2 Clinical Presentation, Diagnosis, and Classification of Acute Myeloid Leukemia

Ridas Juskevicius, Mary Ann Thompson, Aaron Shaver, and David Head

2.1 Introduction

The acute myeloid leukemias (AML) are a diverse set of phenotypically similar diseases characterized by increased myeloblasts replacing the normal bone marrow, with variable involvement of peripheral blood and occasional involvement of extramedullary sites. In some cases, proliferating blasts replace normal hematopoiesis resulting in failure of the marrow to produce normal peripheral blood cells, with tumor burden itself becoming life-threatening. In other cases, while blasts are increased as a percentage of marrow cells, the predominant problem is primary marrow failure (resembling MDS) rather than blast tumor burden. Classification of AML has undergone fundamental changes over the last two decades, in part due to recognition of these varying scenarios [1]. Although not without areas of controversy, the introduction of the World Health Organization (WHO) classification framework in 2001, updated in 2008 and revised in 2016 [2], represents the official international consensus classification of AML, combining these two scenarios under the common heading of AML. The WHO classification of AML is based on clinical, phenotypic, and molecular genetic features with an attempt to define biologically and prognostically distinct entities which have uniform response to therapy. Although genetic heterogeneity of AML has been recognized for several decades, enormous molecular heterogeneity has become apparent only recently with the introduction of new molecular diagnostic methodologies including next-generation sequencing (NGS)-based assays. The massive amount of data generated utilizing these techniques is contributing to improved understanding of the biologic heterogeneity of AML. Incorporation of the data into the classification framework of AML is inevitable, but is still at its early stages, as we are only now beginning to understand the biologic and clinical implications of these newly discovered molecular alterations. In this chapter, we discuss the clinical presentation, diagnosis, and classification of AML, including appropriate diagnostic laboratory studies necessary for diagnosis and subclassification of biologically and clinically relevant types of disease. Understanding the basis for the current WHO classification of AML requires additional knowledge of the myelodysplastic syndromes (MDS) and their relationship to one subset of AML. Finally, we will address monitoring AML minimal residual disease during and after treatment.

R. Juskevicius (✉) · M. A. Thompson
A. Shaver · D. Head
Department of Pathology, Microbiology and Immunology, Vanderbilt University Medical Center, Nashville, TN, USA
e-mail: ridas.juskevicius@vumc.org; maryann.thompson.arildsen@vumc.org; aaron.shaver@vumc.org; david.head@vumc.org

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2.2 Clinical Presentation of AML

The classic onset of the symptoms of acute leukemia is rapid. The patient may have felt ill for only a few weeks prior to seeking medical attention. In other cases, the presentation may be more insidious, with prolonged symptoms related to cytopenias with or without prior diagnosis of underlying MDS. In either case, the most typical presentation is that of symptoms related to bone marrow failure. These include easy bruising and petechiae due to thrombocytopenia, frequent infections due to neutropenia, and/or symptoms related to anemia such as fatigue, pallor, or even cardiovascular effects of profound anemia. In this type of presentation, the primary care physician will typically obtain a complete blood count (CBC), which may show circulating blasts. The number of blasts in peripheral blood may be few or numerous. When blasts are present in the peripheral blood accompanied by anemia and thrombocytopenia in a newly presenting patient, the level of suspicion for acute leukemia is high and a bone marrow biopsy is typically obtained. When blasts are few in number, other morphologic clues on the peripheral smear that may increase the suspicion of marrow replacement by leukemia include leukoerythroblastosis (triad of immature myeloids, nucleated red blood cells, and teardrop red cells), dysplastic changes in neutrophils, and so-called leukemic hiatus where only blasts and few mature segmented neutrophils are present with the absence of other left-shifted myeloid cells that would typically be seen in reactive conditions. All these clues should serve as triggers to obtain a diagnostic bone marrow sample.

Since in the contemporary practice of medicine the initial examination of blood smear takes place in the clinical hematology laboratory, the ability of the hematology technologists to recognize blast morphology is crucial, as they serve as the frontline of diagnosis in patients where the diagnosis of AML may not be suspected. Laboratory quality control (QC) and continuing medical education (CME) activities to reinforce this ability are crucial. The morphologic characteristics of myeloid blasts on the Wright stained peripheral blood smear include immature chromatin ("ground-glass"), increased nuclear:cytoplasmic ratio, and variable granulation to the cytoplasm. The presence of Auer rods, needle-shaped cytoplasmic inclusions resulting from fusion of primary azurophilic granules, is pathognomonic for myeloblasts. In the more frequent absence of Auer rods, flow cytometry must be performed to determine unequivocally the lineage of blasts.

Several clinical manifestations of AML constitute medical emergencies, most notably (1) leukostasis due to hyperleukocytosis and (2) coagulopathy, typically associated with, but not restricted to, acute promyelocytic leukemia (APL). Hyperleukocytosis is usually defined as a white blood cell count greater than 100,000 per μL, but whether leukostasis occurs depends on many factors individual to the patient. Leukostasis is thought to be the result of increased blood viscosity due to the increased cellularity, reduced deformability of the blasts (versus mature cells), and direct and indirect blast–endothelium interaction, all causing occlusion of microvasculature [3]. Both the specific lineage of the increased cells and their rate of rise in the circulation are contributory factors, with monoblasts being the most problematic cell type. Leukostasis should be suspected if the patient has pulmonary, CNS, or cardiovascular symptoms that cannot be explained by other medical conditions: dyspnea, confusion, somnolence, headache, impaired vision, tinnitus, chest pain (myocardial ischemia/infarction), limb ischemia, thrombosis, and priapism [3]. Treatment options include hydration, leukemia-directed chemotherapy, and leukapheresis. The role of the latter is controversial [3, 4]. Hyperleukocytosis may also result in disseminated intravascular coagulation (DIC), which should be considered if the peripheral blood smear demonstrates schistocytes and decreased platelets, and confirmed by checking for decreased fibrinogen, elevated D-dimers, prolonged prothrombin time (PT), and activated partial thromboplastin time (aPTT). DIC occurs in 30–40% of patients with AML and hyperleukocytosis [4]. Finally, hyperleukocytosis may be associated with tumor lysis syndrome (TLS),

which occurs with treatment in approximately 10% of AML patients [4]. Chemistry laboratory values for potassium, phosphorus, calcium, and particularly uric acid should be monitored to detect TLS.

The clinical presentation of acute promyelocytic leukemia (APL) bears particular discussion as the associated coagulopathy may result in life-threatening hemorrhage or thrombosis. The risk of early death from hemorrhage in APL has been estimated at 17–29% in community studies [5], with most cases occurring before institution of treatment. At presentation, mucocutaneous bleeding is common, with immediate risk of hemorrhagic death due to intracranial or pulmonary bleeding. The characteristics of APL blasts on the peripheral blood smear will be described later in this chapter. The presence of low platelets is also obviously significant. Clinical signs are bleeding from gums, epistaxis, GI hemorrhage, and excessive ecchymoses and petechiae. When APL is suspected, coagulation studies including PT, aPTT, D-dimers, and fibrinogen should be obtained. The complex coagulopathy of APL is multifactorial but includes tissue factor (TF)-induced DIC and primary hyperfibrinolysis [5]. APL blasts have increased TF on their surface, which activates factor VII. The resultant factor VIIa activates FIX and FX, leading to thrombin generation, ultimately resulting in fibrin formation. In addition, the promyelocytic blast surface contains Annexin II, which binds plasminogen and tissue plasminogen activator (tPA), promoting plasmin formation and thus fibrinolysis [5]. Immediate treatment with all-trans retinoic acid (ATRA) is required when APL is suspected, before confirmation of the diagnosis with other studies. Treatment with ATRA causes blasts to mature and arrests the coagulopathy. This is essential prior to initiation of chemotherapy, when there will be massive lysis of the blasts. If diagnosis of APL is not subsequently confirmed, ATRA may be stopped with no compromise to other treatment options.

A rare presentation of AML is with myeloid sarcoma, which is defined as a tumor mass consisting of myeloid blasts in which tissue architecture is destroyed, to distinguish it from an area of simple leukemic infiltration [2]. The most common sites are skin, lymph nodes, gastrointestinal tract, bone, soft tissue, and testes. The presentation is usually as a solitary mass [2]. Myeloid sarcoma may be the first, and sometimes the only, early manifestation of AML. It may also be the first manifestation of blast crisis of an underlying myeloproliferative or myelodysplastic syndrome. Another common setting is at relapse, including post-hematopoietic stem cell transplant. Diagnosis depends on morphology (preferably including a Wright stained touch preparation) and immunophenotyping of the myeloid blasts using a combination of flow cytometry and immunohistochemistry. Cytogenetic analysis including FISH may be helpful, particularly if the lesion has monocytic differentiation which often lacks definitive immunologic markers of immaturity. Myeloid sarcoma is most often associated with monocytic differentiation. It has relatively high prevalence in children, which likely reflects a higher incidence of AML with core binding factor abnormalities (t(8;21) and inv16) in this age group, since myeloid sarcomas are prevalent in AML with core binding factor abnormalities [6, 7]. In several series of adults with myeloid sarcoma, there were many cases with a complex karyotype, monosomies, trisomy 8, and translocations involving 11q23 (*KMT2A*) [8, 9]. For diagnostic purposes, the antigens expressed most often in myeloid sarcoma are CD43, CD68, lysozyme, MPO, and CD117 [10]. Immunohistochemistry which includes antibodies to CD4, CD56, CD123, and TCL-1 may be helpful to rule out the possibility of a blastic plasmacytoid dendritic cell neoplasm (which typically is MPO negative, TCL-1 positive, and usually positive for both CD4 and CD56) [11, 12].

A very rare presentation of AML is CNS involvement with the first manifestation being blasts in the CSF, not the peripheral blood. CNS symptomatology suggesting a process involving cranial nerves, spinal cord, or meninges will trigger CSF cytologic examination of a Wright stained cytospin slide, showing blasts and requiring further testing such as flow cytometry to confirm diagnosis. In one study of 12,000 patients

diagnosed with acute leukemia (ALL and AML), only nine patients presented in this way with blasts present in the CSF prior to presence in the peripheral blood [13].

In patients with myeloproliferative or myelodysplastic disease, exacerbation (often insidious) of symptoms (fatigue, bruising, dyspnea), or deterioration of laboratory values (cytopenias, increased peripheral blood blast count, elevation in uric acid or LDH) may be a harbinger of blast crisis with evolution to acute leukemia. In this setting, the blasts are likely to be myeloid. Morphologic review of the peripheral blood smear and a low threshold for obtaining a bone marrow sample are recommended. A caveat about making the diagnosis of AML in this setting is that a leukoerythroblastic smear due to profound hypercellularity or myelofibrosis may have a few blasts on the peripheral blood smear. Therefore, review of the peripheral blood smear should be followed by a bone marrow biopsy. In patients with CML, approximately two-thirds of blast crises are acute myeloid leukemia, whereas one-third are acute lymphoblastic leukemia [2].

2.3 Laboratory Studies for the Diagnosis and Monitoring of AML

2.3.1 Morphology

A good bone marrow aspirate and biopsy sample are essential and require good technique at the bedside in acquisition and in the laboratory in processing the sample. Squash preps are discouraged except in the hands of experienced technologists. Preferable are push preps, performed identically to preparation of peripheral smears, or coverslip preparations. Touch preps should also be performed routinely. If a biopsy is to be obtained, it should be large enough to properly assess marrow characteristics and should be redirected to avoid the preceding aspirate site.

Morphologic evaluation of biopsy samples is the cornerstone of pathologic evaluation and still remains important even with the advent of other ancillary modalities. Review of morphology can focus on low-power, large-scale patterns, or high-power, fine-scale details. Low-power evaluation of the bone marrow sample can help detect patterns of infiltration and assess for disease burden. However, high power examination of individual cell features, often called cytomorphology, is of particular importance in hematopathology and especially in evaluation of AML, since the differential diagnosis often depends on morphologic features present in individual cells, such as Auer rods, cytoplasmic granules, and nuclear features.

The need for both low- and high-power examination of bone marrow specimens helps to explain some of the sample collection strategies employed in the evaluation of leukemias. Taking both aspirate and core biopsy samples of bone marrow, for example, allows evaluation of individual cytomorphology on smeared specimens of aspirate material, as well as evaluation of low power architectural distortion and geographic patterns using the core biopsy specimen. While examination of these two different tissue types historically was performed by different groups of physicians—pathologists were responsible for reviewing core biopsy specimens, and hematologists often reviewed aspirate specimens—modern practice, particularly in the United States, has moved toward combining the review of both specimen types under the auspices of the pathologist, which allows better integration of all sources of diagnostic data into one process and one report.

2.3.2 Immunophenotype

In addition to assessment of light microscopic morphologic features, modern diagnosis requires interpretation of the set of proteins and other markers expressed by the cell, which is referred to as the immunophenotype. In particular, the WHO classification of AML requires correlation with immunophenotype both for excluding other categories of acute leukemia and in aiding in subclassification. Myeloid-specific markers such as myeloperoxidase, or markers of immaturity such as CD34, are important diagnostic adjuncts built directly into the WHO classification system.

Most methods for immunophenotyping employ targeted antibodies (or other molecules with high specificity of binding, such as nucleic acid sequences), whose specific regions react with the phenotypic target of interest. Laboratory techniques for immunophenotyping differ in the method for assaying the binding of these targeted antibodies. While a range of techniques are available, two categories of the most prevalent techniques in the clinical diagnostic setting are tissue-based techniques such as immunohistochemistry (IHC) and in situ hybridization (ISH) and cell-based techniques such as flow cytometry. These categories have overlapping strengths and limitations and are often used in a complementary strategy in the diagnostic setting.

Immunohistochemistry and other tissue-based methods leverage the diagnostic information present in morphologic features of the tumor to help correlate with the immunophenotypic data, particularly in tumor populations that are heterogeneous or mixed with a significant non-neoplastic background population. This is brought about by performing the antibody reaction and subsequent development for visualization in the setting of an intact tissue block, with a counterstain added so that morphologic features can be appreciated at the same time. IHC uses specific antibodies conjugated to a reporting molecule, whose presence is detected by a secondary reaction after the initial antibody binding step. The result is a color change (typically brown or red, depending on the developer) in the cells/areas where the antibody has bound. The result is a pattern of color change on a tissue slide that correlates with the presence of the marker of interest. ISH is a similar technique that uses synthetic DNA/RNA sequences with attached reporter molecules to detect the distribution of complementary nucleic acid sequences, rather than proteins or other antibody targets.

Tissue-based methods like IHC have two primary areas of strength. IHC can be performed on formalin-fixed, paraffin-embedded material. Because of the longevity of this type of material and the lack of a need for viable, fresh specimen, this allows a range of studies to be performed both at the time of the initial acquisition of the material and at any point in the future when re-review of the specimen is needed. For small samples, such as bone marrow biopsies, the small amount of tissue received can be used for both morphologic and immunophenotypic interpretation, without having to triage the sample between two diagnostic techniques. The second area of strength is that, because the IHC stain is performed on the tissue in situ, morphologic correlates can be drawn with areas of abnormal IHC staining. In the case of AML, IHC can be particularly helpful in a heterogeneous, mixed sample where the morphologic features of blasts are striking. This is particularly important for morphologically unusual subclassifications of AML, such as acute promyelocytic leukemia or AML with erythroid or megakaryocytic differentiation. In these cases, IHC allows direct correlation of the phenotypic data with the morphologic diagnostic features.

Immunohistochemistry does have significant drawbacks, which limit its utility in certain situations. The most prominent of these limitations is the necessity to use only one (or at most two) labeled antibodies in a single reaction, due to the relatively limited number of different reporter tags available for routine use. For a neoplastic process such as AML in which it is necessary to assay a complicated immunophenotype with many markers, this requires laborious and error-prone comparison between individual markers tested on different slides. Focal areas of abnormality may not be present on every slide, and scant tissues may be entirely consumed in the process of testing before the entire immunophenotype can be measured. Another limitation is that, in the clinical setting at least, IHC and ISH stains are typically reviewed by eye under the microscope, and therefore evaluation of the results is necessarily qualitative (positive/negative, dim/bright) rather than quantitative. This can be a limitation for some markers of diagnostic or therapeutic importance, such as CD38, where expression is almost ubiquitous, and it is the degree of intensity of expression that is the important clinical consideration [14]. A separate issue with tissue-based techniques like IHC and ISH is the time required to perform the testing.

These techniques require several hours for binding and developing of the specific target molecules, which limits the rate at which diagnostic information can be incorporated. One or two rounds of IHC stains can add 1–2 days to the time required to render a final diagnosis for a case, which can have a clinical impact, especially in settings such as initial diagnosis or initiation of targeted therapy.

The prevalence of flow cytometry in clinical hematolymphoid diagnostics, and in particular in the evaluation of acute leukemia, is due to its ability to address many of the limitations described above for tissue-based immunophenotyping. In turn, flow cytometry itself has many limitations that can be backed up with the use of IHC or ISH. As a technique, flow cytometry shares some similarities with IHC: specific antibodies are linked to reporter molecules, which in the case of the most common form of flow cytometry are fluorophores that emit light at specific wavelengths upon excitation by a laser. These antibodies are allowed to hybridize with the cells of interest, and then exposed to a reporter reaction (in this case, excitation by a laser) which allows for detection of specifically bound antibodies. The major distinction from tissue-based techniques is that flow cytometry is performed on disaggregated, individual cells in suspension in a buffer fluid, rather than on intact sections of tissue. Additionally, multiple different antibodies conjugated to different fluorophores are used at once, allowing the measurement of multiple markers simultaneously on the same cells.

Flow cytometry's differences from tissue-based techniques like IHC lead directly to its advantages and disadvantages. Whereas interpretation of IHC for multiple markers on the same tissue can lead to frustration and ambiguity as multiple slides have to be compared, flow cytometry is a natural system for looking at multiple markers on the same specimen. This is especially important for subclassification within broader categories or for distinction between closely related diseases, where assessment of a complicated set of overlapping immunophenotypes needs to be made using a large battery of specific antibodies. Another advantage of flow cytometry is its ability to reproducibly measure relative quantitative intensity of staining, rather than the crude strong/weak/negative categorization with IHC. An example of the utility of this approach in myeloid neoplasia is in assessment of CD56 on bone marrow myeloid precursors: dim, variable CD56 expression may be seen in a variety of reactive conditions, while uniform brighter expression of CD56 is a much more specific marker of neoplastic abnormality. Properly calibrated flow cytometry can also often detect much lower intensity of staining than IHC, allowing the diagnostician to detect dim aberrant expression of markers not associated with normal populations that help definitively establish the presence of a neoplasm [15]. In the setting of a new presentation of acute leukemia, the rapid turnaround time of flow cytometry is an additional advantage. Total time in the laboratory from processing to data acquisition to analysis can take less than an hour, allowing rapid triage of an unstable patient.

The limitations of flow cytometry primarily stem from the need for individual cells in suspension. The process of disaggregating the cells results in a complete loss of the low-power, geographic context, in contrast to IHC, where the ability to map staining pattern onto morphologic pattern can often be vital to interpreting a complicated sample. The same processing requirements also remove the ability to correlate the immunophenotypic features detected by flow cytometry with specific high-power cytomorphologic findings. As discussed above in the section on immunohistochemistry, this can be relevant in cases with relatively rare leukemic cells with striking morphologic features. Finally, the requirement for disaggregation and suspension means that paraffin-embedded tissue is unsuitable for flow cytometry; fresh aspirate or disaggregated biopsy material, or carefully frozen archival material is required. This limits the utility of flow cytometry for returning to previous cases or as an adjunct test in cases where appropriate material was not reserved at the time of biopsy.

This set of opposing and complementary strengths and limitations has led to the adoption of both IHC/ISH and flow cytometry as routine

clinical tests in hematolymphoid disease, including myeloid neoplasms such as acute myeloid leukemia. Some diagnostic challenges are more suited to one modality over another. Fresh bone marrow aspirate material is an ideal specimen for flow cytometry, and in newly diagnosed disease, the abundant and often relatively homogeneous blast population makes correlation with specific morphologic patterns relatively unimportant. For these reasons, comprehensive flow cytometry panels are used as the first-line immunophenotypic assessment of new leukemia. On the other hand, tissues where disaggregation might be more difficult or not expected at the time of biopsy, such as cutaneous involvement by extramedullary deposits of acute leukemia, are less amenable to flow cytometry and the importance of IHC increases. Another area favoring overlapping use of the two modalities is in diseases such as myeloid leukemias with monocytic differentiation, where the flow cytometry immunophenotypic features are not always helpful for distinguishing between chronic and acute disease, and correlation of immunophenotypic abnormalities with morphologic features may be necessary to definitively establish the disease subtype.

Acute myeloid leukemia is well-studied and illustrative of how a careful analysis of immunophenotype can assist in the diagnostic process, while also serving as a reminder of the necessity of incorporating the immunophenotypic data into a broader context of morphologic and ancillary testing. Specific subtyping of AML can have a massive impact on prognosis and therapy for the patient, and specific subtypes often correlate with immunophenotypic differences. APL is a well-known example: it has profound prognostic implications due to its association with DIC, and it is amenable to a very specialized targeted therapy using retinoic acid derivatives. APL has a striking immunophenotype, often lacking many of the markers generally associated with immature myeloid cells, including CD34 and HLA-DR, while strongly expressing other myeloid phenotypic markers such as CD117 and myeloperoxidase. Detection of a population of leukemic blasts with this immunophenotype can help raise or confirm clinical and morphologic suspicion for APL, leading to proper targeted and supportive management of the patient. Unfortunately, detection of this special phenotype is neither entirely specific nor sensitive for APL. The prominent granules in APL tend to autofluorescence when exposed to laser light, leading to a well-known propensity for the leukemic blasts to show non-specific, non-antibody-mediated fluorescence for a wide range of markers [16], leading to false negatives in the sense that the immunophenotypic pattern of interest is not recognized. Relatively simple techniques exist to identify and account for this autofluorescence but neglecting to employ these techniques can lead to misdiagnosis on immunophenotypic grounds. On the other hand, even if the phenotype is correctly interpreted, it is not entirely specific for APL. Other leukemias may have a similar phenotypic pattern, with a prominent example being *NPM1*-mutated AML, a common category of AML with prognostic and therapeutic consequences much different than APL [17]. Thus, recognition of specific phenotypic patterns can be helpful in guiding the clinician onto the right track, but definitive diagnosis still generally relies on correlation with the entire suite of diagnostic testing, including morphology, cytogenetics, and molecular studies.

2.3.3 Cytogenetics

A frequent and recurrent abnormality in many hematologic neoplasms, including AML, is the presence of large-scale chromosomal abnormalities, including gain or loss of large sections or even entire chromosomes, as well as translocations involving transfer of millions of base pairs of genetic material from one chromosomal section to another. The analysis of chromosomal structure for these classes of large-scale abnormalities is referred to as cytogenetics. Some of the best-established diagnostic categories in AML depend on the detection of cytogenetic abnormalities, most particularly in looking for the presence of balanced translocations, exchange of two portions of chromosomes in a way that

results in no net gain or loss of genetic material, or specific patterns of aneuploidy, gain or loss of chromosomal material in a non-balanced fashion that leads to a change in the total amount of genetic material. For this reason, cytogenetic diagnostic techniques are standard of care in AML. Three of the most common techniques, each with their own advantages and limitations, are conventional karyotyping, fluorescence in situ hybridization (FISH), and comparative genomic hybridization (CGH).

Conventional karyotyping is the oldest and perhaps the most straightforward of these techniques. In karyotyping, cells of interest are stimulated ex vivo with mitogens to induce chromosomal replication and then arrested at metaphase via treatment with cell cycle inhibitors such as colchicine. Cells treated in this fashion have their chromosomal material well organized into chromatids, and proper staining techniques lead to visualization of individual chromosomes, each with a recognizable and unique banding pattern due to alternating stretches of tightly and loosely packed DNA. With appropriate training, these banding patterns can be used to detect and enumerate chromosomes and even to detect whether chromosomal elements have been translocated, amplified, or deleted. This technique is very well suited for the detection of aneuploidy, since the presence or absence of major chromosomal segments is readily apparent. Translocations, duplications, or deletions involving large enough stretches of DNA can also be detected due to abnormalities in the banding pattern. Because the karyotype is analyzed in an untargeted, nonbiased fashion, conventional karyotyping is also an optimal technique for the detection of non-hotspot abnormalities, such as the wide range of aneuploidy that can be seen in AML with myelodysplasia-related changes.

The technique of conventional karyotyping leads to a set of trade-offs that limit its utility in certain areas. While manually enumerating every chromosome in the cell allows the technique to be broadly sensitive to a wide variety of changes, it makes the technique labor intensive and requires specialized training. Because of this, conventional karyotyping in clinical practice is usually limited to 20 or 30 cells from one sample; this is enough to reliably detect abnormalities in samples floridly involved by a neoplastic population but is extremely insensitive for trying to track low-level involvement by disease in the context of therapy or disease evolution. The reliance on banding patterns visible under the microscope also limits the resolution of the assay; changes involving chromosomal regions smaller than several megabases are generally invisible to this technique. Finally, the reliance on the experimental conditions necessary to induce and then arrest mitosis requires the collection of viable, unfixed cells that respond to ex vivo mitogen stimulation. This means that archival or other fixed specimens cannot be analyzed using this technique, limiting it to fresh sample only.

Some of these trade-offs are addressed by FISH, another cytogenetic technique. FISH is performed via base-pair hybridization between target DNA and long (several kilobase) fluorescently labeled nucleic acid probes. The probes and the target DNA are allowed to hybridize, and then fluorescence is assayed under the microscope. Depending on the way the probes are designed, different patterns of fluorescence can be observed which help detect gain or loss of chromosomal segments, as well as translocation or other chromosomal disruptions involving specifically targeted segments of DNA. For example, an increase in the number of signals observed in a single cell from two to three might indicate triploidy (acquisition of another chromosome) or simply focal amplification of the targeted DNA sequence. Translocations can be detected using probes aligned at either side of the common area of breakage; visualization of the probes distant from each other in the nucleus would indicate a translocation involving the targeted area. Due to the relative ease with which this kind of pattern can be interpreted compared to manual staining and karyotyping, a larger number of cells can be assayed with FISH; clinical assays typically assess between 200 and 500 cells per sample. This increases the clinical sensitivity of the test, allowing for better detection of relatively low-level disease involvement. The targeted probes

also allow for better resolution in FISH compared to conventional karyotyping; FISH can detect abnormalities at the level of tens to hundreds of kilobases, which is at least an order of magnitude more sensitive than the megabase-level resolution of conventional karyotyping. Finally, FISH can be performed on non-replicating cells and does not require ex vivo stimulation, meaning that it can be performed on formalin-fixed, paraffin-embedded archival material without the necessity for culturing viable cells.

The targeted nature of FISH's probe-based system leads to its disadvantages as well as its advantages. While conventional karyotyping can detect a wide range of abnormalities, including ones not suspected by the diagnostician before the test was performed, FISH can only detect abnormalities at areas covered by its target sequence, which represents a tiny fraction of the total cytogenetic material. Thus, FISH is poorly suited for detecting a non-specific karyotype and is liable to completely overlook unsuspected findings. FISH performs best when used in a small panel to test for abnormalities high on the pre-test differential diagnosis in new disease or to assess for the presence of a known abnormality in follow-up testing.

A newer technique that addresses some of the limitations of both conventional karyotyping and FISH, while introducing its own complications, is comparative genomic hybridization (CGH). This method can detect copy number alterations and map them to specific chromosomal locations with a relatively high resolution. The most widely available CGH technique in the clinical setting is array CGH, in which the target genome is fluorescently labeled (rather than the synthetic probes, as is the case in FISH) and hybridized to a standardized reference array. After hybridization, the degree of fluorescence is measured for each element of the standardized array, which gives a readout of relative abundance of chromosomal material mapped onto the array. The resolution of the technique is dependent on the number of elements used in the reference array and, with the current techniques, can be decreased to the order of tens or hundreds of kilobases. This allows for a mapping of chromosomal gains or losses with a resolution many orders of magnitude finer than conventional karyotype, with high confidence in the mapping of the abnormal areas to specific chromosomal regions without the need for specialized training in reading banding patterns. Because the procedure requires only genomic DNA from the target sample, archival fixed material can be used as well as fresh material, circumventing one of the other major limitations of conventional karyotyping. And because targets from across the genome are included in the standardized array, the CGH technique lacks the highly targeted "tunnel vision" issues that plague FISH.

Given the importance of recurrent cytogenetic abnormalities in the WHO classification of AML, cytogenetics continues to play a central role in disease diagnosis and classification. Clinically validated targeted FISH panels are readily available for all of the recurrent translocations and inversions. Additionally, FISH probes can be used to track recurrent patterns of aneuploidy, particularly those seen in AML with myelodysplasia-related changes. Conventional karyotyping still plays an important role, particularly at diagnosis, in order to assess for non-standard abnormalities that may not be picked up by targeted FISH probes. Given the greater clinical sensitivity of FISH relative to conventional cytogenetics, follow-up assessment of AML is best performed in conjunction with targeted FISH, while conventional karyotyping in this setting is reserved for the assessment of clonal evolution or, potentially, emergence of new therapy-associated dysplastic clones. Despite the cited advantages of CGH, its role in clinical practice is still evolving, and it is not yet in widespread clinical use for diagnosis or management of AML.

2.3.4 Molecular Genetics

Cytogenetic analysis is a powerful technique but is restricted in its scope to large-scale changes to chromosomal structure. An entirely different scale of genetic alterations occurs at the level of one or a few base pairs: point mutations, small

insertions or deletions, and other fine-scale genetic abnormalities. Testing for these abnormalities requires an entirely different set of tools, one that has exploded in scope and utility in the last decade or two. The results of this kind of molecular genetic analysis are being rapidly assessed and incorporated in classification and prognostic guidelines, with AML serving as a prime example of a disease process for which the entire diagnostic approach has changed as a result of these new techniques. As expected in an emerging field, many techniques are coming to the fore and being incorporated into clinical practice, but they fall into a few major categories: sequence-specific amplification techniques, Sanger sequencing, and next-generation sequencing.

Amplification-based assays are targeted via synthetic nucleic acid probes to particular areas of the genome. They use polymerase chain reaction (PCR) or related techniques to amplify segments of DNA or RNA including the targeted area of interest. Once the target area is amplified, follow-up studies are employed to investigate these amplified fragments, by their size, their hybridization characteristics, their abundance, or their specific sequence. With some systems, the targeted probes can be made to hybridize at hotspots of frequent mutation, so that the mere presence or absence of a product from a test reaction can indicate whether a particular genetic alteration is present. Using techniques such as real-time PCR, quantitative results can be obtained, allowing the levels of specific abnormalities to be followed over time, a technique which has been directly incorporated into the therapeutic strategies for some subtypes of AML [18].

Because of their targeted nature and their relative maturity as some of the first molecular assays developed, amplification-based assays are widely employed in clinical laboratories both in oncologic testing and elsewhere. Only DNA (or RNA, depending on the test type), not intact cells, is required to perform these tests, so testing is usually amenable to validation on fixed and archived material. In addition, the amplification of the sample over many cycles using targeted probes allows sensitive and reliable measurement of abnormalities present only at a very low level in the sample tissue, often at a level several orders of magnitude lower than the most clinically sensitive cytogenetic techniques, and at a level roughly comparable or even more sensitive than flow cytometry [19].

Amplification-based assays share the same Achilles' heel as other targeted techniques like FISH, in that only those abnormalities for which targeted probes have been designed and validated can be assayed. For certain common abnormalities with well-defined hotspots, these techniques are very well-suited and can readily be employed; excellent examples of this type of lesion in AML includes mutations in the *FLT3* and *NPM1* genes, as well as other genes with frequently occurring hotspot mutations such as *IDH1* or *IDH2*. However, the advent of other molecular techniques such as sequencing has revealed the breadth and diversity of non-hotspot molecular genetic changes in neoplastic populations, many of which have already been demonstrated to be clinically relevant. For these classes of mutations, there is no practical, efficient method to employ targeted amplification techniques. A separate issue with this class of assays is that, due to the necessity for binding of the targeted probes used to guide amplification, the tests rely on the presence of the complementary sequences to the probes in the diagnostic sample being assayed. If large-scale changes, such as deletion or extensive mutation, has removed the binding sites, the assay will fail with a false negative or equivocal result.

Sequencing methods directly read the genetic sequence in the area of interest. Sequencing may often be performed as a follow-on technique after some of the amplification-based systems described above, although in more modern, high-throughput incarnations, other systems for selecting areas for sequencing may also be used. Widely used sequencing assays in the previous generation relied on the well-known Sanger technique, named after its developer, which incorporates fluorescent or radio-labeled tagged terminating nucleotides to create an entire array of differently size fragments, with the size and

terminating label of each fragment spelling out the nucleotide present at that position. Sanger and other related previous-generation sequencing assays had the advantage of allowing direct review of the results to help with troubleshooting but were relatively laborious and expensive to scale up even to the level of coverage of a large gene such as *TP53*, much less an entire panel of genes relevant for one tumor type, and certainly were not feasible for projects such as whole exome sequencing.

Next-generation sequencing (NGS) is a blanket term for a family of related techniques which permit rapid upscaling of sequencing efforts to high-throughput environments, in which large, multigene panels and even whole exome or whole genome sequencing can be performed on a patient-by-patient basis in the clinical setting. In most NGS techniques, a large number of small sequencing reactions are typically carried out in parallel, allowing assessment of many targets (or of many samples for fewer targets) simultaneously. Selection of targets to sequence can be done via processing of whole exome or whole genome material, or by using targeted amplification-based or hybrid capture techniques. An important component of NGS techniques is an elaborate software "pipeline" to help filter the results for human review; the sequencing techniques employed are relatively error-prone, so testing errors need to be eliminated, along with the large number of benign variants identified that are unrelated to disease.

The advantages of sequencing in current practice center on the increased volume of data that the assays provide. With NGS techniques, the "tunnel vision" issue that smaller scale targeted techniques create can be minimized through sheer brute force, by sequencing more and more targets. At the current time, routine analysis of whole genome data is not economically or informatically practicable, but large panels (tens to hundreds) of clinically relevant genes can be tested at once, and not just at commonly mutated hotspots. This has led to increasing recognition of the clinical importance of large genes without significant targetable hotspots, such as *TP53*, both in AML and in other hematologic neoplasms. The high level of coverage provided by NGS leads to other benefits as well; in cases where fewer genetic regions need to be examined, the bandwidth of the assay can be used to provide deeper coverage of the smaller number of targets. This leads to better clinical sensitivity of the assay, where smaller abnormal populations can be detected due to the sequencing of many DNA segments from a mixed sample. Thus, NGS assays can be tweaked to provide some combination of broad coverage or high clinical sensitivity, based on the clinical needs.

This flexibility in assay design, as well as the sheer volume of data produced, leads to some of the most confounding challenges facing those who wish to use NGS in the clinical arena. Because the field is still in a state of active evolution, and because everything from the selection of the genes of interest to the method for filtering the resulting data is in a state of innovative flux without well-validated guidelines for standardization, comparison of data collected by different centers or using different techniques is less straightforward than it might appear by simply reviewing the final, synthesized reported information. Even with acceptably standardized and validated assays, the sheer volume of data produced by NGS studies can create its own problems. It can be difficult for clinicians and diagnosticians, either in the clinical trial setting or in the routine care of patients, to separate out genetic variants into clearly benign changes or polymorphisms, clearly disease-associated mutations, and potentially novel abnormalities that may be associated with the patient's disease.

Even for mutations that have been well-demonstrated to be definitively associated with disease, it can be surprisingly difficult to assign a particular diagnostic or therapy-guiding role. AML provides excellent examples of such challenges. Mutations in genes like *NPM1* and *FLT3* (particularly internal tandem duplications or ITDs) were thought to be well-understood on the basis of targeted amplification studies before the wide advent of NGS: *NPM1* mutations were associated with relatively good prognosis, and *FLT3* ITD mutations were associated with relatively poor prognosis, with the effects of *FLT3* to

some degree trumping those of *NPM1* in leukemias where the mutations co-occurred [20, 21]. However, with more data derived from NGS studies, it appears that the picture is muddier, with the prognostic effect of these mutations likely dependent to some degree on the presence or absence of mutations in other genes, including *IDH1*, *IDH2*, and *DNMT3A*, as well as the prevalence of the *FLT3* ITD mutation in the clone [22–24]. The importance of these genetic combinations is still in flux, owing to some degree to the problems of statistical power brought about by the need to measure so many different genetic combinations.

Current strategies for the diagnosis and follow-up of AML rely heavily on molecular genetic techniques. Given the increasing number of genetic targets that are relevant to the classification and prognostication of AML, new diagnosis is best done in conjunction with a multi-gene panel, typically performed via NGS, that assays for a wide range of genes. One potential issue with using a large NGS panel as an upfront diagnostic tool is the potentially long (5–10 days) turnaround time, particularly if the testing is performed at a reference laboratory. The mutational status of some AML-associated genes is playing an increasingly important role in initial therapy, both on clinical trials and in routine practice. For example, given the importance of FDA-approved inhibitors for AML with mutations in *IDH1* [25], *IDH2* [26], or *FLT3* [27], rapid assessment of mutational status for these genes, all of which have mutations primarily focused in hotspots, is necessary. For this reason, faster-turnaround amplification-based methods may be performed in conjunction with more comprehensive NGS panels in order to provide timely access to critically important clinical data.

2.4 Clinical Diagnostic Testing in Residual Disease

The laboratory testing modalities described above have been used in various forms for decades in the assessment and categorization of newly diagnosed oncologic disease, including AML. Diseases could be classified, stratified for prognostic purposes, and triaged for consideration for targeted therapeutic intervention. This remains an important and rapidly growing area for clinical diagnostics, but many of the same testing modalities also present opportunities for analysis in subsequent examination of the patient for residual disease.

Of particular interest to clinicians in hematologic malignancies, including AML, is determining whether the neoplastic clone remains in the patient at various stages of therapy. Older descriptors for disease status like “complete remission” (CR) are relatively insensitive; in AML, CR is defined as a decrease in blasts below 5% of total marrow cells (along with recovery of peripheral counts, return of marrow cellularity and normal hematopoiesis, and absence of extramedullary disease), but without necessarily distinguishing between normal marrow myeloblasts and malignant leukemic blasts. While this classification approach was a reasonable one based on the diagnostic modalities available at the time—primarily morphologic review and some basic immunophenotypic assays such as cytochemistry, and remains in clinical use today, modern methods of disease detection have made it abundantly clear that residual levels of abnormal cells can be readily detected after therapy in some groups of patients. Furthermore, the presence or absence of this low-level involvement can be shown to play a major role in determining prognosis and frank relapse risk. In many cases, the best predictor of relapse is the persistence of abnormal cells after therapy, often at levels of detection below the threshold for classic CR and other categories; this low-level persistent disease is termed “minimal residual disease” (MRD).

The utility of MRD detection was first and most extensively demonstrated in B lymphoblastic leukemia (B-ALL), particularly in the pediatric setting, and flow cytometric assessment of MRD is currently a well-established tool for the prognosis, monitoring, and treatment of B-ALL [28, 29]. The role of MRD testing in AML is an area of active and maturing development, with roles being defined for testing both by flow cytometry and by targeted genetic studies.

In general, detection of MRD by flow cytometry is based on a set of important technical principles. It is critical to be able to distinguish the abnormal leukemic population from normal precursors by its immunophenotypic characteristics. While B-ALL MRD detection has become at least somewhat standardized, approaches to AML MRD detection by flow cytometry are currently somewhat more variable between different centers. Some centers have emphasized more of a holistic different-from-normal approach to analysis, looking at whole populations of maturing myeloid cells and looking for abnormalities in those patterns, while other centers have focused more on looking at specific early populations of cells and evaluating their expression of various combinations of abnormal markers [30]. Both of these approaches are made challenging by the nature of the AML blast populations. AML shows more variation in the "leukemia-associated immunophenotype" (LAIP) at the time of diagnosis than B-ALL does, with less ability to rely on a reproducible gating scheme to routinely isolate the neoplastic population. Additionally, AML blasts show an even greater propensity than B-ALL blasts to alter their LAIP over time, requiring greater vigilance on the part of those monitoring for MRD. For these reasons, many of the largest AML trials have validated a cutoff of 0.1% for MRD detection, rather than the 0.01% used in B-ALL [31].

While flow cytometry is a reliable technique for MRD detection, able to be used in the vast majority of cases, the difficulty and relative lack of reproducibility due to the heavy analytic requirements of the assay have led to the use of molecular genetic techniques for MRD detection. As described in the previous sections, amplification-based targeted genetic assays can detect extremely small abnormal populations in a mixed sample. For those patients who have genetic lesions at diagnosis amenable to this type of testing, molecular methods for MRD detection are of great utility. *NPM1*-mutated AML is a prime example of this approach in myeloid disease; the detection of *NPM1* mutations by targeted amplification has been shown to be a powerful MRD detection tool [32]. Caveats apply to this approach, however; *NPM1*-mutated AML may relapse as a *NPM1*-negative clone [33], leading to potential false-negative results for MRD assays based on tracking of a single genetic lesion. The same problem arises for many other genetic abnormalities that could be tracked in patients, meaning genetic techniques for MRD detection must often be complemented with other tools, such as flow cytometry. The presence of clonal rearrangements of the *TCR* and *IGH* loci in T- and B-lymphoblastic leukemias that are almost never lost during clonal evolution provides a powerful tool for MRD monitoring in those diseases using specialized NGS panels with high clinical sensitivity [34, 35], but a similar common and invariant abnormality in AML has not yet been identified.

These methods for developing tools for MRD detection have been applied to AML with significant success [19]. Flow cytometry and molecular genetic MRD detection at the end of induction chemotherapy have been shown to be important for prognosis of AML patients to a much greater degree than standard CR status, both in the setting of conventional therapy [36, 37] and in allogeneic stem cell transplant (SCT) [38]. In the specific setting of SCT, MRD detection at the time of transplant has been shown to be strongly associated with the risk of relapse after transplant [39], potentially helping to determine which patients should be eligible for transplant and which should be triaged for additional pre-transplant therapy.

2.5 AML Classification

2.5.1 The WHO Classification Framework

Acute myeloid leukemia (AML) is a heterogeneous disease clinically, morphologically, and genetically. In the latter half of the twentieth century, the FAB classification was in general use for the classification of AML. This classification, a standardization of the historical approach to classification, used morphologic, cytochemical, and later flow cytometric features to classify AML

based on how leukemic blasts recapitulate normal hematopoiesis (lineage of differentiation and level of maturation of blasts). While useful for laboratory description of leukemic blasts, by the end of the twentieth century, this approach had become obsolete for clinical practice and of limited utility for correlation with rapidly expanding cytogenetic, molecular genetics, and biologic knowledge.

An alternate approach was proposed in 1995 [1] which grouped most patients with AML into two broad biologically and clinically meaningful, although imprecisely defined, groups: de novo AML (DN-AML), meaning patients with no antecedent marrow abnormalities (not to be confused with the clinical usage of that term), and secondary AML (s-AML), meaning patients with antecedent hematopoietic disease, irrespective of whether it was recognized clinically (Fig. 2.1). DN-AML cases occur more frequently in younger patients, with a median age in the 30s and a relatively flat incidence curve for population at risk, implying a relatively simple pathogenesis.

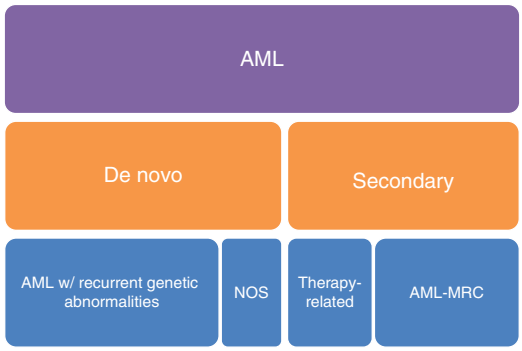

Fig. 2.1 Approximate representation of the broad biologically and clinically relevant categories of AML as they map on to the WHO 2016 classification framework. These categories are currently imprecisely defined by clinical features and currently available diagnostic methods. The proportional distribution of the de novo and secondary AML cases depends on demographics of the population at risk (i.e., de novo AML is more common in children and young to middle-aged adults with an incidence rate that is relatively flat throughout life. On the other hand, secondary AML with most cases corresponding to AML-MRC in the WHO classification framework is most common in the elderly patients comprising most AML cases beyond 60 years of age with a median age in the 70s)

s-AML cases tend to occur in older patients, with a median age over 60 years and an exponential incidence curve implying a random multistep pathogenesis. This approach was adopted in the WHO classification of 2001, the third edition of the World Health Organization Classification of Tumours of Haematopoietic and Lymphoid Tissues, as "AML with recurrent genetic abnormalities" (AML-RGA) and "AML with myelodysplasia-related changes" (AML-MRC) respectively, with subsequent elaboration in the 4 th edition and in a revision (2017) (Table 2.1) [2, 40, 41]. This classification was designed to be

Table 2.1 WHO classification of acute myeloid leukemia

AML with recurrent genetic abnormalities (mostly de novo AML)
AML with t(8;21)(q22;q22.1); *RUNX1-RUNX1T1*
AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11*
Acute promyelocytic leukemia (APL) with t(15;17)(q22;q12); *PML-RARA*
AML with t(9;11)(p21.3;q23.3); *MLLT3-KMT2A*
AML with t(6;9)(p23;q34.1); *DEK-NUP214*
AML with inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); *GATA2,MECOM*
AML (megakaryoblastic) with t(1;22)(p13.3;q13.3); *RBM15-MKL1*
Provisional entity: AML with BCR-ABL1
AML with mutated *NPM1*
AML with biallelic mutations of *CEBPA*
Provisional entity: AML with mutated RUNX1
AML with myelodysplasia-related changes (mostly secondary AML)
Therapy-related myeloid neoplasms (mostly secondary AML)
AML, NOS
AML with minimal differentiation
AML without maturation
AML with maturation
Acute myelomonocytic leukemia
Acute monoblastic/monocytic leukemia
Pure erythroid leukemia
Acute megakaryoblastic leukemia
Acute basophilic leukemia
Acute panmyelosis with myelofibrosis
Myeloid sarcoma
Myeloid proliferations related to Down syndrome
Transient abnormal myelopoiesis (TAM)
Myeloid leukemia associated with Down syndrome

flexible to allow incorporation of new entities as they are recognized. It differs fundamentally from the historical (FAB) approach.

In the WHO classification, AML-RGA encompasses most cases of DN-AML, including cases with recurring balanced translocations. WHO AML-MRC encompasses most cases of s-AML, which are cases with often multiple genetic and mostly unbalanced chromosomal abnormalities, with background features frequently suggesting underlying myelodysplastic syndrome (MDS). Distinction between these pathogenetically different categories of AML is not always straightforward but is important clinically for therapeutic decisions. AML-RGA (DN-AML) usually has normal background hematopoiesis at presentation and in remission, with normalization of peripheral blood counts (complete remission). Patients with AML with recurrent genetic abnormalities tend to be younger and have relatively simple genomic aberrations. Most cytogenetically and molecularly defined subtypes under this category are recognized as distinct clinicopathologic entities and comprise approximately 65–70% of all AML cases [23, 42, 43] (Fig. 2.2). This is in contrast to AML-MRC (s-AML) which tends to occur in older patients, have MDS-like background hematopoiesis, with poor marrow reserve, the probability of reversion to clonal hematopoiesis (i.e., MDS), and persistent cytopenias during "remission" (sometimes called complete remission with incomplete recovery of counts [CRi]). AML-MRC also has a high frequency of resistance to conventional chemotherapy at presentation.

AML arising in the setting of prior cytotoxic therapy is classified under the WHO category as "Therapy-related myeloid neoplasms" [2]. The most common cause of therapy-related myeloid neoplasms is treatment that causes DNA interstrand crosslinks or DNA double-stranded breaks (alkylating agents, platinum derivatives, nitrosoureas, or ionizing radiation). AML cases in this group are typically associated with unbalanced chromosomal aberrations and major gains or losses of chromosomes and are an iatrogenic model of AML-MRC (Table 2.2). A second cause of therapy-related myeloid neoplasms is topoisomerase II inhibitor therapy, which is typically associated with balanced chromosomal translocations (especially involving the genes *KMT2A/MLL* and *RUNX1*); this subset appears to be an iatrogenic model of AML with recurrent genetic abnormalities. An uncommon third type of therapy-related AML, seen in patients receiving any mix of complex chemotherapy and/or radiation, is a several log increase in the incidence of the common balanced translocations of AML-RGA [44].

The precise biology of DN-AML and s-AML has been the subject of recent genomic investigation [45]. Most AML with recurrent genetic abnormalities is characterized by a single balanced translocation and a low number of other gene mutations, most frequently activating mutations in the signaling genes including *NRAS*, *FLT3*, *KIT*, other tyrosine kinases, and protein tyrosine phosphatases. Also included in this group are cases with a normal karyotype and mutations in *NPM1* or (biallelic) *CEBPA* genes.

Whole-genome sequencing studies have shown that progression from MDS to s-AML involves sequential acquisition of mutations at the stem cell level resulting in survival and proliferation advantages [46, 47]. At the MDS stage most differentiated cells contain identical mutations, indicating marrow involvement by a clonal process. At the AML stage, several clones defined by acquisition of new sets of mutations are present, as well the original set of stem cell mutations, indicating clonal evolution. The new mutations tend to be in genes involved in adhesion, cell death, cell cycle regulation, differentiation, metabolism, motility, signaling, transcription, and transporter proteins [47, 48].

An investigation of the genetic basis of AML ontogeny comparing the spectrum of genetic lesions in well-defined s-AML patients (including therapy-related disease) and DN-AML identified three distinct mutually exclusive patterns of mutations [45]. First, three abnormalities significantly under-represented in AML-MRC (s-AML) are *NPM1* mutations, *KMT2A*(*MLL*)/11q23 rearrangements, and core binding factor (CBF) rearrangements (so-called de novo-type alterations); it should be noted that by definition *NPM1*

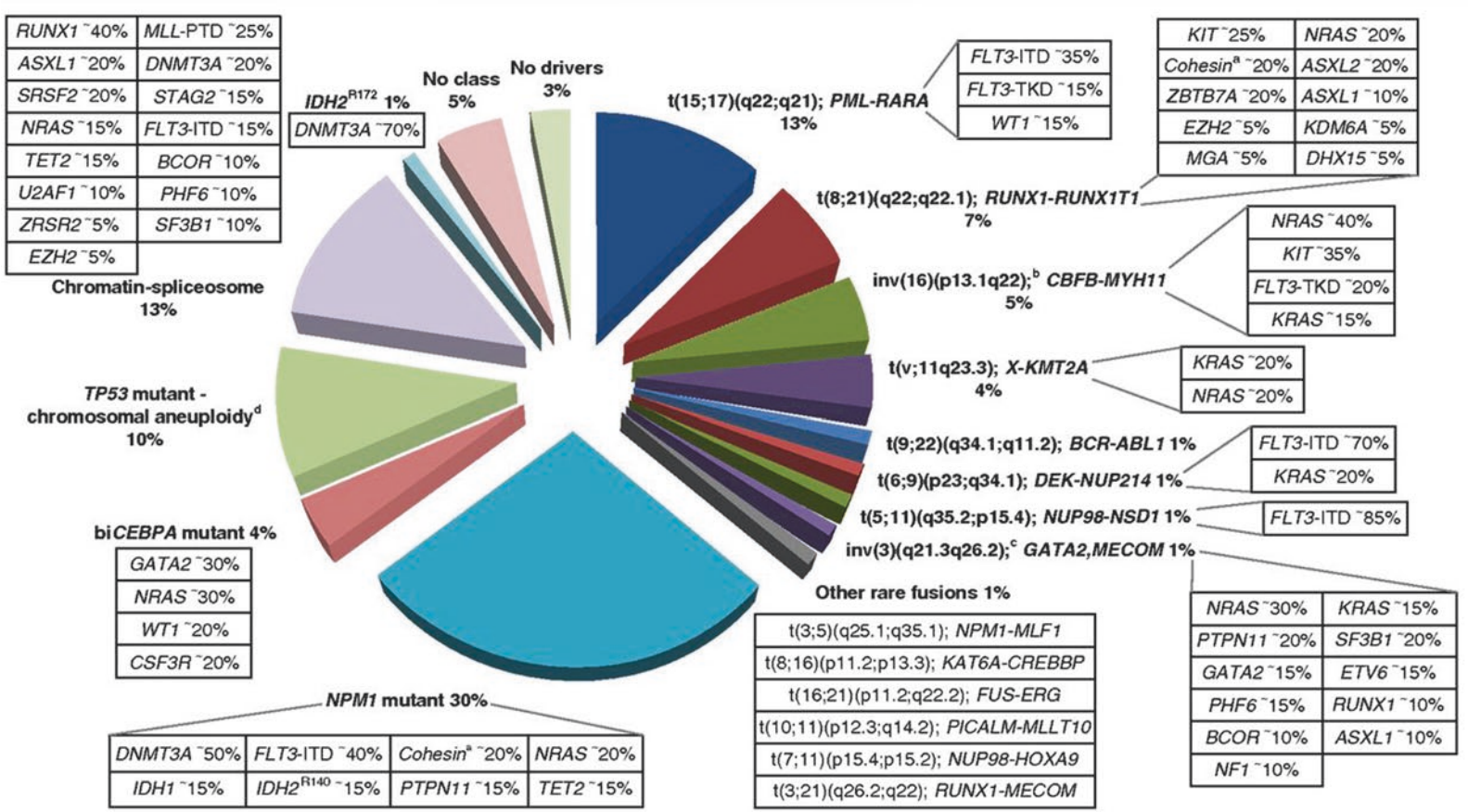

Fig. 2.2 Proportional distribution of AML cases associated with specific genetic lesions including most cases of de novo AML due to gene fusions and mutations with class definitions based on the study by Pappaemmanuil et al. [23]. For each AML class indicated in the pie chart frequent co-occurring mutations are shown in associated boxes. (Republished with permission of *Blood* from Döhner H, Estey E, Grimwade D, et al. Diagnosis and management of AML in adults: 2017 ELN recommendations from an international expert panel. *Blood*. 129:424–447, 2017; permission conveyed through Copyright Clearance Center, Inc.)

Table 2.2 Two major classes of therapy-related AML

	Alkylating agent class	Topo II inhibitor class
Cytogenetics	Del(5q), −7/del(7q), complex	Balanced translocations involving 11q23, 21q22, others
Frequency	~70%	~30%
Latency	Long (5–7 years)	Short (2–3 years)
Preceded by MDS phase	Typically, yes	No
Implicated medications	• Alkylating agents: bendamustine, busulfan, carmustine, chlorambucil, cyclophosphamide, dacarbazine, lomustine, melphalan, mitomycin C, nitrogen mustard, procarbazine, thiotepa • Platinum-based agents: cisplatin, carboplatin • Antimetabolite agents: azathioprine, fludarabine	• Anthracyclines: daunorubicin, epirubicin, doxorubicin • Other topoisomerase II inhibitors: etoposide, teniposide, amsacrine, mitoxantrone

mutations are placed in AML-RGA in the WHO classification and that AML with *PML/RARA* was not included in the study. Second, mutations (referred to as "secondary-type") in eight genes mostly belonging to spliceosome and chromatin modifier functional classes (*SRSF2*, *SF3B1*, *U2AF1*, *ZRSR2*, *ASXL1*, *EZH2*, *BCOR*, and *STAG2*) appear to be highly specific (>95%) for AML-MRC (s-AML). The mutations in these genes are commonly seen in MDS, appear early in leukemogenesis, and persist in clonal remissions. Third, mutations in *TP53* are associated with a distinct clinical phenotype including complex karyotype, therapy resistance, and very poor survival [45]. No distinct genomic patterns were specific for WHO defined therapy-related AML, perhaps because these cases appear to be comprised of several different entities, as discussed above. These cases were distributed throughout the three mutational patterns mentioned above. This information suggests a genetic framework for future classification of AML into biological, pathogenetic, and clinically relevant groups.

2.5.2 AML with Recurrent Genetic Abnormalities

Biologically, the flat incidence curve of most AML-RGA suggests a single rate-limiting pathogenetic step (not a single step, but a single rate-limiting step) in development of disease (Fig. 2.3). To the extent that the molecular pathogenesis of AML-RGA has been clarified, most cases are characterized by one of a series of recurring genetic abnormalities that block differentiation of hematopoietic precursors, and a superimposed additional molecular abnormality(−ies) that drives proliferation.

2.5.2.1 Acute Promyelocytic Leukemia with *PML-RARA* and Variant Translocations

Acute promyelocytic leukemia (APL) with PML-RARA has a predominance of abnormal promyelocytes with characteristic nuclear morphology and cytoplasmic granulation. In AML it represents the best correlation of genetics with morphology. There is a spectrum from hypergranular to microgranular blast cell morphology in different patients. Recognition of the morphologic features of APL is extremely important for the early diagnosis and institution of targeted therapy with ATRA for this type of otherwise favorable prognosis AML, in order to prevent early and potentially serious complications of coagulopathy. The typical variant of APL has distinctive hypergranular blasts (abnormal promyelocytes) with prominent azurophilic granules which tend to obscure the boundary between the nucleus and the cytoplasm. They have distinctively shaped bilobed or grooved nuclei sometimes resembling an apple core. Some, but not all, cases have frequent Auer rods, and occasional cells with multiple Auer rods which sometimes are seen in bundles (so-

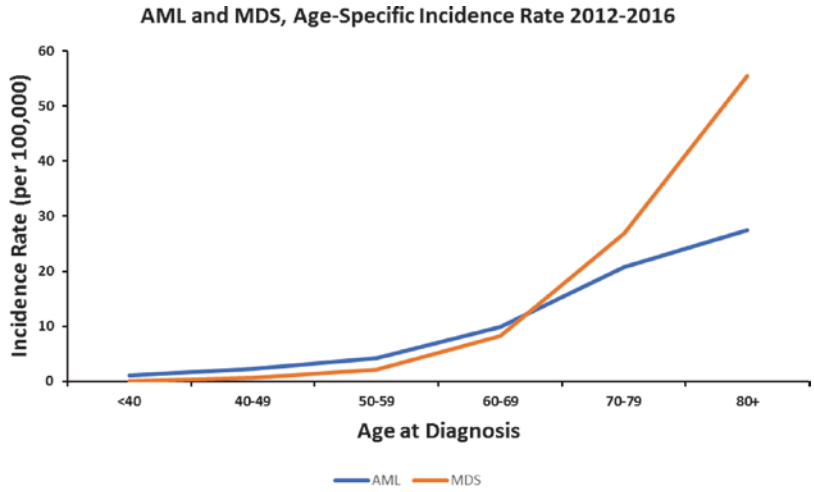

Fig. 2.3 Age-specific incidence rate of AML and MDS based on SEER data. MDS and secondary AML with most cases corresponding to AML-MRC in the WHO framework are most common in the elderly patients comprising most AML cases beyond 60 years of age with a median age in the 70s. While AML-MRC does occur in children and young adults, its incidence for population at risk comprises an exponential curve with progressive age, accounting for this feature of the incidence curve of AML as a whole

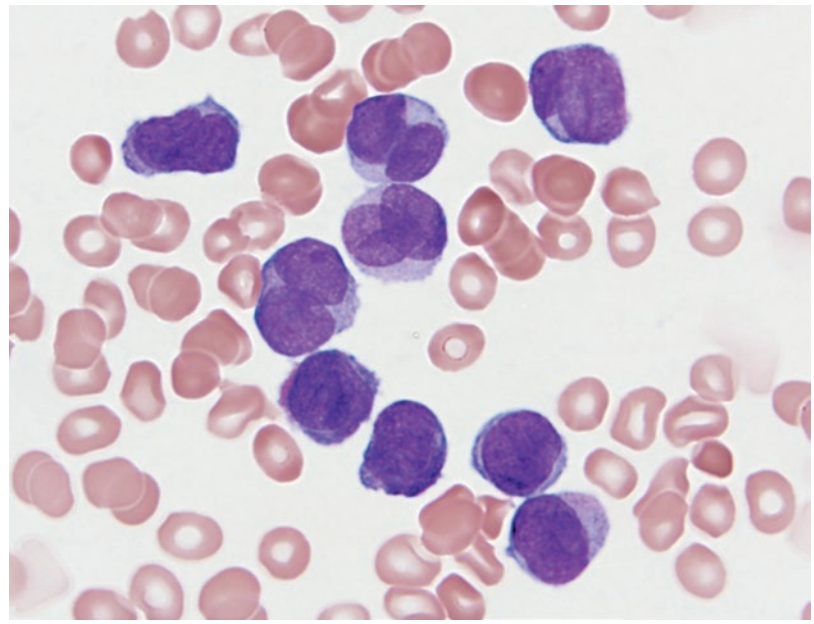

Fig. 2.4 Microgranular (hypogranular) APL (peripheral blood smear stained with Wright's stain at 1000× original magnification). Abnormal promyelocytes in this variant have few obvious cytoplasmic granules on Romanowsky stain but retain the characteristic distinctively shaped bilobed or grooved nuclei sometimes resembling an apple core

called faggot cells). The hypergranular variant often has low WBC counts and a low number of circulating blasts. The microgranular (hypogranular) variant (Fig. 2.4) may be more difficult to recognize, especially by an inexperienced observer, as the abnormal promyelocytes in this variant have few obvious granules with Romanowsky staining; however, even in the microgranular variant, the abnormal promyelocytes have similar nuclear features to the typical variant and if carefully searched for, at least a few typical cells with dense cytoplasmic granulation can be found, especially in the region of the perinuclear hof. These blasts still have the capacity to

release thrombogenic substances, as in the hypergranular variant. Patients with the microgranular variant tend to have a higher number of blasts in the peripheral blood.

By flow cytometry, there is a bright expression of myeloperoxidase and CD33 with variable expression of CD13 and CD117 and frequent expression of CD64 by the APL blast cells, which are typically negative for CD34 and HLA-DR. However, in the microgranular variant CD34 is frequently expressed, and there may be aberrant expression of CD2 [2]. Of note, as noted previously, APL blasts often display autofluorescence, complicating interpretation of data. An autofluorescence control should be run to correct for this problem [2, 49–51].

The successful treatment of APL with retinoic acid (RA) is a fascinating example of the potential power of targeted clinical application of molecular findings. As the association between t(15;17) (q22;q21) and APL was known, the near simultaneous reports [52] that oral all-trans retinoic acid (ATRA) induced complete remission in APL and that the retinoic acid receptor alpha gene (*RARa*) was located at 17q21 [53] led quickly to the demonstration that t(15;17) involves *RARa* and a previously unrecognized partner, *PML* [50, 51, 54].

The t(15;17) fuses the 5′ portion of the *PML* (ProMyelocytic Leukemia) gene at 15q24.1 and the 3′ portion of the *RARA* gene at 17q21.2. The breakpoint in *RARA* is invariant in intron 2, incorporating in the fusion protein the C-terminal portion of RARA including its DNA-binding, ligand-binding, dimerization, and repression domains. There are three possible breakpoint regions in PML. The most common bcr-1 in intron 6 includes the first six exons of *PML* and is designated PML(L)- RARA [55]. The second bcr-3 in intron 3 generates a shorter transcript, PML(S)-RARA. The third bcr-2 occurs within exon 6. RT-PCR using a single 3′ *RARA* primer and 2 *PML* primers to encompass the breakpoint sequences in introns 3 and 6 detects all three transcripts. FISH will also detect all *PML-RARA* fusion gene variants. These variant *RARA* breaks have no apparent clinical significance.

RARA is half (with retinoid X receptor or RXR) of a heterodimer ligand-dependent nuclear membrane receptor which mediates the cellular effects of RA. The heterodimer binds to RA response elements (RAREs) in the promoters of many genes important in myeloid differentiation. In the absence of RA, wild-type RARA/RXR on RAREs binds to the co-repressor proteins SMRT, N-CoR, mSin3, and histone deacetylases. Deacetylation of histone at the promoter, mediated by this complex, results in transcriptional repression, blocking cellular differentiation. Physiologic concentration of retinoic acid (10^{-8} M) causes a conformational change of the receptor, release of co-repressors, and recruitment of a co-activator complex (SRC-1) which associates with histone acetyltransferases [56]. This new complex mediates acetylation of histones at the promoter, relaxes chromatin conformation, and allows transcription to proceed (reviewed in [57]), resulting in cellular differentiation.

PML/RARA also heterodimerizes with RXR and binds to RAREs, competing with RARA/RXR in a dominant negative manner. In the absence of ligand, PML/RARA (via its 3′ RARA portion) binds co-repressor proteins similarly to RARA but requires pharmacologic concentration of retinoic acid, in the form of ATRA (10^{-6} M), to release them and bind to the co-activator complex. This is the mechanistic basis for the induction of differentiation of leukemic cells in APL with pharmacologic dosage of ATRA [56, 58].

Other translocations involving the RARA locus on 17q21.2 have been described. Studies of APL with variant RARA translocation t(11;17) (q23.2;q21.2); *ZBTB16-RARA* [58, 59] has furthered understanding of the mechanism of response of APL to ATRA. Patients with t(11;17) AML are resistant to treatment with pharmacologic dosage ATRA. The fusion partner gene *ZBTB16* on chromosome 11q23 encodes the promyelocytic leukemia zinc finger (PLZF) protein, a transcriptional repressor that contributes a second co-repressor binding site to the fusion protein. Although pharmacologic dosage ATRA induces release of co-repressors from the RARA portion of the fusion protein, those binding to PLZF are unaffected [56, 57, 60]. Addition of

Trichostatin A, which inhibits the deacetylase activity of PLZF-associated co-repressors [58, 61], allows induction of differentiation to proceed. Leukemia with *ZBTB16-RARA* has some morphological differences from typical APL, with blasts showing more regular nuclei. In some cases, the "blasts" approach the appearance of myelocytes [59]. Typically, Auer rods are absent. There may be an increased proportion of neutrophils with pseudo Pelger–Huet appearance [2]. Another rare fusion partner with RARA is *STAT5B* at 17q21.2. Similar to APL with *ZBTB16-RARA*, APL with *STAT5B-RARA* appears to be resistant to ATRA [2].

Finally, two additional partners involved in variant translocations in APL, both of which are ATRA responsive, are *NPM1* in t(5;17)(q35;q21) [62] and *NUMA1* in t(11;17)(q13.4;q21.2) [63]. All translocation partners of *RARA* encode proteins with multimerization domains, and all appear to contribute a block of differentiation to leukemia pathogenesis.

Wild-type PML protein is normally localized in subnuclear PML oncogenic domains (PODs), also called nuclear bodies (NBs), in which other nuclear factors colocalize [64]. PML may act as a tumor suppressor protein and is involved in growth suppression as well as in induction of apoptosis (reviewed in Ref. [57]). Although it does not bind DNA directly, it influences transcription by interacting with both the transcriptional activator CBP [65] and transcriptional repressor HDACs, possibly within the NBs. The protein encoded by the *PML-RARA* fusion transcript resulting from the t(15;17) translocation is delocalized from the NBs to a multigranular nuclear pattern with nucleolar exclusion [66].

Whole genome sequencing of de novo and relapsed APL patients has demonstrated approximately 8 non-silent somatic mutations per exome [67]. In de novo APL cases, mutations in *FLT3*, *WT1*, *NRAS*, and *KRAS* were predominant. In relapsed APL, there were frequent mutations in *RARA* and *PML*, with *RARA* mutations predominating in cases with a history of ATRA treatment and *PML* mutations associated with arsenic trioxide treatment. In addition, in relapsed APL, there were mutations in *ARID1A* and *ARID1B*, members of the SWI/SNF chromatin remodeling complex [67].

Although by convention an arbitrary level of 20% blasts is required for diagnosis of AML, the presence of the recurrent translocation t(15;17) is diagnostic of APL even when present in a small percentage of cells. Of note if the clinical setting is post chemo/radiotherapy, diagnosis should be therapy-related myeloid neoplasm as the main classification of the patient's disease [2].

2.5.2.2 AML with Core Binding Factor Translocations

Core binding factor (CBF) AML refers to AML-RGA characterized by the recurring structural abnormalities t(8;21)(q22;q22), involving *RUNX1(CBFA1)* and *RUNX1T1*, and inv(16)(p13.1q22), involving *CBFB* and *MYH11*. Together, these comprise 30% of pediatric AML cases after infancy and 15% of adult AML cases [68]. The *RUNX1* (Runt-related transcription factor 1, formerly called *AML1*) gene was cloned from the t(8;21)(q22;q22) breakpoint [69, 70]. In addition to involvement in this translocation, it is also mutated in another 3% of AML. The activity of the murine counterpart of RUNX1 was first described as part of the core binding factor complex (CBF), which binds to a core enhancer sequence of the Molony leukemia virus long terminal repeat (LTR) [71]. A second component of CBF, the non-DNA binding CBFβ, was subsequently found to be involved in AML-RGA with inversion 16 [72]. Finally, the fusion partner of *RUNX1* in t(8;21), named *RUNX1T1*, formerly *ETO* (eight-twenty-one), also encodes a transcriptional regulator [73]. The wild-type CBF complex recruits additional transcription factors, regulates hematopoietic differentiation, and is essential for hematopoietic development. Gene deletion of either *Runx1* [74] or *Cbfβ* [75] in mice results in fetal death at E11.5–12.5. These embryos lack all fetal hematopoiesis. Further transgenic experiments have demonstrated that RUNX1 is essential for the development of hematopoietic stem cells in the aorta/gonadal/mesodermal (AGM) region, the source of definitive hematopoiesis [76]. The essential role of RUNX1 in hematopoietic development appears

to be through its function as a transcriptional activator. *RUNX1*, located at chromosome 21q22.3, is encoded by 12 exons over 260 kb of DNA. The N-terminal portion of the protein is the DNA binding domain. This region is mutated in familial platelet disorder (FPD) and in AML associated with *RUNX1* mutations [77, 78]. CBF*β* interacts via this domain and changes the conformation of RUNX1 to increase DNA binding affinity [79].

The blasts in AML with t(8;21) *RUNX1T1/RUNX1* translocation display a degree of granulocytic differentiation, with salmon pink coloration associated with the perinuclear hof (Fig. 2.5), but exceptions are frequent (about 20% of cases).

In the t(8;21) translocation, the *RUNX1* gene is fused to the *RUNX1T1* gene on chromosome 8. The RUNX1-RUNX1T1 protein specifically binds to the same DNA binding site as RUNX1, heterodimerizes with CBF*β* [80] and acts as a dominant negative inhibitor of wild-type RUNX1. RUNX1-RUNX1T1 also functions as an active transcriptional repressor [81] by associating with class I histone deacetylases (HDACs) via RUNX1T1 [82]. Targets of RUNX1-RUNX1T1 repression are presumed to include genes important for granulocyte differentiation. In addition, RUNX1-RUNX1T1 represses the tumor suppressor genes *P14ARF* and *NF1* [83, 84]. P14ARF stabilizes TP53 by antagonizing MDM2, an inhibitor of TP53 [85]. Therefore, repression of *P14ARF* reduces the checkpoint control path of TP53 and may be a key event in t(8;21) leukemogenesis.

AML with inv(16) or t(16;16), the *CBFB/MYH9* translocation, present in about 8% of AML cases, correlates frequently (50–60% of cases) with myelomonocytic differentiation and with dysplastic eosinophils containing immature basophilic as well as eosinophilic granules (basoeosinophils) (historic FAB category M4Eo) (Fig. 2.6) [86].

This cytogenetic abnormality fuses the first 165aa of *CBFβ* to the C-terminal of *SMMHC* encoding its coiled-coil region [87]. The CBF*β*/SMMHC fusion protein associates with mSin3a and HDAC8 and interacts with RUNX1 to form a transcriptional repressor complex [88].

A number of experiments demonstrate that the CBF translocations are necessary but not sufficient for induction of leukemia. Support for the hypothesis that genetic mutations besides a mutant *RUNX1* locus are necessary for development of acute leukemia comes from the study of patients with familial platelet disorder with propensity to develop AML (FPD/AML). These patients have mutations in one allele of *RUNX1*

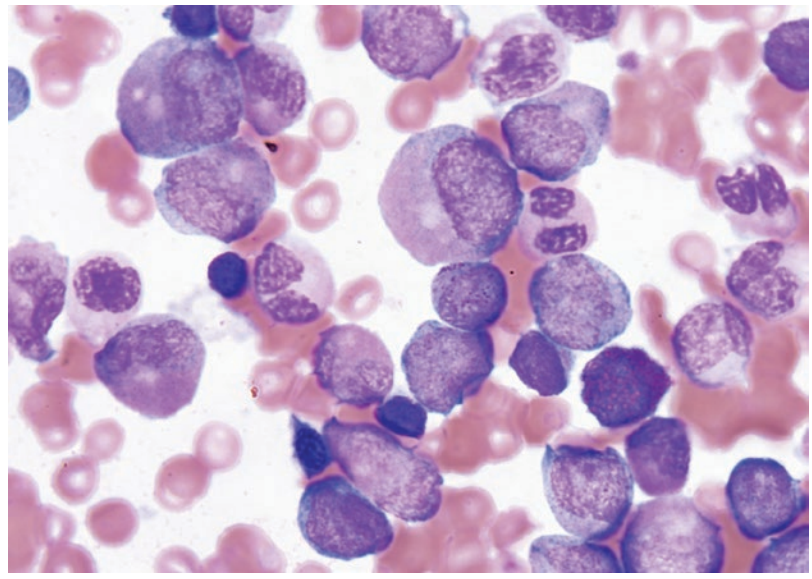

Fig. 2.5 AML with t(8;21);*RUNX1-RUNX1T1* (bone marrow aspirate smear stained with Wright's stain at 1000× original magnification). There is some degree of granulocytic maturation with characteristic salmon-coloration of the cytoplasm in perinuclear hof area

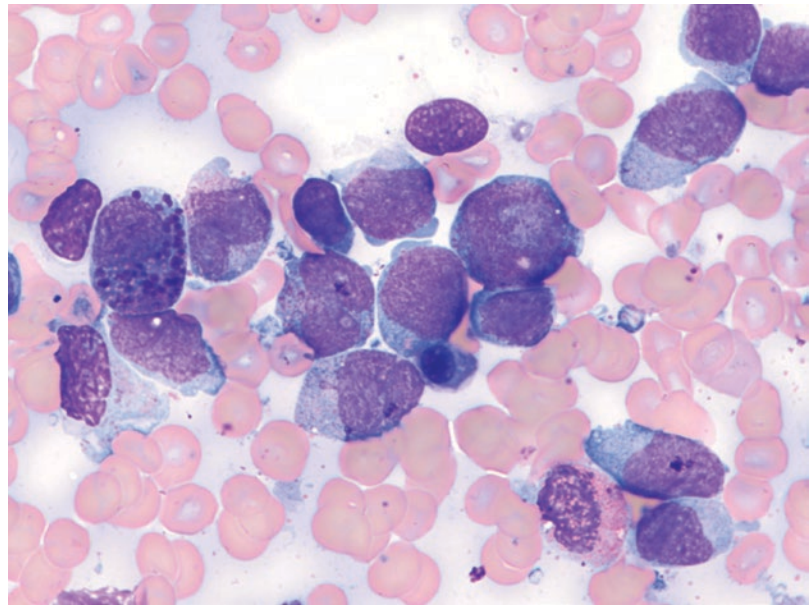

Fig. 2.6 AML with inv16 (bone marrow aspirate smear stained with Wright's stain at 1000× original magnification). There is myelomonocytic differentiation with dysplastic eosinophils containing immature basophilic as well as eosinophilic granules (baso-eosinophils)

[89]. They have defective platelets and progressive pancytopenia and develop myelodysplasia and a high incidence of AML with age. However, secondary mutations appear to be necessary before progression to AML occurs. Another set of studies also support this hypothesis. Guthrie spot studies (drop of blood obtained at birth and stored) have shown that many children up to age 10 years with AML with t(8;21) or inv(16) [and also t(15;17)] have identical translocations (including sequencing across intronic fusions) in their Guthrie spots and are asymptomatic for years before developing AML, again indicating that the translocations are necessary but not sufficient for induction of AML [90, 91].

The presence of additional mutations in CBF leukemia has been addressed directly by NGS experiments. In one experiment comparing patients with *RUNX1-RUNX1T1* and *CBFB-MYH11*, an average of 11.86 somatic mutations with functional consequences were present in *RUNX1-RUNX1T1* cases and 7.74 somatic mutations were present in *CBFB-MYH11* cases [68]. About 66% of mutations were in kinase pathway genes such as *NRAS, KIT, FLT3, KRAS, PTPN11, NF1*, and *CCND2*. *KIT* mutations were found in 45% of t(8;21) and 33% of inv(16) cases, with a mutant allelic ratio of 35% or greater required to confer a worse prognosis [68]. Similar findings were present in a subsequent study performing NGS on a series of 331 patients with t(8;21) [92]. Additional mutations in kinase pathway genes were detected in 63.4% of cases; additional mutations were also detected in genes encoding epigenetic regulators (45% of cases), as well as cohesion complex members, MYC-related regulators, and spliceosome complex proteins. Loss of a sex chromosome was the most common karyotypic abnormality, besides the defining t(8;21). A reduced complete remission (CR) rate was associated with del(7q), *FLT3*-ITD (high allele burden), and *JAK2* mutations. The factors most strongly associated with poor prognosis were a *cKIT* mutation in >25% of cells (KIT^{high}) and *JAK2* mutations. These results suggest that RTK inhibitors may be effective ancillary treatment alternatives for t(8;21) leukemia [92].

As with t(15;17) APL, although by convention an arbitrary level of 20% blasts is required for diagnosis of AML, the presence of the recurrent translocations t(8;21) and inv(16) or t(16;16) is diagnostic of acute myeloid leukemia even when present in a small percentage of cells. Of note, if the clinical setting is post chemo/radiotherapy, diagnosis should be therapy-related myeloid neo-

plasm as the main classification of the patient's disease [2].

2.5.2.3 AML with t(9;11)(p21.3;q23.3); *KMT2A-MLLT3*

KMT2A (previously named mixed lineage leukemia or *MLL*), which maps to chromosome 11q23.3, is a transcriptional regulator and chromatin remodeling gene frequently rearranged in AML-RGA (as well as in ALL) and characteristically rearranged in infant leukemia, epipodophyllotoxin therapy-related leukemia, and frequently in mixed phenotype leukemia [93]. *KMT2A* translocations are seen in leukemias of both myeloid and lymphoid origin, hence the older name (*MLL*). In AML, t(9;11) involving *MLLT3* (*AF9*) is the most common translocation partner and appears to define a distinct disease entity. Unusually however, compared to other recurring translocations in AML, there are multiple variant *KMT2A* translocation partners, with more than 70 partner genes characterized. This type of AML can occur at any age. It comprises the large majority of AML in infants up to 18 months of age. Although *KMT2A* translocations involving some of the partner genes such as *MLLT1* (*ENL*), *MLLT10* (*AF10*), *MLLT4* (*AF6*), or *ELL* predominantly occur in AML, they can also be seen in ALL. Cases have been described as switching from AML to ALL and vice versa with treatment.

The *KMT2A* gene is the mammalian homolog of *trithorax*, a *Drosophila* transcriptional regulator that encodes a methyltransferase and positively regulates homeobox (*HOX*) genes, a large family of genes involved in the regulation of development and essential for growth and differentiation [93, 94]. Wild-type KMT2A regulates *HOX* gene expression by methylation of histone H3 lysine (H3K4), resulting in transcriptional activation; this action requires the KMT2A SET domain, a domain shared by a number of transcriptional regulators with histone methyltransferase activity [95]. The multiplicity of fusion partners of *KMT2A* has been perplexing. Three of the most common fusion partners, *MLLT3*(*AF9*), *AF10*, and *MLLT1*(*ENL*), associate with DOT1L, a histone methyltransferase with different activity than wild-type KMT2A. DOT1L (in a complex, which consists of DOT1L, AF10, MLLT6(AF17), and MLLT1(ENL)) methylates histone H3 lysine 79(H3K79) [96, 97], which is also associated with transcriptional activation [93, 98]. Thus, many of the fusion partners of *KMT2A* normally associate in complexes that regulate transcription through histone methylation. *HOX* genes are expressed highly during early development, but then are downregulated during hematopoiesis. The end result of the altered methylation of these transcriptional regulatory complexes by the KMT2A-fusion proteins is thought to be abnormally sustained *HOX* gene expression [93]. Small molecule inhibitors of DOT1L are under development, with success in mouse models of *KMT2A* leukemia. One such molecule, pinometostat (EPZ-5676), has been evaluated in a phase I clinical trial in adult patients with advanced acute leukemia with *KMT2A* rearrangements and has shown clinically meaningful responses and modest efficacy as a single agent [99, 100].

KMT2A rearrangements are associated with several unique types of leukemia. In infant acute leukemia (birth to 18 months, both AML and ALL), there is a 60–80% incidence of 11q23.3 rearrangements [101]. In secondary acute leukemias (both AML and ALL) developing after treatment with DNA topoisomerase II inhibitors (epipodophyllotoxins), there is a 70–90% incidence of *KMT2A* rearrangements, particularly t(4;11)(q21;q23.3) and t(9;11)(p21–22;q23.3) [102, 103]. Topoisomerase II is involved in unwinding of DNA during replication and transcription by producing double-stranded nicks in DNA, after which the ends are rejoined by a ligase activity of topoisomerase II. Topoisomerase II inhibitors block this ligase function, and DNA-free ends accumulate, triggering apoptotic events. There are 11 possible topoisomerase II consensus binding sites in *KMT2A* breakpoint cluster areas [104]. Incorrect religation of DNA-free ends in these areas due to inhibition of topoisomerase II religase activity may explain the association of topoisomerase II inhibitors and translocations involving *KMT2A*. Interestingly, infant leukemia with *KMT2A* translocations has a similar distri-

bution of breakpoints to cases following epipodophyllotoxin treatment, whereas sporadic cases of *KMT2A* acute leukemia have more random breakpoints [105]. This observation has triggered speculation that in utero exposure to environmental topoisomerase II inhibitors such as flavonoids may have a role in the etiology of infant leukemia [106]. In addition, this hypothesis may be supported by the fact that in utero exposure to a common class of antibiotics with anti-bacterial topoisomerase II (gyrase) activity (fluoroquinolones), which have been shown to cross react with human topoisomerase II, results in an increased risk of leukemia development in infants and young children [107].

The latency of development of leukemia appears to be shorter for *KMT2A* rearrangements than for other leukemogenic rearrangements. Similarly, therapy-related leukemias based on *KMT2A* rearrangement occur sooner after therapy than those occurring after alkylating agents or radiation [103, 108]. This suggests that the oncogenic fusion protein produced by the *KMT2A* rearrangement can deregulate the cell without the accumulation of many secondary mutations.

Overexpression of *MECOM* (*EVI1*) is common in AMLs with *KMT2A* rearrangements, being seen in approximately 40% of cases with t(9;11). Secondary chromosomal abnormalities are commonly seen in AML with t(9;11) (p21.3;q23.3), with trisomy 8 most frequently observed in *MECOM* negative cases; the secondary translocations do not appear to influence prognosis.

AML with t(9;11) and other *KMT2A* fusions frequently has myelomonocytic and monoblastic morphology and immunophenotype including strong expression of CD33, CD65, CD4, and HLA-DR and low to variable expression of CD13, CD14, CD117, and CD34.

Controversial points in placing *KMT2A* rearranged AML cases in the WHO classification include occurrence of the same rearrangements in non-random settings, including MDS and therapy-related neoplasms discussed above. Diagnosis of AML-RGA with *KMT2A* translocations should be limited to de novo AML cases [2]. AML arising in the context of prior cytotoxic therapy should be classified as therapy-related disease with a *KMT2A* rearrangement. Likewise, AML with myelodysplasia-related changes and a *KMT2A* translocation should be diagnosed as AML with myelodysplasia-related changes. Furthermore, although t(9;11) cases with <20% blasts are not currently classified as AML, it is suggested that in the right clinical setting they should be treated as AML [2]. AML with t(9;11) has an intermediate survival which is superior to that of AML with other 11q23.3 translocations [109, 110]. Overexpression of *MECOM* associates with poor prognosis [111].

2.5.2.4 AML with Biallelic Mutation of CEBPA

CCAAT/enhancer binding protein-alpha (CEBPA) is a transcription factor that is required for granulocytic differentiation [112, 113] as demonstrated in *Cebpa* knockout mice lacking mature granulocytes [114]. CEBPA transactivates the genes for G-CSF and GM-CSF receptors and several granulocyte-specific proteins. Genetic aberrations in other myeloid leukemia-associated genes often lead to *CEBPA* down-regulation. Furthermore, the *CEBPA* promoter is methylated in half of AML cases [115]. Biallelic mutations in *CEBPA* have been identified in about 4–9% of children and young adult patients with AML [116, 117]. The biallelic mutation in CEBPA is associated with a specific gene expression pattern that is different from single mutations. It is now recognized that the favorable prognosis associated with CEBPA mutation in AML is related to biallelic mutations, a requirement to assign a case to this category [118, 119]. If biallelic CEBPA mutations are found in AML, especially in younger patients, an investigation should be undertaken of the possibility of a germline mutation, which would change the diagnosis to germline AML predisposition syndrome [120].

About 5–9% of AML cases with CEBPA mutations have *FLT3*-ITD mutations [121–123]. *GATA2* mutations are found in approximately 39% of cases [124]. Most cases (>70%) have a normal karyotype; of karyotypic abnormalities, del(9q) is commonly seen (similar to AML with

mutated NPM1); this does not appear to influence prognosis [122].

AML with biallelic mutation of *CEBPA* has no specific or distinguishing morphologic features and shows a myeloid phenotype with a possibly higher frequency of expression of HLA-DR, CD7 and CD15, and no expression of monocytic markers such as CD14 and CD64. Variable dysplasia is present in a significant minority of cases, similar to AML with mutated NPM1; it does not adversely influence prognosis [125].

2.5.2.5 AML with Mutated NPM1

AML with mutated *NPM1* occurs most commonly in adults and the elderly, typically without preceding abnormalities, and typically has a normal karyotype. *NPM1* mutation is one of the most common recurrent genetic aberrations in AML (35% of adult AML with a normal karyotype with lesser frequency in the pediatric population), and only rarely occurs in other myeloid neoplasms such as MDS or MDS/MPN [126] (Fig. 2.2). AML with mutated NPM1 requires other mutations prior to acquisition of the NPM1 mutation. While placed in AML-RGA in the WHO classification, these features share much in common with AML-MRC, rather than other types of AML-RGA.

NPM1 (nucleophosmin) is a molecular chaperone that shuttles between cytoplasm and nucleus [127]. It is essential for cell survival [128] with several major functions, including ribosome biogenesis, regulation of centrosome duplication during the cell cycle, chromatin remodeling, potentiating the p53 stress response, interaction with tumor suppressor proteins, and DNA repair functions (reviewed in [129–132]). *NPM1* mutations are stably expressed, being retained in leukemic blasts at relapse in the majority of cases [33, 133, 134]. By inspection of variant allele frequencies, it has been shown that *NPM1* mutations precede *FLT3* mutations that often co-occur in AML.

Wild-type NPM1 is composed of 294 amino acids and has two nuclear localization signals (NLS), two nuclear export signals (NES) that mediate the nuclear-cytoplasmic shuttling of wild-type NPM1, and a nucleolar localization signal (NoLS) at the C-terminal end containing two tryptophan residues at positions 288 and 290 that are critical for retaining NPM1 in the nucleolus. *NPM1* mutations occur in the portion of the gene previously known as exon 12, with over 50 mutations described and named alphabetically in the order of discovery (types A, B, C, D, etc.). All the subtypes share an identical biological consequence, leading to generation of a mutant NPM1 protein with four extra amino acids [132, 135, 136]. The mutant NPM1 appears to function in a dominant negative manner through heterodimerization with normal NPM1 to cause relocation of some normal NPM1, as well as the mutant NPM1, from its normal predominantly nucleolar location to the cytoplasm [137]. This can be detected in tissue sections by immunohistochemistry and is predictive of *NPM1* mutation [136]. The mutation is always heterozygous; homozygous mutation is embryonic lethal [128, 138].

NPM1 mutation was initially considered a founder event in leukemogenesis, because it usually is maintained at relapse. However, it is now thought that *NPM1* mutation occurs later in AML development due to its absence in preleukemic hematopoietic stem cells, and the fact that in 10% of patients the *NPM1* mutation is lost at relapse while further chromosomal and molecular changes are acquired [33, 134]. One of the mechanisms by which mutant NPM1 may promote leukemogenesis is by destabilization of proteins regulating the TP53 response. In addition, cytoplasmic NPM1c retains its ability to bind to cytoplasmic caspases 6 and 8, which may inhibit apoptosis, also enhancing leukemogenesis. Furthermore, NPM1 may interact with a protein that is part of the E3 ubiquitin ligase that degrades MYC protein; mutant cytoplasmic NPM1c disrupts this activity, thus indirectly increasing the levels of MYC protein [130]. It has also been reported recently that *HOX* overexpression is directly dependent on mutant *NPM1* and maintains the leukemic state in *NPM1* mutated AML. It has also been shown that relocalization or degradation of mutant NPM1 induces differentiation of AML cells, potentially providing the rationale for a novel therapeutic strategy [139].

NPM1 mutations occur most frequently in conjunction with *FLT3*-ITD and *DNMT3A* mutations, but mutations in *TET2*, *IDH1*, and *IDH2* also commonly co-occur [22, 130, 131, 140]. Patients with *NPM1* mutations in the absence of *FLT3*-ITD mutations with high variant allele frequency appear to have a favorable response to chemotherapy [136, 137]. This may be due to the role of wild-type NPM1 in DNA repair; if *NPM1* is mutated there is less efficient repair of DNA damage induced by chemotherapy [131]. Younger patients with a normal karyotype and no concurrent *FLT3*-ITD mutation have prognosis comparable to that of CBF AMLs and may not benefit from allogeneic stem cell transplantation in first remission [141]. Co-occurrence of *NPM1*, *FLT3*-ITD, and *DNMT3A* mutations has been associated with very poor outcome [142].

AML with mutated *NPM1* strongly associates with myelomonocytic or monocytic morphology, but other morphologic types occur including AML without maturation and pure erythroid leukemia. Some cases have characteristic morphology with nuclei showing cup-shaped or thumb imprint-like indentations (Fig. 2.7). This morphology may raise the differential diagnosis of APL, especially in the presence of the similar immunophenotypic features to APL (lack of CD34 and HLA-DR expression on blasts). Blasts otherwise show an immature myeloid or monocytic profile (CD36+, CD64+, CD14+). Multilineage dysplasia is seen in up to 25% of cases but appears to have no prognostic significance [143, 144].

2.5.3 Rare Subtypes of AML-RGA

2.5.3.1 AML with t(6;9)(p23;q34.1); *DEK-NUP214*

The t(6;9)(p23;q34.1) occurs in 0.7–1.8% of AML cases and occurs both in later childhood and adults. The t(6;9) results in a fusion of *DEK* on chromosome 6 and *NUP214* on chromosome 9. This fusion protein acts aberrantly, altering nuclear transport by binding to soluble transport factors [145]. *FLT3*-ITD mutations are common in this entity [146–148]. There are no specific distinguishing morphologic features of blast cells in the AML with t(6;9)(p23;q34.1) but blood and marrow basophilia, generally uncommon in AML, is seen in more than half of cases [146,

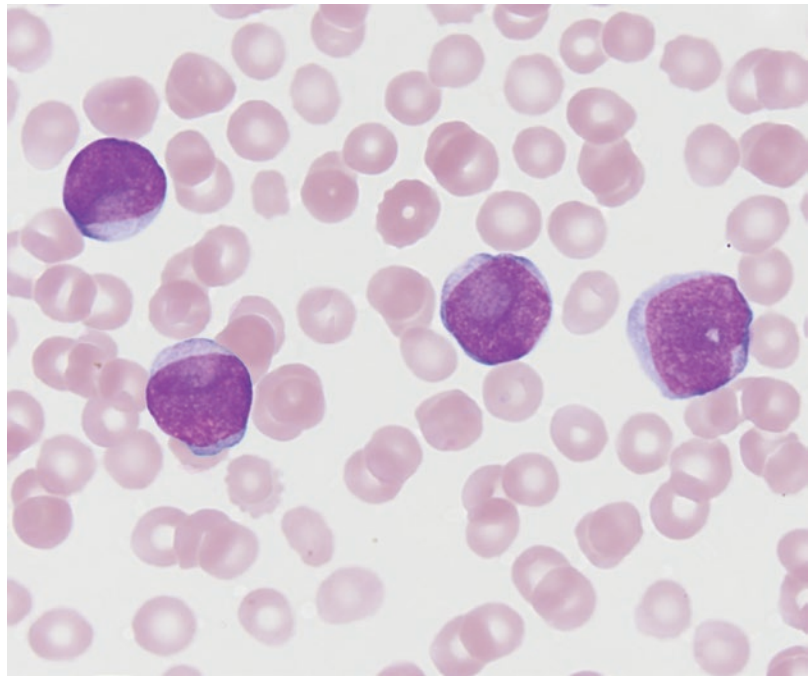

Fig. 2.7 *NPM1* mutated AML (peripheral blood smear stained with Wright's stain at 1000× original magnification). Some cases have characteristic morphology with nuclei showing cup-shaped or thumb imprint-like indentations. This morphology may raise the differential diagnosis of APL, especially in the presence of the similar immunophenotypic features to APL (lack of CD34 and HLA-DR expression on blasts)

149]. Some cases with t(6;9)(p23;q34.1) may have less than 20% blasts and are not currently classified as AML, but in an appropriate clinical setting, these patients may be treated as AML. The blasts cells have a non-specific myeloid phenotype, with co-expression of TdT seen in approximately 50% of cases. Basophils can be detected by flow cytometry as a separate population positive for CD123, CD33, and CD38 but negative for HLA-DR. The prognosis of AML with t(6;9)(p23;q34.1) is generally poor, with a high white cell count predictive of shorter overall survival [146, 148].

2.5.3.2 AML with inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); *GATA2, MECOM*

It is now recognized that inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2) in this type of AML does not represent a fusion gene but results in repositioning a distal *GATA2* enhancer to activate the oncogene *MECOM* at 3q26.2 (also known as *EVI1*), simultaneously resulting in GATA2 haploinsufficiency [150, 151]. These cases tend to occur in older patients with a median age approximating 60 years, suggesting differences with other AML-RGA subtypes. Secondary chromosomal abnormalities are common, with monosomy 7, del 5q, and complex karyotypes frequently seen. Mutations in genes activating RAS tyrosine kinase signaling pathways are present in most cases: *NRAS* (27% of cases), *PTPN11* (20%), *FLT3* (13%), *KRAS* (11%), *NF1* (9%), *CBL* (7%), and *KIT* (2%). Other commonly mutated genes are *GATA2*, *RUNX1*, and *SF3B1* [152]. This type of AML accounts for 1–2% of AMLs and is characterized by normal or elevated platelet counts and increased, dysplastic megakaryocytes typically with hypolobated or unilobed nuclei [153] (Fig. 2.8). Multilineage dysplasia in the non-blast marrow cells is also common, and marrow eosinophils and basophils may be increased [154, 155]. Bone marrow blasts have variable morphology with no specific characteristics. The blasts typically have a non-specific myeloid phenotype with aberrant expression of the T-associated marker CD7 and megakaryocytic markers CD41 and CD61 in a subset of cases. Cases with <20% blasts are not currently classified as AML; however, this is controversial since the outcome for patients with <20% or > 20% blasts are equally poor in this very aggressive disease with very short survival [156].

2.5.3.3 AML with t(1;22)(p13.3;q13.1); *RBM15-MKL1*

AML with t(1;22)(p13.3;q13.1) is characterized by blasts with megakaryocytic differentiation by morphology and immunophenotype. This type of AML-RGA is rare (<1% of all AML cases). It

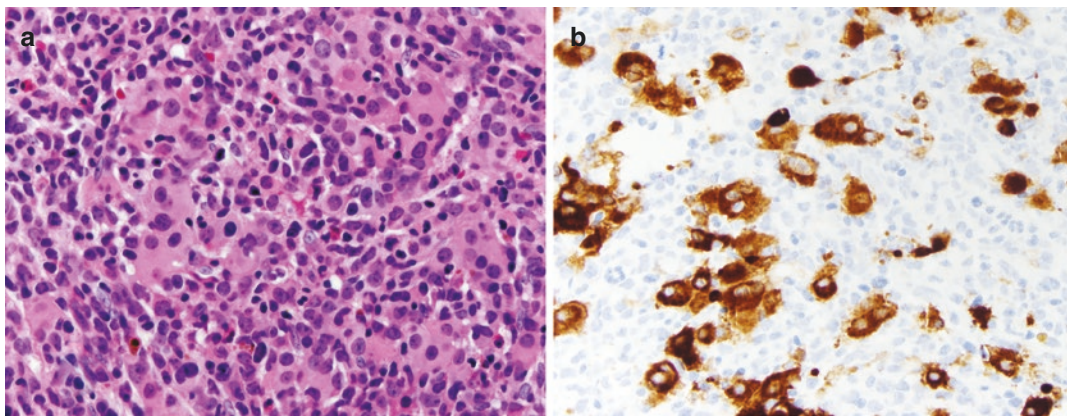

Fig. 2.8 AML with inv3/t(3;3) (bone marrow biopsy sections at 400× original magnification). H&E stained section showing numerous abnormal megakaryocytes with small hypolobated or unilobed nuclei (**a**), which are highlighted with CD61 immunohistochemical stain (**b**)

occurs almost exclusively in infants without Down syndrome in the first 6 months of life, presenting with marked organomegaly (commonly hepatosplenomegaly) due to leukemic infiltrates [157, 158]. Typically, the bone marrow is inaspirable due to dense marrow fibrosis. Careful search of the peripheral smear for blasts may be helpful in this diagnosis. In sections of biopsies of the marrow or liver, the blast infiltration mimics metastatic small round cell tumor, with cohesive clusters of small round cells in tissue or vascular spaces [157, 158]. There may be micromegakaryocytes associated with the megakaryoblast infiltrate; dysplasia in other cell lines is uncommon. Megakaryoblasts may be present in small numbers in the peripheral smear, facilitating diagnosis if recognized. Phenotypically the megakaryoblasts are often negative for CD34, CD45, HLA-DR, MPO, and lymphoid markers by flow cytometry and immunohistochemistry, contributing to mistaken diagnosis as a small round cell tumor, NOS. The blasts may show variable expression of myeloid markers CD13 and CD33, and if tested, are positive for megakaryocyte markers (CD41, CD61, and CD42b).

2.5.3.4 Provisional Categories of AML-RGA (WHO 2016)

Two provisional categories of AML-RGA are recognized in the 2016 WHO classification [2]. These categories exclude cases that meet criteria for other recognized types of AML.

De novo *AML with BCR-ABL1* cases may be difficult to distinguish from blast phase CML, especially without adequate clinical information, but the significance of this targetable fusion warrants the recognition of this entity [159, 160]. Preliminary data indicate that other molecular abnormalities may allow distinction of AML-RGA with *BCR-ABL1* from blast phase CML.

AML with mutated RUNX1 appears to represent a biologically distinct group of AMLs with some studies reporting worse prognosis versus other types of AML. Similar to biallelic CEBPA mutations, a subset of these patients may have germline mutations of *RUNX1*, and germline and family studies should be performed when these mutations are detected [161–164].

2.5.4 Myelodysplastic Syndromes (MDS) in Relationship to AML-MRC

Myelodysplastic syndromes (MDS) are a heterogeneous group of clonal stem cell disorders characterized by ineffective hematopoiesis manifesting with low blood counts, typically normo- or hypercellular BM, and a variable degree of morphologic dysplasia in hematopoietic elements. The diagnosis of MDS relies on identifying and quantifying morphologic dysplasia, quantifying the proportion of blast cells, and/or demonstrating characteristic MDS-associated cytogenetic abnormalities [2]. This diagnosis can be challenging, especially in low-grade MDS (see below) with no increase in blasts, as the morphologic dysplasia may be subtle and subjective, and many patients (45–50%) lack characteristic cytogenetic abnormalities [2]. MDS is still a poorly understood set of diseases pathogenetically related to AML-MRC [2]. Distinction of MDS from AML is currently based, with exceptions, on an arbitrary marrow blast percentage, lowered from the historical ≥30% to ≥20% in the WHO classification; this is not based on an understanding of biological differences in the two sets of diseases [2]. For discussion of AML pathogenesis, the MDS subtypes in the WHO classification can be consolidated into two types: low-grade MDS (MDS with single lineage dysplasia, MDS with ring sideroblasts, MDS with multilineage dysplasia, MDS with isolated del(5q)), and high-grade MDS (MDS with excess blasts). High-grade MDS (see below) is usually fatal with or without progression to AML-MRC. Understanding the biology and pathogenesis of MDS remains elusive but would seem to be critical for improving the differential diagnosis of AML-MRC versus MDS and versus DN-AML and would possibly contribute to improved treatment strategies for AML-MRC and MDS, and possibly to prevention of progression of MDS to AML-MRC.

A variety of data (including progressive genetic damage, acquired structural and functional abnormalities in hematopoietic cells, and the high rate of transformation to AML) suggest

that high-grade MDS is a mutator phenotype with inherent genetic instability. AML-MRC appears to require both a block of differentiation and a drive to proliferate, but it is doubtful either of these events is the biologic basis of MDS. A solitary drive to proliferate (with associated inhibition of programed cell death) is the apparent cause of chronic myelogenous leukemia (reviewed in [165]) and the other myeloproliferative neoplasms (MPN) [166–168]; these diseases differ from MDS in being proliferative, lacking MDS-type morphologic dysplasia, and lacking MDS-type cytogenetic abnormalities. A solitary block of differentiation in hematopoietic progenitors has no clinical phenotype, except a possible propensity to be transformed by acquisition of a second genetic event that drives proliferation [90]. Both clinical and transgenic examples of each of these possibilities lack the phenotype of MDS, although acquisition of both appears to be required for MDS to transform to AML-MRC. A plausible hypothesis is that subsets of high-grade MDS represent acquisition of one of these events superimposed on the underlying biology of low-grade MDS. MDS with excess blasts (MDS-EB) is characterized by increased but relatively stable numbers of marrow blasts, with shortened survival of patients due to complications of MDS and with an increased propensity to progress to AML; hypothetically, MDS-EB may represent a block of myeloid differentiation superimposed on the underlying biology of low-grade MDS. The MDS/MPN diseases (CMML, JMML, and atypical CML) have mixed features of MDS (dysplasia, shared genetic abnormalities) and MPN (proliferation) [2]. A drive to proliferate through mutations leading to increased active RAS has been demonstrated in many MDS/MPN cases (inactivating *ATM* mutations; activating *RAS*, *PTPN1*, and *NF-1* mutations) [2], yet the cases also share morphology and cytogenetic abnormalities with MDS. In a possibly informative clinical scenario, septic patients with MDS may develop a reversible leukemoid reaction, often with monocytosis and mimicking CMML (apparently due to a physiologic proliferative drive superimposed on MDS); with successful treatment of sepsis patients revert to MDS. A second example of this possibility, patients with MDS with ring sideroblasts that progress to MDS/MPN with thrombocytosis often have coincidental acquisition of an activating *JAK2* mutation [2, 169]. Thus, while available data suggest that acquisition of both a drive to proliferate and a block of differentiation may contribute to MDS progression, and both are necessary for progression to AML-MRC, neither represents the underlying pathogenesis of MDS. The most tenable hypothesis is that the underlying pathogenic abnormality of high-grade MDS is its mutator phenotype, which causes random genetic damage, including in some cases acquisition of a drive to proliferate, with resultant progression to AML-MRC [46, 48].

While high-grade MDS is a mutator phenotype, at least some low-grade MDS is not. Both MDS with isolated del(5q) and MDS with ring sideroblasts (MDS-RS), especially cases with *SF3B1* mutation, if defined stringently using WHO criteria, have very low rates of progression to AML with survival approaching age-matched peers [169–171]. While 5q− is one of the most common cytogenetic abnormalities in MDS, in most cases secondary to an underlying mutator phenotype, the specific subtype of MDS with isolated del(5q) may represent emergence of a 5q− clone with an associated clonal survival advantage, but a stable biologic state with no underlying mutator phenotype. MDS-RS defined as mostly unilineage erythroid abnormalities and the presence of mutations in the spliceosome gene *SF3B1*, may have a similar pathogenesis with additional superimposed consequences of mitochondrial damage due to iron loading [171, 172].

Progression of MDS to AML-MRC does not represent a continuum, but rather stepwise acquisition of specific genetic events is required for transformation and should equate with a rapid rate of accumulation of primitive precursors (blasts) in the marrow, differing qualitatively from MDS [46, 48] (Fig. 2.9). Thus, separation of MDS and AML-MRC often requires clinical–pathological judgment; it should be based on evidence of transformation with a distinct change in the rate of accumulation and the percentage of blasts, not

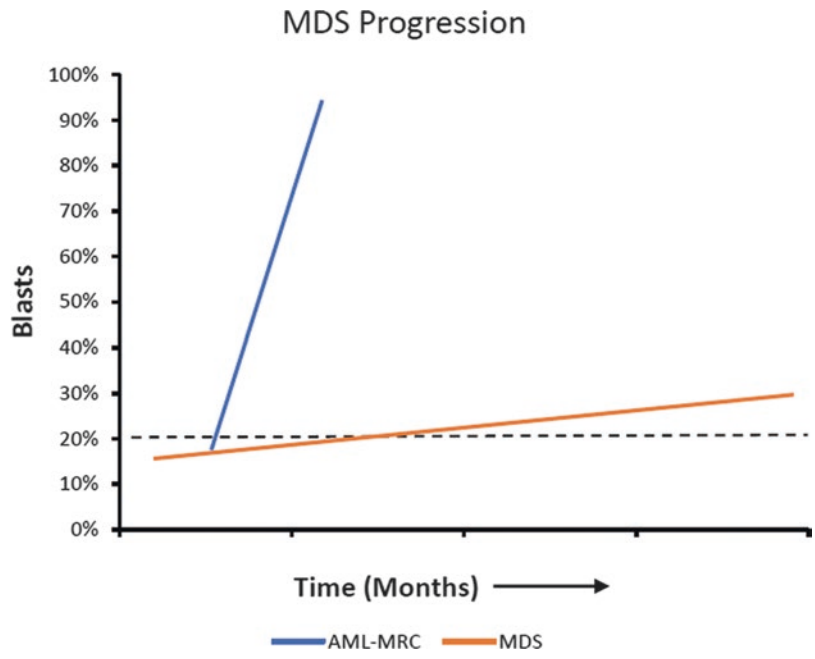

Fig. 2.9 Increasing proportion of blasts over time in MDS. Transformation to AML is denoted by rapid increase in bone marrow blasts (blue line). In contrast, if the blast proportion rises slowly over months exceeding or fluctuating around the arbitrary threshold of 20%, the situation should be considered to be a persistent MDS (orange line)

just on a marrow blast % rising slowly above an arbitrary threshold (whether 20% or 30%). While it is safe to assume that a high marrow blast % (>40–50%) represents transformation to AML-MRC, lower levels require clinical interpretation as to whether the patient's disease has shifted from primary marrow failure to proliferating blasts. If initial data are inconclusive, a repeat marrow examination after an interval may clarify whether the basic disease process has changed from one of marrow failure (MDS) to a proliferative state (AML) (Fig. 2.9). As our knowledge of these diseases increases, separation of MDS and AML-MRC may eventually include demonstration of specific genetic events leading to transformation [46, 48]. Finally, this perspective should not be interpreted to mean that MDS with a high blast % is a favorable disease; it is lethal with short median survival, but lacking transformation is resistant to cytotoxic chemotherapy.

2.5.5 AML with Myelodysplasia-Related Changes

AML with myelodysplasia-related changes (AML-MRC) is diagnosed when AML is preceded by MDS or MDS/MPN, there is an MDS-related cytogenetic abnormality, and/or there is otherwise unexplained multilineage morphologic dysplasia (Table 2.1). There should be no history of prior exposure to cytotoxic drugs or radiation therapy (which define therapy-related AML), and genetic abnormalities that define AML-RGA subtypes must be absent [2]. AML-MRC differs biologically and clinically from AML-RGA. Patients who fulfill criteria for AML-MRC can present clinically as "de novo" disease, in the sense of no prior diagnosed clinical abnormality, but with clinical, epidemiologic, genetic, treatment response, and prognosis data similar to other AML-MRC cases [45, 126]. It is reasonable to postulate that most clinically "de-novo" cases of AML-MRC have prior subclinical marrow disease only coming to medical attention at the time of progression to AML.

AML-MRC occurs most commonly in elderly patients, comprising the majority of AML cases beyond age of 60 years. It occurs at low frequency in children and young adults, with increasing incidence with age giving an exponential incidence curve for AML as a whole (Fig. 2.3). The exponential incidence curve of AML-MRC sug-

gests several random events are required for transformation in this set of AML.

AML-MRC is characterized by a series of cytogenetic changes shared with MDS. Despite extended efforts, the genes affected and the biologic impact of these cytogenetic changes (e.g., −7, 5q–, +8, 20q–, +21) remain incompletely understood. In most cases, these cytogenetic changes appear to be related to progression of MDS, rather than its initiation, as they are absent in up to 60% of MDS cases at presentation. AML-MRC also shares a subset of gene mutations with MDS [173, 174]. Recent NGS studies have found that mutations in a subset of spliceosome and chromatin modification genes are highly specific for AML-MRC. Similar genetic analyses may allow more precise definition of these diseases in the future [45, 175]. *TP53* mutations, which are also more frequent in secondary AML and MDS, usually associate with complex karyotypes and predict poor survival [176]. Some of these genetic abnormalities are now the target of specific treatment strategies (e.g. *IDH1/2* mutations, *FLT3*-ITD, *TP53* mutations) [43, 177].

Unlike AML-RGA, AML-MRC lacks clearly defined syndromes with specific morphologic, cytogenetic, or molecular genetic signatures. The genetic abnormalities cited above that have directed therapy lack specific morphologic or cytogenetic features to characterize them, short of molecular genetic identification. As discussed previously, some subtypes of AML currently placed debatably in WHO AML-RGA [AML with mutated NPM1, AML with inv(3) or t(3;3)] share features with AML-MRC.

2.5.6 AML, Not Otherwise Specified (the Historical Approach to AML Classification)

For much of the twentieth-century classification of AML was based on how leukemic blasts recapitulate normal hematopoiesis. Classification depended upon whether blasts in a given case had the appearance of myeloblasts, monoblasts, megakaryoblasts, promyelocytes, or erythroblasts and whether they appeared minimally, moderately, or well differentiated. This approach was formalized by the French-American-British (FAB) working group in a series of papers beginning in 1976, allowing analysis of its relevance. In the FAB classification, M0 designates AML with minimal morphologic or cytochemical differentiation, M1–2 AML with minimal or moderate granulocytic differentiation, M3 acute promyelocytic leukemia (APL), M4 AML with mixed myelomonocytic differentiation, M5a and M5b monoblastic leukemia with minimal or moderate differentiation, M6a myeloid leukemia with dysplastic background erythropoiesis, M6b acute erythroblastic leukemia, and M7 acute megakaryoblastic leukemia. Unfortunately, subsequent analyses showed a general lack of clinical and biological relevance to this approach. Analysis of 5848 patients with newly diagnosed AML demonstrated that when data on NPM1 and CEBPA mutations was available, the FAB classification added no further prognostic information [178]. The approach remains a useful shorthand descriptor of myeloblast morphology. The FAB categories are substantially retained in the WHO classification under the heading of AML, NOS (Table 2.1). AML, NOS was originally included for use in cases where cytogenetic and molecular data are not available for classification, as in developing countries where access to laboratories performing these tests is limited. This was controversial as these categories lack clear biologic and clinical relevance, and there is an ever-diminishing number of newly diagnosed AML cases that cannot be subclassified in other categories (Fig. 2.2). Despite these limitations, it is useful to revisit several categories that have unique morphologic or clinical presentations.

AML with Monocytic Differentiation: AML with monocytic differentiation may arise as AML-RGA, AML-MRC, or from a precedent chronic myelomonocytic leukemia (CMML). In the latter case, there may be progressive variation in the percentages of monocytes, promonocytes, and monoblasts over time, creating difficulty in determining when to call progression to AML. Monocytic precursors may not have distinctive immaturity markers by flow

cytometry, so morphologic review of the peripheral blood and bone marrow aspirate, and morphologic and immunohistochemical examination of the bone marrow biopsy/particle, are critical to the final designation of AML. In the peripheral blood and bone marrow aspirate, there may be a mixture of monocytes and monocytic precursors with varying maturity. Mature monocytes usually have irregular indented nuclei and abundant cytoplasm with pseudopods and cytoplasmic vacuoles. Promonocytes have delicate nuclear folds that one can see through, more immature chromatin, and blue cytoplasm which is more circumscribed than the mature monocyte (Fig. 2.10). These are considered "blast equivalents" in the calculation of the percentage of immature cells in the blood or bone marrow aspirate [2]. Finally, monoblasts have immature "ground glass" chromatin, nucleoli, folded nuclei, and variable amounts of cytoplasm, but usually with an increased nuclear:cytoplasmic (N:C) ratio (Fig. 2.10). Monoblasts are often negative for the immaturity markers CD34 and CD117. On aspirate and peripheral smears, cytochemical stains are occasionally performed to identify blasts as monocytic; alpha naphthyl esterase staining will stain cytoplasmic granules in monocytic cells; this stain is markedly reduced with fluoride treatment. Immunohistochemistry performed on the bone marrow biopsy/particle may include antibodies to MPO (negative in most monoblasts) and lysozyme (positive in monocytic cells, but not specific to monoblasts).

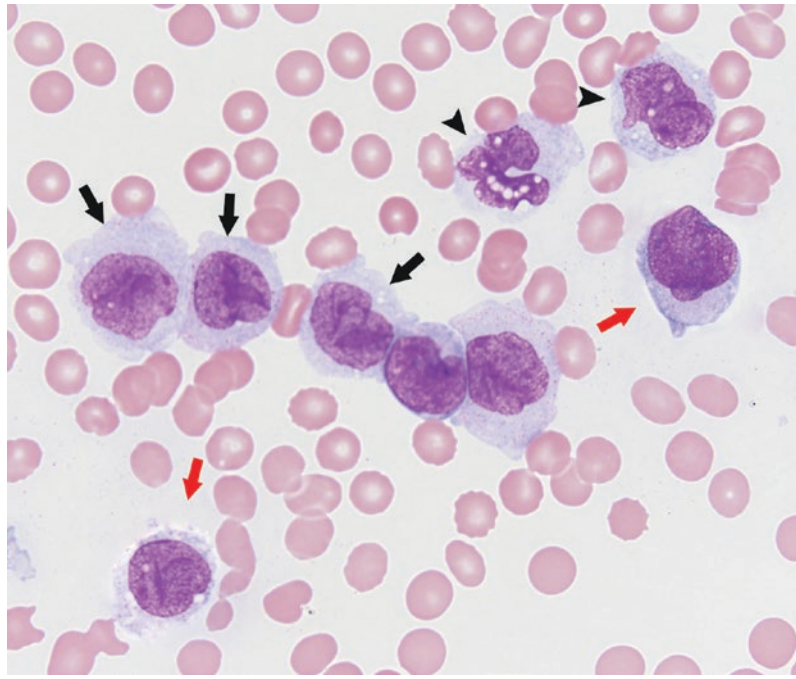

Fig. 2.10 AML with monocytic differentiation (peripheral blood smear stained with Wright's stain at 1000× original magnification). Mature monocytes usually have irregular indented nuclei and abundant cytoplasm with cytoplasmic vacuoles (cells in the top right indicated with black arrowheads). Promonocytes have delicate nuclear folds that one can see through, more immature chromatin, and variable amount of pale blue cytoplasm (a row of cells in the middle-left indicated with black arrows). These are considered "blast equivalents" in the calculation of the percentage of immature cells. Monoblasts (cells in the middle right and lower left indicated with red arrows) have immature "ground glass" chromatin, occasionally folded nuclei, variably prominent nucleoli, and an increased N:C ratio. Due to continuous morphologic spectrum of these monocytic cells, the recognition and enumeration of the promonocytes may be difficult and somewhat subjective especially in situations where smear and staining quality are less than optimal

Acute Erythroid Leukemia: Acute erythroid leukemia in the current WHO classification is limited to what was previously called "pure" erythroleukemia, FAB M6b, or Di Guglielmo's leukemia. It is rare and is defined as a neoplastic proliferation of immature precursors of the erythroid lineage where 80% of bone marrow cells are erythroid, with ≥30% proerythroblasts and no myeloblast population [2]. Blasts have morphologic features of proerythroblasts: large round nuclei with multiple nucleoli, deep basophilic cytoplasm, and cytoplasmic vacuoles. Immature chromatin and the cytoplasmic vacuoles are the main features that distinguish neoplastic from normal proerythroblasts. The vacuoles are often positive on a PAS cytochemical stain performed on an aspirate smear. On bone marrow biopsy/particle sections, immunohistochemistry using antibodies for E-cadherin and CD71 is recommended. Some morphologic features are shared with acute megakaryoblastic leukemia; however, the main differential to be aware of is similarity to benign conditions such as erythroid hyperplasia in response to erythroid growth factor or associated with megaloblastic anemia. A common clinical feature is profound anemia. Cytogenetic studies usually indicate a complex karyotype [2], which corresponds to the prevalence of *TP53* mutations in this leukemia [179].

Acute Megakaryoblastic Leukemia: Acute megakaryoblastic leukemia, also rare, is defined as an acute leukemia with greater than 20% blasts, greater than 50% of which are megakaryocytic in lineage. This category excludes cases of AML with myelodysplasia-related changes or therapy-related AML and also does not include cases with the recurrent cytogenetic abnormalities t(1;22) (p13.3;q13.1) and inv3(q21.3q26.2), or t(3;3)(q21.3;q26.2). Each of these leukemias with recurrent cytogenetic abnormalities are listed separately in the WHO classification [2]. Here we will mention general characteristics of megakaryocytic blasts. Megakaryoblasts are medium to large blasts with a high N:C ratio and basophilic cytoplasm which often has characteristic cytoplasmic blebs. On the aspirate smear and tissue sections, megakaryoblasts may form cohesive clusters and therefore may easily be mistaken for carcinoma, if marrow material is limited. Careful search of the peripheral smear for blasts may be helpful in this differential diagnosis. On the aspirate smear, alpha naphthyl acetate esterase stains megakaryoblasts and, in contrast to monocytic cells, is not quenched by fluoride. Immunohistochemistry with megakaryocyte-specific antibodies, including CD42b and CD61, is recommended, although these markers may be negative in poorly differentiated megakaryoblasts. Often the marrow is markedly fibrotic, resulting in hypocellular aspirate smears and compromising diagnosis.

A unique setting of acute megakaryoblastic leukemia is in the context of Down syndrome. Children with Down syndrome have a markedly increased incidence of acute myeloid leukemia, and over 50% of cases have megakaryoblastic differentiation (discussed in more detail below).

2.5.7 Therapy-Related AML

Therapy-related AML (t-AML) is classified within a distinct WHO category of therapy-related myeloid neoplasms (t-MN) which also includes therapy-related MDS (t-MDS) [2]. This is a distinct and well-recognized clinical syndrome which occurs as a late complication of cytotoxic chemotherapy for a primary neoplastic or non-neoplastic process [180, 181]. The incidence of t-AML is approximately 7% but is currently rising due to an increasing number of cancer survivors at risk [182–184]. There are two major biologic classes of t-AML (Table 2.2) (reviewed in [185]). The more common class is associated with prior exposure to alkylating agents and/or radiation therapy and occurs typically after 5–7 years. This type is usually preceded by an MDS phase and is associated with MDS type cytogenetic changes including abnormalities of chromosomes 5 and 7, complex karyotype and high frequency of *TP53* mutations. t-AML in general is associated with more adverse genetic lesions, and *TP53* may be the most commonly mutated gene in t-MDS and t-AML [186]. t-AML after therapy with topoisomerase II inhibitors has a shorter latency,

occurring 1–3 years after the exposure, typically with no antecedent MDS, and is associated with balanced translocations frequently involving *KMT2A (MLL)* at 11q23, *RUNX1* at 21q22 and other balanced translocations including *PML/RARA*. The precise distinction between these two classes may not always be possible or practical due to the use of multi-agent chemotherapy often in combination with radiation therapy. Genetically and phenotypically, these two classes of t-AML resemble AML-MRC and AML-RGA, respectively, with no distinctive genomic patterns that are specific for t-AML [45, 185]. Some recent studies have identified increased prevalence of CHIP, including mutations in the *TP53* pathway, in patients who eventually develop t-AML after treatment for other malignancy, suggesting that the hematopoietic progenitor cells with mutations in the *TP53* pathway may undergo selective unrepaired damage by chemotherapy, eventually leading to t-AML [185, 187]. In addition, some cases of t-AML have been shown to be associated with germline mutations in cancer susceptibility genes involving DNA damage response pathways such as *BRCA1, BRCA2, TP53,* and *CHEK2* [185, 188, 189]. The overall prognosis of patients with t-AML is poor, mainly due to consequences of prior therapy for the primary disease and to enrichment of this type of AML with adverse disease-related features.

2.5.8 Germline Predisposition to AML

The expanding availability of detailed molecular data in AML, and its integration with clinical and laboratory data, has led to recognition of predisposing germline mutations in a growing number of genes in patients with AML and other myeloid disorders [190]. Although these cases are currently considered to be rare, as more data accumulate, these neoplasms may be found to be more common than currently appreciated. As with myeloid disorders occurring in inherited syndromes associated with DNA damage (e.g., Fanconi anemia, dyskeratosis congenita), the recognition of these newly characterized autosomal dominant disorders arising from germline mutations will be essential for proper clinical management, long-term follow-up, and genetic counseling [191, 192]. Some of these patients may present with AML or MDS with no prior history of significant organ dysfunction or pre-existing hematologic disorder, as seen in myeloid neoplasms associated with germline mutations in *CEBPA* or *DDX41*. Other patients may present with a pre-existing platelet disorder (e.g. mutations in *RUNX1*, *ANKRD26* or *ETV6*) [2]. Still others may present with additional non-hematological phenotypic abnormalities (e.g., germline *GATA2* mutation, inherited marrow failure disorders) [193]. Given the important clinical management considerations including donor selection for allogeneic stem cell transplantation, it is critical to distinguish diseases arising because of germline predisposition from those arising spontaneously or secondary to chemical or environmental exposures. Targeted myeloid gene sequencing panels are increasingly utilized as part of the routine diagnostic work up of AML and MDS cases. These panels include increasing numbers of known genes associated with germline predisposition syndromes. Types of mutations and mutant allele frequencies, in a context of a patient's clinical and family history, may raise suspicion of a germline predisposition, with a recommendation for confirmatory germline testing. An illustration of this is AML with germline *CEBPA* mutation. In these cases, the *CEBPA* mutation is biallelic with the germline mutation usually found in the 5′ end of one allele and a somatic mutation at the 3′ end of the other allele [120, 194]. Therefore, when a patient presents with a new diagnosis of AML with biallelic *CEBPA* mutations, testing should be undertaken to rule out a germline mutation, which if present would result in reclassification of the case, alter the clinical management of the patient, and lead to genetic counseling. A similar example is diagnosis of AML with mutation in *RUNX1* at high variant allele frequency (close to 50%, implying a constitutional abnormality), with similar consequences to the discussion of *CEBPA*.

2.5.9 Myeloid Leukemia Associated with Down Syndrome [195]

An unusual form of AML and MDS occurs with high frequency in children with Down syndrome (DS) under 4 years of age. The two are lumped together in the WHO classification as myeloid leukemia associated with Down syndrome (DS AML) because of similar excellent responses to chemotherapy, differing markedly from AML and MDS in non-DS children. The incidence of this set of disease is approximately 300–400 times that of MDS and AML variants in non-DS children. There is no increase in the incidence of standard non-DS AML and MDS in DS patients. DS AML may be preceded by transient abnormal myelopoiesis (TAM) in the neonatal period. TAM and DS AML both have a high frequency of mutations of *GATA1*, of interest since GATA1 protein mediates differentiation of erythroid and megakaryocytic precursors. About one third of TAM patients later develop DS AML; these patients usually have the same *GATA1* mutation as was present with TAM. Both MDS and AML in DS patients have unusual and characteristic features which appear to correspond to disruption of function of GATA1. Patients typically present with peripheral cytopenias and/or circulating or increased marrow blasts. Dysplasia is present and essentially restricted to erythroid and megakaryocytic precursors. Erythroids demonstrate megaloblastoid change and frequent hyperplasia. Megakaryocytes demonstrate hyperplasia, clustering, and hypolobate, often multiple nuclei, frequently including unusual morphology (peripherally displaced nuclei and a large central cytoplasmic inclusion giving the megakaryocyte the appearance of a Touton giant cell, or in smaller cells signet-like morphology) [195]. In AML cases, and if blasts are increased in MDS cases, blast lineage is most frequently megakaryoblastic, but erythroblastic, mixed erythroblastic/megakaryoblastic, and undifferentiated cases also occur [195]. Clinical evaluation is similar to that followed in non-DS cases, to include examination of peripheral blood and bone marrow samples, including blast characterization by IHC and flow cytometry. Blasts have a characteristic antigen expression pattern, with a near 100% frequency of positivity for CD33, CD117, CD38, and CD7, lower frequencies for CD13 and CD34, and variable expression of sublineage antigens depending on blast differentiation (CD41, CD61, and CD42b for megakaryocytic differentiation; CD36, CD71, and glycophorin A for erythroid differentiation). In 10% of DS AML cases, blasts are undifferentiated, lacking sublineage differentiation markers. Cases demonstrating significant myeloid dysplasia or myeloid blast differentiation may represent infrequent cases of standard non-DS AML. Given the expected ratio of DS to non-DS AML in DS patients, such cases should be reviewed by an experienced hematopathologist. With cytogenetic testing patients by definition must have constitutional +21 or mosaic +21. Additional abnormalities such as +8 are seen, but do not appear to affect prognosis. Presence of translocations typical of childhood AML in non-DS patients are not seen; such karyotypes may indicate that standard non-DS type disease should be confirmed by FISH testing and should be reviewed by an expert cytogeneticist. MRD testing in follow-up samples may be beneficial for predicting outcome. While *GATA1* mutations are usually present, such information is not required for clinical purposes, and comprehensive testing is difficult as mutations are not localized and require extensive sequencing not currently feasible in clinical laboratories. Other mutations have been demonstrated with advances in molecular testing, but no clinical significance has been shown. After 4 years of age, the incidence of MDS and AML in DS patients decreases markedly (to the approximate level of disease in non-DS children), patients lack *GATA1* mutations, usual subtypes of childhood disease become prevalent, and prognosis reverts to that of standard non-DS disease.

2.6 Summary

An array of testing to include morphology, flow cytometric immunophenotyping, cytogenetics, and increasingly molecular genetics is necessary

Table 2.3 Risk groups in adult AML based on cytogenetic and molecular analysis

Risk profile	Genetic abnormality	Other mutations
Favorable	t(8;21)(q22;q22.1); *RUNX1-RUNX1T1* inv(16)(p13.1;q22) *or* t(16;16)(p13.1;q22); *CBFB-MYH11* Mutated *NPM1* without *FLT3*-ITD or with *FLT3*-ITD$^{low\ b}$ Biallelic mutated *CEBPA*	Any mutation or combination thereof not classified as intermediate or adverse
Intermediate	Mutated *NPM1* and *FLT3*-ITD$^{high\ b}$ Wild-type *NPM1* without *FLT3*-ITD or with *FLT3*-ITD$^{low\ b}$ (without adverse-risk genetic lesions) t(9;11);(p21.3;q23.3); *MLLT3-KMT2A(MLL)* Cytogenetic abnormality not classified as favorable or adverse	Any mutation or combination thereof not classified as favorable or adverse
Adverse	t(6;9);(p23;q34.1); *DEK-NUP214* t(v;11q23.3); *KMT2A (MLL)* rearranged t(9;22)(q34.1;q11.2); *BCR-ABL1* inv(3)(q21.3q26.2 or t(3;3)(q21.3;q26.2); *GATA2,MECOM(EVI1)* −5 or del(5q); −7; −17/abn(17p) Complex karyotype, monosomal karyotype[a] Wild-type *NPM1* and *FLT3*-ITD$^{high\ b}$ Mutated *RUNX1* Mutated *ASXL1* Mutated *TP53*	Any mutation or combination thereof not classified as favorable or intermediate

Adapted from Dohner, H., Estey, E., Grimwade, D. et al. (2017). "Diagnosis and management of AML in adults: 2017 ELN recommendations from an international expert panel." *Blood* 129(4): 424–447

[a]Complex karyotype defined as three or more unrelated chromosome abnormalities in the absence of one of the WHO-designated recurring translocations or inversions. Monosomal karyotype defined by the presence of one single monosomy (excluding loss of X or Y) in association with at least one additional monosomy or structural chromosome abnormality (excluding core-binding factor AML) [196]

[b]Low, low allelic ratio (<0.5); high, high allelic ratio (>0.5); semiquantitative assessment of *FLT3*-ITD allelic ratio (using DNA fragment analysis) is determined as ratio of the area under the curve "*FLT3*-ITD" divided by area under the curve "*FLT3*-wild-type"; recent studies indicate that AML with *NPM1* mutation and *FLT3*-ITD low allelic ratio may also have a more favorable prognosis [197–199]

to diagnose and subclassify AML as well as assign the patients into clinically meaningful risk categories (Table 2.3). Careful attention is required to assure an adequate sample is obtained at diagnosis to accomplish this array of testing. Expanding knowledge of the genetic basis of AML will continue to complicate diagnostic requirements and classification, as increased understanding of biology progresses to therapy directed at specific genetic targets in AML. The most important diagnostic and classification issues are:

- Distinction of AML-RGA from AML-MRC (because of the fundamental biologic differences in the two sets of disease).
- Recognition of specific molecular/genetic subsets of disease amenable to targeted therapy (e.g., currently AML with *PML-RARA*; AML with *IDH1/2*, *FLT3*-ITD, or *TP53* mutations).
- Recognition of specific molecular/genetic subsets of disease requiring specific prognostication-driven treatment strategies (CBF AML, AML with mutated *NPM1*, AML with biallelic *CEBPA* mutation, AML with *TP53* mutation).
- Recognition of specific molecular/genetic subsets of disease requiring possible genetic counseling (AML with germline predisposition).

Accumulating molecular genetic data generated by NGS-based and other technologies will continue to refine our understanding of the biology of AML, provide novel insights into its

pathogenesis, lead to new treatments for subsets of disease, and require an expanding array of laboratory testing. Incorporation of these developments into AML classification will be challenging and may eventually lead to fundamental revisions of AML classification [23, 45, 200–202]. The need for monitoring early response by MRD assessment with adjustment of treatment will require improvements in flow cytometry and/or application of molecular approaches such as NGS-based methods. A role for long-term monitoring of MRD remains to be established, except in APL. There remains a need for better understanding of the pathogenesis of MDS and AML-MRC, as we currently lack precise diagnostic methods and treatments for most patients with these entities outside of allogeneic stem cell transplantation.

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