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REVIEW

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# How I investigate difficult cells at the optical microscope

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## Abstract

Blood cell morphological identification on the peripheral blood and bone marrow films remains a cornerstone for the diagnosis of hematological neoplasms to be integrated with immunophenotyping, molecular genetics, and histopathology. Although standardization is still far from being achieved, with high interobserver variability, in recent years, several classification approaches, from the 1976 FAB to the 2016 WHO classification, have provided hematologists with detailed morphological descriptions for a large number of diseases. Counting blasts and detecting dysplastic specimens are two cornerstones of morphological diagnosis. This review deals with identifying difficult cells, with particular reference of those with relevant diagnostic implications.

**KEYWORDS**

blast, leukemia, morphology, myelodyplasia, WHO classification

## 1 | INTRODUCTION

According to the international WHO guidelines on the classification of hematological neoplasms,[1-3] morphology remains a tool in hematological disorders' diagnostic workup. It should be integrated with flow cytometry, cytogenetics/molecular genetics, histology, and clinical information. Blood cell identification at the optical microscope (OM) represents the first diagnostic screening in the majority of hematological disorders, with a high predictivity in both myeloid- and lymphoid-neoplasms involving peripheral blood (PB) and bone marrow (BM). The leading causes of the high interobserver variability are biological differences in patient samples and individual differences in the morphological skill of observers, as well as lack of harmonization in the terminology and lack of standardization in preparation and staining of BP and BM aspirate smears.[4,5] A complete cell-matched percentage around 50%-60% has been reported in the literature.[4,5] This variability determines discrepancies in the final report under the OM. The present paper aims to describe the Author's approach to the cytomorphological diagnosis of some difficult nucleated cells to complement the indication provided by published guidelines. This review only deals with the differential morphological features that are, in my opinion, useful to identify and differentiate cells that have similar and confounding general appearance. The readers have to bear in mind the cytomorphological features of normal and abnormal PB and BM cells, extensively described elsewhere.[3,6-8]

Moreover, the new instrumental parameters (ie, myeloperoxidase [MPO] activity, cell scatter properties at different angles, platelet, and red cell indices), can be of great help for the selection and an enhanced interpretation of morphological aspects at the time of blood film review.[9,10]

## 2 | BACKGROUND

Qualitative and quantitative criteria are included in the WHO worldwide-adopted guidelines for the diagnosis of hematological neoplasms[1-3] and the ICSH recommendations for the PB cell identification and counting[4]: application of these rules guarantees reproducibility of the morphologic diagnosis.[5,6] At diagnosis, the manual differential count should be performed on PB, out of 200 nucleated white blood cells (WBC) if whenever possible,[11,12] or of an adequate number of cells in cytopenic samples or for routine purposes if normal samples prevail. This author recommends, on the other hand, to carry out the myelogram on BM aspirate smears out of at least 500 cells: WHO recommends higher count in hypercellular samples,[3] to better fit cell distribution on smears, while ICSH Guidelines consider a appropriate number of 300 cells if the

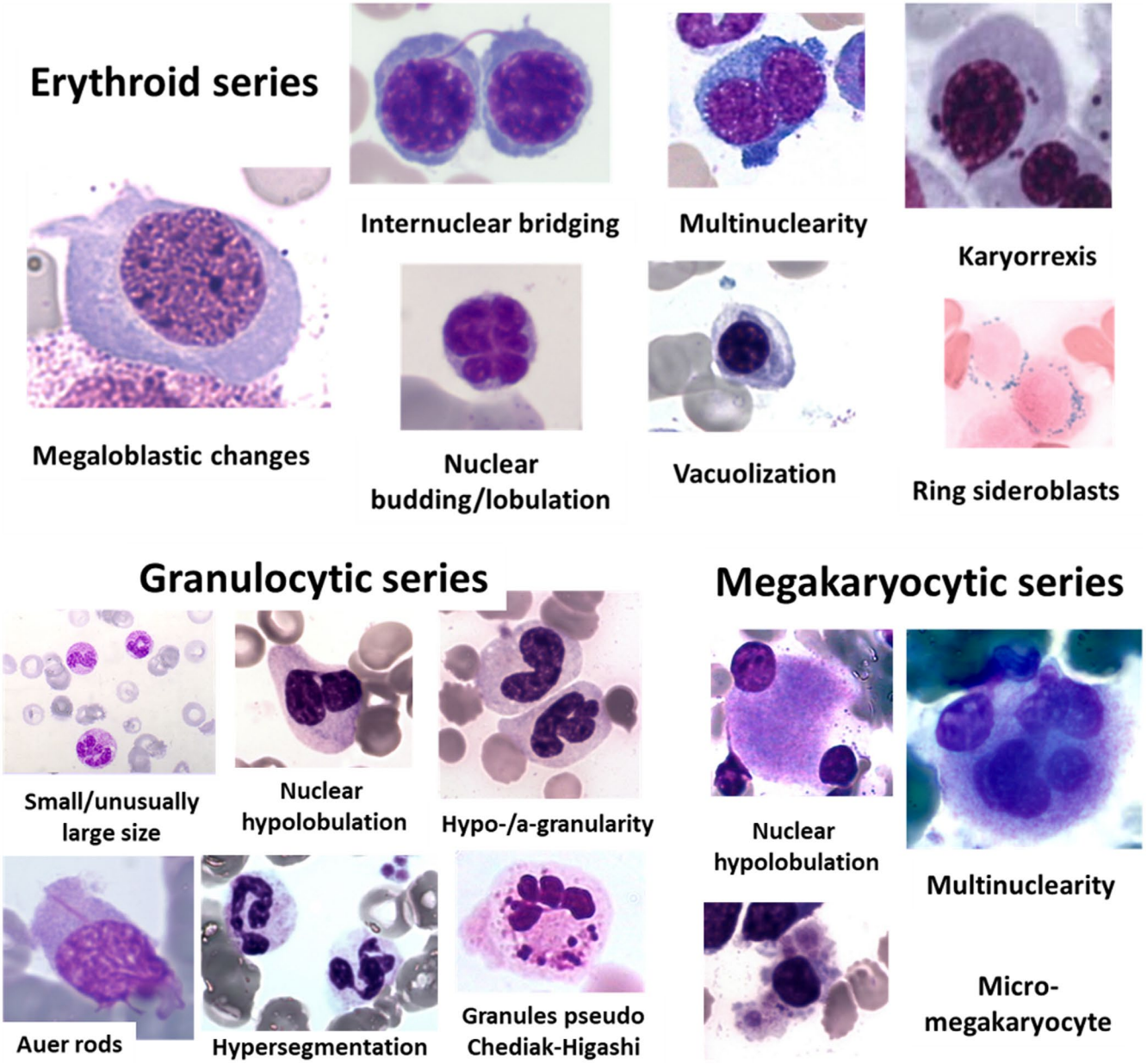


**FIGURE 1** Myelodysplastic features in cells belonging to the main maturative lineages (also see Table 1)

**TABLE 1** Morphologic dysplastic features that should be considered to provide the percentage of dysplastic cells for each lineage (see also Figure 1)

| Series | Displastic features |
|---|---|
| Erythroid | Megaloblastic changes, Nuclear budding/lobulation, Internuclear bridging, Karyorrhexis, Multinuclearity, Vacuolization, Ring sideroblasts |
| Granulocytic | Small/unusually large size, Hypolobulation, Hypersegmentation, Hypo/Agranularity, Pseudo-Chediak-Higashi granules, Auer rods |
| Megakaryocytic | Micromegakaryocytes, Nuclear hypolobulation, Multinuclearity |

count is not performed for the diagnosis.[13] Finally, good laboratory practice includes observing at least two separate smears to confirm the observation's validity under the OM.[11,12] All the types of nucleated cells on PB smears should be evaluated, identified, and included in specific classes to generate the WBC differential count. Blasts, promyelocytes, immature granulocytes, promonocytes,

immature monocytes, mast cells, reactive and abnormal/atypical lymphocytes, when present, should be counted and included in the differential in addition to the five WBC normal classes.[4,11] Nucleated red cells, bare nuclei of megakaryocytes, and nonhematological cells, on the other hand, should be enumerated and commented on separately. Qualitative recommendations on PB evaluation include detecting and describing abnormal morphologic features of WBC, red blood cells (RBC), and platelets (PLT). In the appropriate context, an additional quantitative criterion is the quantification of the percentage of hypo/agranular dysplastic neutrophils.[5] As far as BM smears reporting is concerned, this author recommends to include in the myelogram should consist of the following categories of cells: blasts, promonocytes, promyelocytes, myelocytes, metamyelocytes, band neutrophils, segmented neutrophils, eosinophils, basophils, cells of the monocytic series, mast cells, lymphoid cells, erythroid precursors; megakaryocytes, and other low-frequency cells, such as osteoblasts, osteoclasts, and macrophages, are not included in the myelogram. These cells will be separately included in the final report whether increased or abnormal. Blast percentage at the OM remains crucial for diagnosis, subclassification, prognosis, and disease progression assessment. Accuracy and adherence to the rules are mandatory for interobserver agreement and harmonization. The threshold of ≥20% of blasts separates myelodysplastic syndromes (MDS) from the acute myeloid leukemia (AML), except for the subgroup of AML with recurrent cytogenetic alteration.[3] In this subset, the molecular/genetic lesions drive the diagnosis, irrespective of the number of blasts. An additional quantitative criterion is the enumeration of the percentage of dysplastic cells for the definition of lineage dysplasia. The percentage should be obtained from at least 200 cells in the granulocytic and erythroid lineage to define dysgranulopoiesis and diserythropoiesis, respectively, and from 30 megakaryocytes for the definition of dysmegakaryocytopoiesis Table 1.[3]

This percentage threshold is 10% or 50% for each lineage in the diagnostic pathway of MDS and AML with myelodysplasia-related changes (AML-MRC), respectively Figure 1.[3] Table 2 and Figure 2, respectively, list and provide examples of the morphologic criteria and cell images to identify blasts, promyelocytes, megakaryoblasts, immature, and mature monocytes.

Besides, the WHO classification identifies the category of blast equivalent cells, which includes promonocytes, abnormal promyelocytes, and proerythroblasts[3]: these cells should be included within the blast count in the appropriate diagnostic workup for monocytic clonal proliferation, promyelocytic leukemia, and pure erythroid leukemia, respectively. Adherence to the guidelines ensures reproducibility between operators and reduces the risk of variation in diagnosis. Agreements among observers in reporting normal samples and reporting hypercellular pathological pictures with an unequivocal morphology are usually satisfactory, with an appropriate consensus on the final morphological diagnosis. Disagreements increase in several contexts, such as in the presence of:

- Clonal cells similar to normal or reactive nonclonal cells (ie, cases with monocytic expansion).
- Clonal cells of different lineages (abnormal microgranular promyelocytes versus acute monocytic leukemia or acute monoblastic leukemia).

Disagreements, together with the risk of a misleading diagnosis or prognostication, also increase in the presence of clonal cells at low frequency at diagnostic threshold levels (ie, cases with blast count ≥5% or ≥9% in MDS diagnostic workup). At low counts, reproducibility is also low. In such cases, this author increases as much as possible the number of counted cells and usually resorts to a second observer before completion of the final report to reduce imprecision, according to the international guidelines.[11,13]

**TABLE 2** Morphologic criteria for definition and identification of granular and agranular blasts, promyelocytes, megakaryoblasts, monoblasts, promonocytes, immature, and mature monocytes (see also Figure 2)

| | Size | Nucleus | Chromatin | Nucleoli | Cytoplasm |
|---|---|---|---|---|---|
| Blast, agranular | Small to medium | High N/C ratio | Fine | Present | Basophilic, No Granules, Absent Golgi Zone |
| Blast, granular | Medium to large | variable N/C ratio | Fine | Variable | Granules few to numerous, absent Golgi zone |
| Promyelocyte | 12-20 μm | Eccentric | Intermediate | Rare to none | Granules few to numerous, clear Golgi zone |
| Megakaryoblast | 10-30 μm | Round/indented, irregular | Fine, reticular | One to three | Basophilic, agranular |
| Monoblast | 20-30 μm | Round/oval | Delicate/lace-like | Prominent | Basophilic, possible rare azurophilic granules |
| Promonocyte | Larger than monocyte | Convoluted/ indented | Delicate/lace-like | Prominent | Variable basophilia variable azurophilic granules |
| Monocyte, immature/ atypical/ abnormal | Smaller than monocyte | Convoluted/ indented | Condensed > than promonocyte | Rare | Basophilia < promonocyte >mature monocyte |
| Monocyte, mature | 20-30 μm | Lobulated/ indented | Condensed | No visible | Gray, occasional azurophilic granules and/or vacuoles |

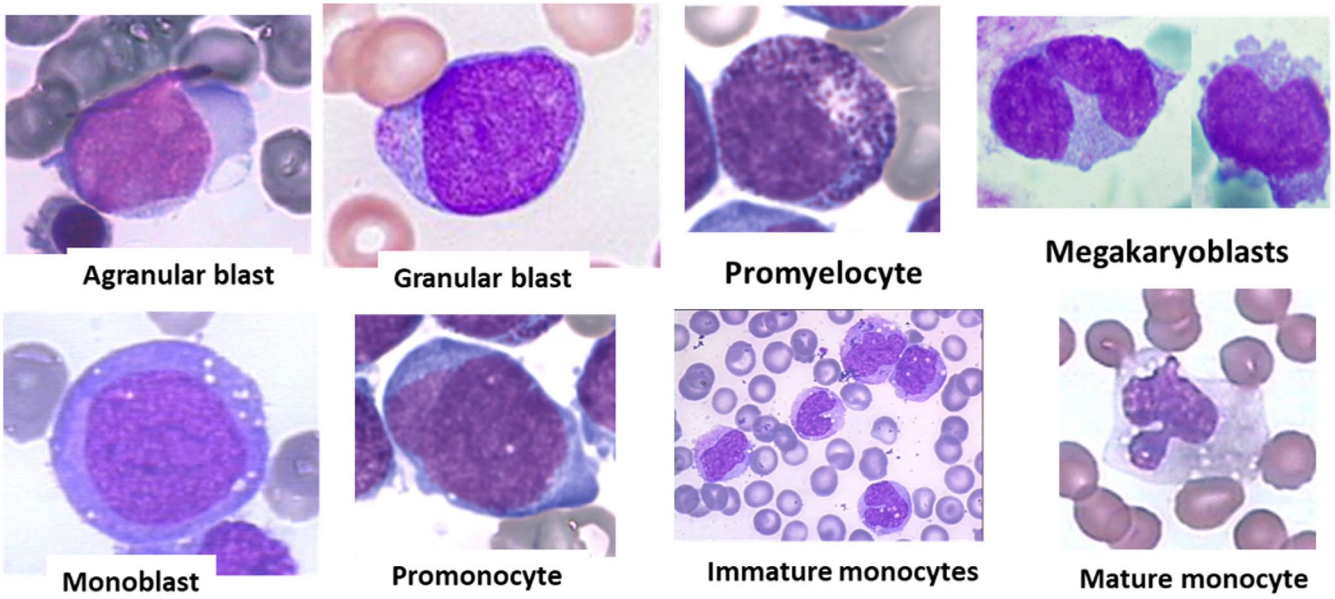


**FIGURE 2** Morphologic criteria for the identification of blasts, promyelocytes, megakaryoblasts, immature, and mature monocytes (see also Table 2)

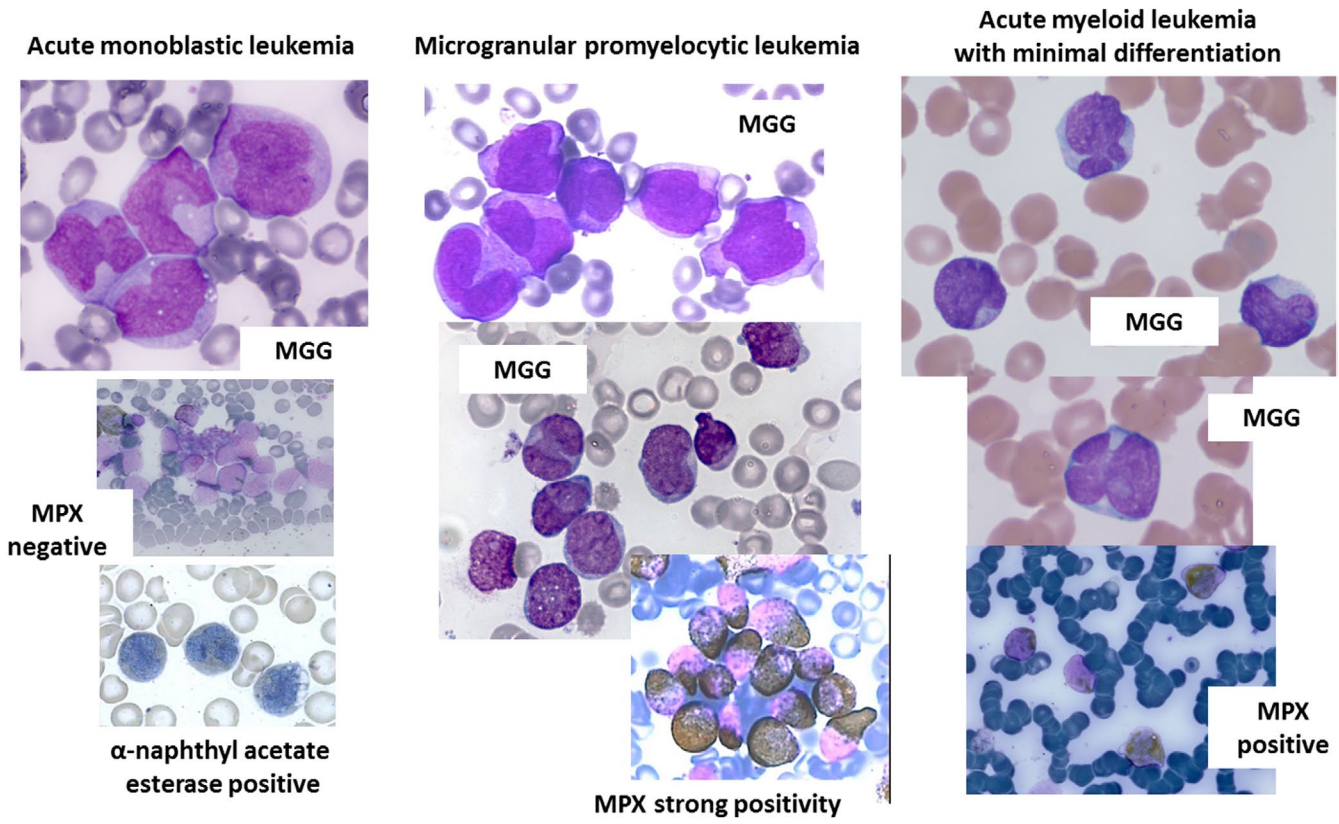


**FIGURE 3** Myeloid blasts of different leukemia subtypes, sharing similar nuclear outlines in the absence of evident cytoplasmic granules

## 3 | DIFFICULT CELLS

Some categories of cells are difficult to be identified at the OM. The wrong identification of such diagnostic cells is often due to similarity in some features shared with clonal cells of different lineage or nonpathologically relevant cells, substantially increases the risk of a diagnostic error. As underlined in the previous section, the final harmonized qualitative and quantitative cell identification

at OM starts from sharing a methodological approach according to the international guidelines.[4,13] With panoptical stains, we appreciate cell dimension, shape, and chromatin pattern of the nucleus, the presence/absence of detectable nucleoli, cytoplasmic color, and granule content, the nucleo/cytoplasmic (N/C) ratio. The use of some basic cytochemical stainings remains an integral part of the cell identification at the OM. Basic cytochemistry includes at least four different methods: (a) MPO, necessary to discriminate granulocytic and monocytic cells from lymphoid; (b) nonspecific α-naphthyl acetate esterases sensitive to sodium fluoride, which are found in monocytes, megakaryocytes and PLT; (c) toluidine blue, which stains purple granules containing heparin and histamine in basophils and mast cells, based on the principle of metachromasia; (d) Prussian blue stain, to detect hemosiderin, iron and iron-containing granules. Finally, the meticulous and systematic evaluation of the general cellular context is unavoidable for the appropriate morphologic identification of these critical cells. In summary, morphologic cell identification requires the knowledge of:

- the archetypal morphology of normal and abnormal blood cells,
- the peculiar morphological features shared by different cells,
- the quantitative and qualitative methodological approach according to international guidelines,
- evaluation within the context of the company the cells keep.

The following sections suggests a possible approach to investigating the most frequently observed difficult/critical cells at OM.

## 4 | LARGE BLASTS WITH RENIFORM OR BILOBED NUCLEI AND A FEW CYTOPLASMIC GRANULES

The finding on smears stained with panoptical stains of abnormal cells of medium to large size with bilobed, multilobed or reniform nuclei showing immature chromatin pattern, without or with only a few fine azurophilic granules in the cytoplasm, should immediately trigger the morphological differential diagnosis between:

- Myeloblasts
- Blasts of monocytic lineage
- Abnormal microgranular promyelocytes (Figure 3).[14-16]

Abnormal microgranular promyelocytes, importantly, have to be identified as blast equivalent in the diagnostic workup of acute promyelocytic leukemia. In these cases, the misdiagnosis can impact the patient's survival[16]: a prompt appropriate morphologic diagnosis is mandatory. The critical point is to know in advance which types of cells share morphologic features of bilobed/reniform nuclear shape, immature chromatin pattern, and barely visible fine azurophilic granules in a light basophilic cytoplasm. The diagnostic confirmation is obtained by performing cytochemical staining for MPO, which is always heavily positive in microgranular promyelocytes. In laboratories where cytochemistry is not immediately available, the clinician must be immediately informed of the suspected diagnosis, waiting for immunophenotype confirmation.

## 5 | CELLS OF THE MONOCYTE LINEAGE

In my experience, morphological details that are useful to identify and classify the monocyte lineage cells are often better visible on PB than on BM aspirates. According to the recent guidelines,[3,17] promonocytes should be considered blast equivalent in the appropriate diagnostic setting, which is leukemias associated with clonal expansion of the monocytic series: acute monoblastic leukemia, acute monocytic leukemia, acute myelomonocytic leukemia, and chronic myelomonocytic leukemia. The promonocytic percentage should be merged with the blast percentage in the final PB and BM report in these patients. In these diseases, smears are usually strongly hypercellular and present with overcrowded microscopic fields, not easy to interpret. The suggested morphological workup is as follows:

- to confirm the monocytic lineage expansion with cytochemistry and, after this,
- to look for the easily identifiable cells of the monocytic lineage, such as monoblasts and mature monocytes.

Monoblasts are large cells, with abundant cytoplasm, light gray to deep blue with possible rare azurophilic granules, round nucleus with delicate lacy chromatin with one or more large prominent nucleoli.

Equally large cells presenting with the same chromatin pattern but with convoluted/indented nuclear shape and variable granule content in the cytoplasm are the promonocytes.[7] In these contexts, the count of promonocytes should be added to the blast count (monoblasts plus myeloblasts and megakaryoblasts, when present): this explains the denomination of promonocytes as "blast equivalent".

Mature monocytes are smaller than monoblasts, with lobulated/indented nucleus with condensed chromatin, without visible nucleoli, lower N/C ratio, and a gray cytoplasm with granules and vacuoles. Cells of the monocytic series that do not fit the morphological features of mature monocytes, promonocytes, and monoblasts should be included in the group of immature/atypical/abnormal monocytes. In the final myelogram, as blasts-equivalent, promonocytes should be included in the blast count, together with monoblasts. All the remaining cells of the monocytic lineage, on the other hand, should be merged within the count of maturating monocytic series: the final number or percentage, rather than the often impossible precise identification of the maturity level, is needed for the diagnostic workup.

## 6 | MPO-NEGATIVE BLASTS

Blast cells, both in the PB and in BM specimens, show a generally immature chromatin pattern, which can variably range from lacy/dispersed to relatively compact, depending on the type of blasts. As described above, monoblasts are large cells with delicate, lacy chromatin with nucleoli, while some lymphoblasts are small, with scant cytoplasm and partially condensed chromatin without any evidence of nucleoli. In these cases, the observation in spread out and not crowded microscopic fields in PB films permits a better appreciation of the chromatin network. Monomorphism of the leukemic cells, associated with the detection of normal features and quantitative reduction of the granulocytic precursors with preserved maturation, may suggest the blast cells' lymphoid origin. Unfortunately, on the other hand, no morphological evidence is sufficient, in these cases, to provide an unequivocal final diagnosis to report. At the OM, only a sure finding of MPO positivity allows reliable identification of the blasts as belonging to the myeloid lineage. MPO negativity, on the opposite, does not exclude the presence of myeloid blasts with minimal differentiation. Immunophenotyping is the only valuable diagnostic tool in the absence of positive morphologic and cytochemical markers. In the evaluation of BM smears, and only very rarely in PB films, a correct morphologic differential diagnosis has to take into account, for, the possible presence of infiltration by solid cancer cells mimicking small lymphoblasts, such as it happens in metastatic diffusion of neuroblastoma, rhabdomyosarcoma, or small cell carcinoma of the lung (Figure 4).[7]

## 7 | SMALL CELLS WITH BILOBED NUCLEI

Bilobed nuclei are a powerful morphologic feature, easy to identify even if not univocal in its diagnostic significance. Cells with similar size and belonging to different lineages, including lymphoid cells and megakaryocytes, can be identified through the following characteristic[3,7]:

- dyserythropoietic erythroblasts: they are easily identifiable through the evaluation of the erythroid context, peculiarities in chromatin condensation, the cytoplasmic color, and the size at different stages of maturation;
- centrocytes: a coffee bean appearance well defines their nucleus; the cytoplasm is exceptionally scanty, almost invisible or recognized only around the nuclear notch;
- bilobed lymphocytes: the nucleus is shaped like a saddlebag and the cytoplasm is usually plentiful;
- bilobed micromegakaryocytes: typical of some cases of MDS, micromegakaryocytes can even circulate in PB and be observed in PB film; in BM aspirates they are morphologically heterogeneous: dense chromatin and pink granular cytoplasm, with occasional peripheral images of platelet formation, are the most typical features;
- hairy cells: larger than lymphocytes, eccentric and bean- or kidney-shaped nuclei with homogeneous or spongy chromatin, ample pale cytoplasm, whose periphery is typically fringed with villous projections, and sometimes with rare azurophilic granules; the typical villi are often better visible on PB films, in which however the number of hairy cells can be small;

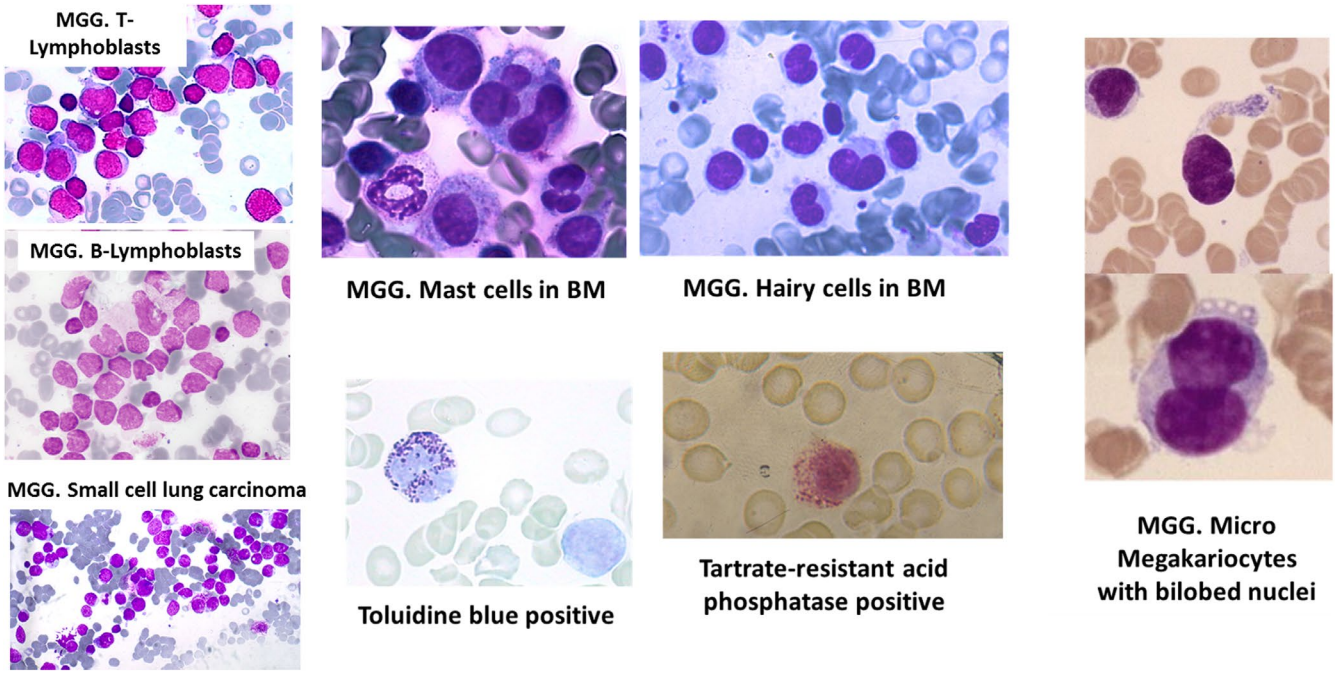


**FIGURE 4** Bone marrow cells of different lineages, sharing similar morphologic features: undifferentiated blasts with similar morphology on the left column; cells with analogous size and bilobed nucleus. Positivities to toluidine blue and tartrate-resistant acid phosphatase allow the differentiation of mast cells from hairy cells

- mast cells: in normal BM aspirates, mast cells are relatively large with an almost not visible nucleus, covered by abundant metachromatic granules; in systemic mastocytosis, on the other hand, hypogranular mast cells can resemble hairy cells.

The morphologic diagnostic criteria used for differentiating on BM smears mast cells from hairy cells can appear elusive[7] and can sometimes be better appreciated on PB films (in the case of hairy cells). There can be substantial morphologic similarity at the level of single cells (Figure 4 ), both in the nuclear shape and in size and aspect of the cytoplasm. A careful observation of the distribution of these cells on the BM aspirate immediately highlights the tendency of hairy cells and mast cells to aggregate. The wise evaluation of the BM context and the company the cells keep help to differentiate the two lineages. A positive toluidine blue staining allows the identification of mast cells. The same is true for tartrate-resistant acid phosphatase positivity toward hairy cells. Together with morphology and cytochemistry, immunophenotyping remains the appropriate diagnostic tool.

## 8 | FINAL COMMENTS

In this era of development of the technologies applied to oncohematological diagnostics, morphology alone in most cases is not the diagnostic gold standard. It has intrinsically limited accuracy and is an observer-dependent method. However, it constitutes the first diagnostic approach after the quantitative full blood count and allows a real-time orientation to the diagnosis. In the context of hematological emergencies, the immediate and precise identification in the PB of cells such as promyelocytes can save the patient's life, in the same way as an accurate count of sickle cells or schistocytes (not covered in this review) does. Even at the BM level, morphological examination still plays a basic diagnostic and prognostic role in acute leukemias (AL) and MDS.[18-20] The training of a morphologist takes a long time. The tendency to immediately resort to semi-automated, second-level diagnostic methods, from immunophenotype/bone biopsy/genetics/molecular investigations to gene sequencing, is quite widespread. One of the consequences of such an attitude is the inexorable loss of specific morphological skills. The debate is, however, still open and lively.[21-24]

In the author's opinion, two final considerations have to be shared. Less developed countries do not have access to new and sophisticated technologies, so that the OM remains the only diagnostic possibility. To maintain and pass on knowledge and competence is, therefore, a duty. A new solution comes today from technology: the use of digitized images, both as single-cell images and as scans of whole slides, allows the training of new morphologists and the diffusion of new morphological pictures throughout the web. Finally, only at the OM can blood cells be evaluated individually, allowing the real-time application of the diagnostic algorithms published by WHO for AL and MDS, the detection of parasites and specific RBCs morphological anomalies (not covered, because of space constrictions, in the remit of the paper's title) and the prediction of clonality versus a reactive cell pattern.

## 9 | KEY REMARKS

The identification of cells under a microscope primarily requires the study and knowledge of the quantitative and qualitative morphological characteristics of the various hematological cell lines, normal, and abnormal.

In accord with international guidelines, a systematic approach to the smear review is the best way to generate a harmonized and reproducible report.

Awareness of the objective diagnostic limits of morphology, with the consequent use of additional and complementary diagnostic tools, is the best prevention of diagnostic errors.

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