed to a comorbid condition
- Leukocytosis $\geq 11 \times 10^9 /L$
- Palpable splenomegaly
- Lactate dehydrogenase level above the upper limit of the institutional reference range

marrow hypercellularity with trilineage growth” (Table 2). In pre-PMF, it is distinguished from granulocytic hyperplasia and megakaryocytic hyperplasia (Table 3).

Regarding the details of each hematopoietic cell lineage, erythropoiesis usually produces normoblastic cells and erythroid precursors form large erythroblastic islands or sheets. The granulocytic lineage appears morphologically normal. Megakaryocytes are increased in number, particularly in cases with an excess of platelets, and frequently have hypersegmented nuclei. Cases with high platelet counts and low Hb or Hct values may mimic ET at onset. Megakaryocytes tend to form loose clusters or lie close to the bone trabeculae, and often exhibit a significant degree of pleomorphism, resulting in a mixture of sizes. Most megakaryocytes have normally folded or deeply lobed nuclei, and usually lack significant atypia, although a minority may have bulbous nuclei and other nuclear abnormalities, particularly when associated with a minor increase in reticulin.³ These morphological characteristics pose a problem in the differentiation of ET and pre-PMF. For example, in age-adjusted hypercellularity, ET is distinguished by relatively unremarkable hypercellularity. Pre-PMF is also distinguished by the observation of numerous megakaryocytes with marked nuclear lobulation and isolated cluster formation.³ Moreover, when small megakaryocytes appear along with micromegakaryocytes, the possibility of myelodysplastic syndrome (MDS) or MDS/MPNs should be considered.

Primary myelofibrosis, prefibrotic/early stage; overt fibrotic stage

Tables 3 and 4 present the diagnostic criteria in the revised WHO classification. There was a modification of the major criteria regarding the pre-fibrotic/early stage. In addition to megakaryocytic proliferation and atypia, “without reticulin fibrosis grade >1, accompanied by increased age-adjusted bone marrow cellularity, granulocytic proliferation, and often decreased erythropoiesis” was added. On the other hand, MF in the overt fibrotic stage was described in

Table 4. Diagnostic criteria for primary myelofibrosis, overt fibrotic stage

Major criteria
1. Megakaryocytic proliferation and atypia, accompanied by reticulin and/or collagen fibrosis grades 2 or 3
2. WHO criteria for <i>BCR-ABL1</i> -positive chronic myeloid leukemia, polycythemia vera, essential thrombocythemia, myelodysplastic syndromes, or other myeloid neoplasms are not met
3. <i>JAK2</i> , <i>CALR</i> , or <i>MPL</i> mutation or presence of another clonal marker or absence of reactive myelofibrosis
Minor criteria
Presence of at least one of the following, confirmed in 2 consecutive determinations:
- Anemia not attributed to a comorbid condition
- Leukocytosis $\geq 11 \times 10^9 /L$
- Palpable splenomegaly
- Lactate dehydrogenase level above the upper limit of the institutional reference range
- Leukoerythroblastosis

Table 5. Diagnostic criteria for essential thrombocythemia

Major criteria
1. Platelet count $\geq 450 \times 10^9 /L$
2. Bone marrow biopsy showing proliferation mainly of the megakaryocytic lineage, with increased numbers of enlarged, mature megakaryocytes with hyperlobulated nuclei; no significant increase or left shift in neutrophil granulopoiesis or erythropoiesis; very rarely a minor (grade 1 ^a increase in reticulin fibers)
3. WHO criteria for <i>BCR-ABL1</i> -positive chronic myeloid leukemia, polycythemia vera, primary myelofibrosis, or other myeloid neoplasms are not met
Minor criterion
Presence of a clonal marker or absence of evidence of reactive thrombocytosis

greater detail, including “accompanied by reticulin and/or collagen fibrosis grades 2 or 3.” Regarding somatic mutations, *JAK2*, *CALR*, or *MPL* mutation was added to the pre-fibrotic/early stage and overt fibrotic stage. In addition, leukocytosis in peripheral blood was added to the minor criteria.

The degree of fibrosis in PMF correlates with the state of disease progression such as decreased peripheral blood white blood cell count, Hb level, platelet count, and serum lactate dehydrogenase value, as well as splenomegaly. The prognosis differs according to each grade; therefore, evaluation of fibrosis is important.^{1,3}

Essential thrombocythemia

Table 5 presents the diagnostic criteria in the revised WHO classification. In addition, “very rarely, a minor (grade 1) increase in reticulin fibers” was added to the major criteria. Regarding somatic mutations, in addition to the *JAK2* mutation, *CALR* and *MPL* mutations were added.

IMMUNOHISTOCHEMISTRY

In ET and PMF patients without *JAK2* or *MPL* mutations, a somatic mutation of *CALRETICULIN* (*CALR*) has been recently discovered.^{16,17} Tefferi *et al.* proposed that the

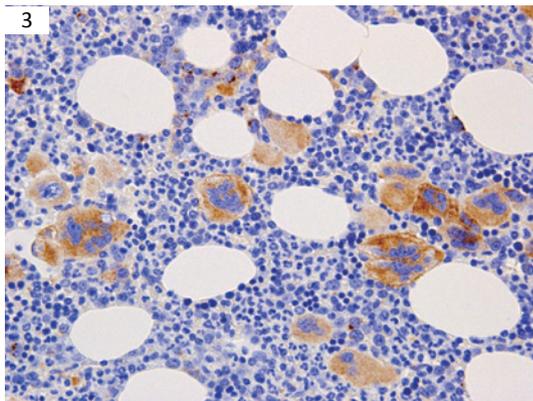


Fig. 3. Immunostaining by CAL2 monoclonal antibodies (ET patient). The megakaryocytic cytoplasm is positive, and the *CALR* mutation was identified upon mutation search in this patient.

CALR mutation should be added to the WHO diagnostic criteria at the time of the revision.¹⁸ In the revised WHO classification, it has been added to the diagnostic criteria for both ET and PMF.^{2,3} Recently, Stein H *et al.* prepared monoclonal antibodies that recognize mutated *CALR*, and on immunostaining, positive findings were obtained for the cytoplasm of megakaryocytes in bone marrow biopsy specimens of patients with MPN-NOS, ET, and PMF. Using the molecular biology assay with Sanger sequencing, 100% specificity was confirmed.¹⁹ Considering that several similar reports have been published,^{20,21} immunostaining by CAL2 monoclonal antibodies is a promising and simple method to screen for mutations. At our institution, when *BCR-ABL1*-negative MPNs are suspected, in addition to trilineage hematopoietic cell markers (erythroid lineage: CD71; granulocytic lineage: myeloperoxidase, megakaryocytic lineage: CD42b; and hematopoietic stem cells: CD34), immunostaining by CAL2 monoclonal antibodies is performed (Fig. 3).

FUTURE OUTLOOK

In the histological examination of bone marrow specimens, megakaryocytic dysplasia is usually evaluated for myelodysplastic syndromes, in addition to evaluation for *BCR-ABL1*-negative MPNs addressed in the present report. Abnormal cell proliferation or bone marrow infiltration of malignant lymphoma for plasma cell myelomas and bone marrow metastasis of carcinomas are examined during the evaluation of bone marrow fibrosis in conjunction with each of these diseases. Moreover, it has been reported that the immunostaining of p53 as a surrogate marker of the *TP53* mutation is useful in MDS.²²⁻²⁶ This is similar for the detection of chromosomal abnormalities in plasma cell myeloma using cyclin D1 and FGFR3.

In the present revisions of the WHO classification, greater emphasis was placed on morphological evaluation using biopsy specimens for *BCR-ABL1*-negative MPNs. As noted above, problems in the morphological evaluation of pre-fibrotic PMF and ET include the evaluation of morphological features and bone marrow cell density of

megakaryocytes, and the evaluation of MF, as well as the reproducibility. In addition, in *BCR-ABL1*-negative MPNs, it has been reported that the presence of *JAK2* mutation correlates with the morphology of megakaryocytes.²⁷ In the future, examining the relationship between mutations and the megakaryocytic morphology may enable new descriptions to be added with regards to the diagnostic criteria for *BCR-ABL1*-negative MPNs, and the relationship between morphological evaluations and somatic mutations. In recent years, it has been reported that in patients with *BCR-ABL1*-negative MPNs accompanied by MF, fibrosis improved after using chemotherapeutic agents such as JAK2 inhibitors.^{28,29} In the future, bone marrow biopsy may be employed in the evaluation or treatment of *BCR-ABL1*-negative MPNs.

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CONFLICT OF INTEREST

The author indicated no conflict of interest.

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