Differential Diagnosis and Testing for Hematologic Malignancies

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Most hematologic malignancies are heterogeneous, meaning they have variable phenotypes, clinical presentation, and outlook for progression and survival—all of which also evolve over time, even within an individual patient. The types of treatments and options within drug classes are also rapidly evolving as new pathways and targets are characterized and exploited for therapeutic benefit. New diagnostic tests are helping clinicians not only confirm a diagnosis, but also understand the nuances of the patient's disease, especially mutations that may be targetable by a growing number of antineoplastic agents. Discussing these newer approaches to diagnostics at JADPRO Live 2018 were Sandra E. Kurtin, PhD, ANP-C, AOCN®, a nurse practitioner at the University of Arizona Cancer Center, and Jennifer Knight, MD, of Greenville Health System and the University of South Carolina School of Medicine—Greenville.

"Staying up to date with this has become critical in managing our patients with hematologic malignancies," said Dr. Kurtin. It is important not only to understand the various diagnostic tests and what they can provide, but to know how to apply them appropriately. "It's important that we use these tests to ask questions that get us the information we need, and that we are not necessarily ordering tests just to get every bit of information, including information that may not be actionable. We have the opportunity to optimize our diagnostic process in a way that benefits the patient and advances the science, but we need to ask the right questions."

DIAGNOSTIC TESTING

The diagnostic process begins with tissue sampling. "Plan ahead. Know what questions you need to ask for a suspected diagnosis or for evaluating response to treatment or disease progression. Ask how many samples you need, and what type of medium or tube to use, then obtain a good sample. For instance, there are more than 30 different types of lymphoma, and in order to make that diagnosis you need a good amount of tissue. This is why an excisional biopsy is preferred," Dr. Kurtin noted.

Bone Marrow Aspirate

Material obtained from a bone marrow biopsy includes the core, the aspirate, the clot section, and touch preparations. The aspirate yields in-

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formation about cytoplastic and nuclear morphology, the aim being to examine cells for maturation or the presence of dyspoiesis, the blasts percentage, presence of auer rods, ringed sideroblasts, iron stores, and cell surface antigens (using flow cytometry). Cytogenetics, polymerase chain reaction (PCR), and gene expression profiling can be done on the aspirate, and chimerisms can be obtained for allogeneic stem cell transplant recipients using the aspirate. Leftover aspirate can be allowed to clot and then processed for morphologic evaluation, Dr. Kurtin explained.

Describing the process of bone marrow biopsy and the importance of each component, Dr. Kurtin said, "We are looking for spicules, the little cells that indicate we are in the marrow space. The excess of that first pull goes for a clot section, which can also provide a lot of information. Then we do a core, which is decalcified in the lab. Before sending it off, we do touch preps, and this gives information about the architecture of the marrow." The architecture reveals the myeloid-vs.-erythroid ratio; location of cell lines; presence of immature precursor cells, iron stores, fibrosis, and malignant disease; and general bone marrow morphology.

Immunohistochemistry

Dr. Kurtin then described what can be seen on immunohistochemistry. Showing a slide she labeled "the tapestry of B lymphocyte disorders" (Lebien & Tedder, 2008), she noted that to understand the differences among the squares (samples), the hematopathologist prepares stains and "algorithms" to get a sense of morphology. "Are the cells big or small? Are they white blood cells? Are they red blood cells? Are they platelets? It really takes a trained eye to be able to detect what the primary diagnosis might be."

Flow Cytometry

Flow cytometry uses large panels to characterize the neoplastic population. It identifies lineage and immunophenotype, the "zip code for cell of origin." It detects aberrant antigenic expression patterns and is useful for detecting blasts. Especially informative is the detection of clusters of differentiation (CDs), which are cellular proteins expressed by particular malignancies. For example, CD20 helps define a cell as a B cell. It is present on almost all B-cell lymphomas and can be targeted by the anti-CD20 antibodies. CD30 is expressed by classical Hodgkin lymphoma cells and is targeted by brentuximab vedotin (Adcetris). "Flow cytometry is not necessarily diagnostic by itself, but it helps us paint the picture of what this disease might be," Dr. Kurtin pointed out.

Flow cytometry can be performed on blood, body fluids, bone marrow aspirate, and lymphoid tissue, but samples must be fresh. It is also useful in evaluating residual disease or progression, and it is a test that can be done in a few hours.

Metaphase Cytogenetics

Metaphase cytogenetics provides karyotypes for the individual patient (the normal male karyotype is 46, XY[20]). Cells are grown in culture, arrested in metaphase, and stained. This requires fresh tissue and several days. With a few exceptions, bone marrow aspirate is required. The process is labor intensive and requires expertise.

Metaphase cytogenetics is informative as to the numeric or structural abnormalities of the tumor, revealing, for example, hyperdiploidy, trisomy, deletions, translocations, and inversions. The complexity of this scenario is reflected in Table 1, which shows the World Health Organization criteria for acute myeloid leukemia (AML) with myelodysplasia-related changes (Arber et al., 2016).

"If you can't make the diagnosis based on the other details of morphology or other testing, there are certain cytogenetic abnormalities that by definition imply what the diagnosis is," she said. "Again, it's about asking the right question and running the right tests."

Fluorescence in Situ Hybridization

Fluorescence in situ hybridization (FISH) offers even more precision. FISH is a process that vividly paints chromosomes or a portion of chromosomes with fluorescent molecules. It identifies chromosomal abnormalities that may not be detectable by metaphase cytogenetics, aids in gene mapping, analyzes chromosome structural aberrations, and determines ploidy.

"Basically, this is another way to identify chromosomal abnormalities that may be missed by metaphase cytogenetics," Dr. Kurtin indicated. For example, in multiple myeloma, one sees fully matured plasma cells, but they are not dividing.

Table 1. Metaphase Cytogenetics

May detect numeric or structural abnormalities
- Numeric
  - » Ploidy
    - Hyperdiploid: > 46 chromosomes
    - Hypodiploid: < 46 chromosomes
  - » Trisomies: change in the number of individual chromosomes
- Structural
  - » Deletions (del)
  - » Duplications (+)
  - » Translocations (t)
  - » Inversions (inv)
  - » Rings or markers

Cytogenetic abnormalities sufficient to diagnose AML with MRC when > PB or BM blasts and prior therapy have been excluded
- Complex karyotype (3 or more abnormalities)
- Unbalanced abnormalities
  - » 27/del(7q)
  - » del(5q)/t(5q)
  - » i(17q)/t(17p)
  - » 213/del(13q)
  - » del(11q)
  - » del(12p)/t(12p)
  - » idic(X)(q13)
- Balanced abnormalities
  - » t(11;16)(q23.3;p13.3)
  - » t(3;21)(q26.2;q22.1)
  - » t(1;3)(p36.3;q21.2)
  - » t(2;11)(p21;q23.3)
  - » t(5;12)(q32;p13.2)
  - » t(5;7)(q32;q11.2)
  - » t(5;17)(q32;p13.2)
  - » t(5;10)(q32;q21.2)
  - » t(3;5)(q25.3;q35.1)

Note. AML = acute myeloid leukemia; MRC = myelodysplasia-related changes; PB = punch biopsy; BM = bone marrow. Information from Arber et al. (2016); Pittaluga, Barry, & Raffeld (2017).

Cytogenetics may appear normal, but FISH can detect some important cytogenetic abnormalities, such as deletions 13q or 17p.

While FISH has relatively low sensitivity and examines fairly large structural abnormalities, compared to karyotyping, it is more sensitive and detects smaller abnormalities. It is especially useful for following patients over time. "The drawback is, you need to know what you're 'fishing' for," Dr. Kurtin pointed out. FISH needs to be ordered for a specific diagnosis, because specific panels are required for different diseases.

Polymerase Chain Reaction

"Polymerase chain reaction (PCR) is the next-level test. It amplifies nucleic acid sequences and can detect structural abnormalities as well as single-base-pair abnormalities," she continued. "PCR is highly sensitive, it's fast, it's qualitative and quantitative, and it can be done on fresh tissue or on FFPE (formalin-fixed paraffin-embedded) tissue. But again, it's not a general panel. You need to know what you're looking for. You have to be able to ask the right question."

Next-Generation Sequencing

Next-generation sequencing (NGS) is the "big, new, fancy test that everyone thinks they want," she continued. NGS can simultaneously sequence millions of DNA molecules in parallel from random, unselected, or genetic regions of interest. There is a hefty upfront cost to develop the technology and reagents, otherwise known as the "library," but the prepacked analysis software and reagents are inexpensive. Not all such tests are reimbursed. The US Food and Drug Administration has recently approved NGS for detecting minimal residual disease in B-cell acute lymphoblastic leukemia (ALL) and multiple myeloma.

CASE STUDY

Dr. Knight then presented several cases for which diagnosis, prognosis, and treatment were determined using these tests. One case involved a 68-year-old man admitted for significant anemia (hemoglobin < 6.0 g/dL) who had a presumed diagnosis of an acute gastrointestinal (GI) bleed. A recent colonoscopy was normal, essentially ruling out the lower GI tract as the source, and upper endoscopy showed no abnormalities, excluding the upper GI tract as the source. Laboratory tests showed that in addition to low hemoglobin he also had a low white cell count (2.3 Th/mm^3), low platelet count (84 Th/mm^3), and significant neutropenia and circulating blasts. "This patient was highly suspicious for acute leukemia," Dr. Knight said.

A bone marrow biopsy not only revealed blasts but also promonocytes, which are blast-equivalents. With the immunophenotype confirmed by flow cytometry, the patient was diagnosed with AML with monocytic differentiation. Acute myeloid leukemia is a complex, dynamic disease, characterized by multiple somatically acquired driver mutations, coexisting competing clones, and disease evolution over time. Genetic testing is needed not only to obtain the final subclassification but also for prognostic and therapeutic information.

On an NGS panel, no mutations were detected from among *FLT3, CEBPA, IDH1/2, NPM1, TP53, KRAS,* and *NRAS*. Testing did not exclude the presence, however, of a mutation occurring at an allele frequency that was less than 5% to 10% (the established detection limit), or other alterations in untested gene regions. The oncology team had a single-gene *FLT3* mutation analysis also done separately, in order to obtain results sooner than the turnaround time for the NGS panel (typically a 2–3 weeks), in order to initiate midostaurin, if appropriate. This was, in fact, suspicious for a low-level *FLT3-ITD* mutation (detected below the level of assay sensitivity and well below what NGS testing could detect). Karyotyping revealed a poor-prognostic feature, monosomy 7. This information was helpful in designing treatments for the patient.

GENETIC TESTING

Molecular studies are becoming increasingly important. For working up AML, a disease in which mutations greatly affect prognosis and treatment, the National Comprehensive Cancer Network (NCCN) Guidelines recommend cytogenetic analyses via karyotype, with or without FISH (NCCN, 2018). Currently, the NCCN lists 8 mutations that persons with acute leukemia should be tested for: *c-KIT, FLT3-ITD, FLT3-TKD, NPM1, CEBPA, IDH1, IDH2*, and *TP53* (NCCN, 2018). "But the NCCN acknowledges that the field is evolving rapidly, and there are other platforms, including NGS, to look for additional mutations that may affect prognosis for your patient, therapeutic decisions, and eligibility for clinical trials," Dr. Knight said. "The question then becomes whether you should test all of your AML patients with NGS panels."

In Dr. Knight's opinion, the use of NGS should be driven by certain considerations:

- Whether there is the potential for diagnostically significant, prognostically significant, or therapeutically significant genetic mutations in the patient
- Whether the detected mutations can be used to track minimal residual disease (MRD)
- Whether the turnaround time makes testing and use of the results feasible
- The type of specimen that is needed (some allow FFPE as well as bone marrow aspirate and peripheral blood)
- Insurance reimbursement for the test

Many commercial panels are available, and some examples are provided in Table 2. They test for different genes, accept different tissue samples, and have different turnaround times.

Further explaining these proposed considerations, Dr. Knight noted that some mutations define subtypes of AML, such as t(15:17), and some, such as those associated with clonal hematopoiesis of indeterminate potential (*ASXL1* is one), carry less diagnostic specificity. As for prognostic significance of the mutations, this can be affected by an abnormal karyotype. A simple karyotype is still necessary. Additionally, combinations of mutations and allele frequency may also carry different prognostic messages. "Is the proportion of mutated leukemic cells prognostically significant? Does it matter if one or both alleles are mutated? For *CEBPA*, for example, a biallelic mutation is associated with good outcomes, but for *FLT3,* a mutated-to-wild-type ratio greater than 0.5 is associated with worse prognosis," she indicated. "For many genes, information is still being gathered on the significance of allele frequency."

Dr. Kurtin weighed in on the topic of *FLT3* mutations and the complicated nature of testing by NGS. She noted that *FLT3-ITD* mutations can be detected by NGS, but some other types of *FLT3* mutations cannot. Single-gene mutation analysis for *FLT3* may be a better choice. "This is an example of the science rushing ahead of our ability for clinical utilization of the information," she said. "There are nuances to NGS testing. This is really an evolving science."

Turnaround time for the NGS test often matters to clinicians. As more targeted drugs become approved, it becomes more important to identify the mutations and start patients on targeted treat-

Table 2. Multigene Panels

Test name (lab)	Genes tested	TAT (specimen)
NeoTYPE AML Prognostic Profile (NeoGenomics)	28 genes: *ASXL1, BCOR, BRAF, CEBPA, CSF3R, DNMT3A, ETV6, EZH2, FLT3, HRAS, IDH1, IDH2, JAK2* including *V617F* and exons 12+14, *KIT, KMT2A (MLL), KRAS, NPM1, NRAS, PDGFRA, PHF6, PTPN11, RUNX1, SETBP1, STAG2, TET2, TP53,* and *WT1*	14 days (PB, BM, FFPE)
IntelliGEN Myeloid (Lab Corp)	50 genes: *ABL1, ASXL1, BCOR, BCORL1, BRAF, CALR, CBL, CDKN2A, CEBPA, CSF3R, CUX1, DNMT3A, ETV6, EZH2, FBXW7, FLT3, GATA1, GATA2, IDH1, IDH2, IKZF1, JAK2, JAK3, KDM6A, KIT, MKT2A, KRAS, MPL, NF1, NOTCH1, NPM1, NRAS, PDGFRA, PHF6, PML, PTEN, PTPN11, RAD21, RUNX1, SETBP1, SF3B1, SMC1A, SMC3, SRSF2, STAG2, TAT2, TP53, U2AF1, WT1,* and *ZRSR2*	Not provided (PB, BM)
Myeloid Molecular Profile (Genoptix)	44 genes: *ASXL1, BCOR, BRAF, CALR, CBL, CEBPA, CSF3R, DDX41, DNMT3A, ETNK1, ETV6, EZH2, GATA2, GNAS, GNB1, IDH1, IDH2, JAK2, KIT, KRAS, MPL, NF1, NPM1, NRAS, PDGFRA, PHF6, PPM1D, PTPN11, RAD21, RUNX1, SETBP1, SF3B1, SH2B3, SMC1A, SMC3, SRSF2, STAG2, STAT3, STAT5B, TET2, TP53, U2AF1, WT1,* and *ZRSR2*	10–12 days (PB, BM)
FoundationOneHeme (Foundation Medicine)	> 400 genes	2 weeks (PB, BM, FFPE)

Note. TAT = turnaround time; PB = punch biopsy; BM = bone marrow; FFPE = formalin-fixed paraffin-embedded tissue. Information obtained from individual lab websites on 9/13/18.

ment as soon as possible, she explained. Prompt treatment can be hampered by a turnaround time of 2 weeks, which is standard for many tests. "If you are considering a targeted drug for induction therapy, an NGS panel might not be the way to go," Dr. Knight advised.

MINIMAL RESIDUAL DISEASE

Minimal residual disease in AML is emerging as an important prognostic factor. "MRD in AML is a different beast than it is in ALL and myeloma," Dr. Knight commented. Some mutations, such as those in *NPM1*, can be used to track MRD over time, while some others, such as *FLT3*, cannot. For many mutations, however, the answer is not clear, and this is a hot area of research. A recent study published in *The New England Journal of Medicine* (Jongen-Lavrencic et al., 2018) described a different strategy: Rather than tracking individual mutations over time, the NGS panel could be viewed "as a whole" and any mutation arising during the disease trajectory could be called MRD positive. Other issues to be sorted out with tracking MRD are the significance of allele frequency, optimal limits of detection, and optimal timing for testing. At this time, the benefit of changing treatment based on MRD positivity remains unknown. To Dr. Knight, "That's the ultimate question and goal."

Disclosure

Dr. Kurtin has consulted for Amgen, Celgene, DSI, Genentech/AbbVie, Incyte, Janssen/Pharmacyclics, Jazz Pharmaceuticals, and Takeda. Dr. Knight has nothing to disclose.

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