With molecular MPN testing, think positive

Karen Titus

March 2015—If molecular tests for myeloproliferative neoplasms ever decide to write their autobiography, they could easily do a riff on the business bestseller *Getting to Yes*.

For myeloproliferative neoplasms, morphologic and clinical findings should guide molecular analysis, which can often be a helpful way to clinch the diagnosis, says Dr. David Czuchlewski (left), of TriCore Reference Laboratories, with Mohammad Vasef, MD, TriCore's director of molecular diagnostics.

Diagnosing and monitoring MPNs produce more than their fair share of negative results. But in many cases, that "no" news is not necessarily good news, or even any news at all. Maybe the mutation truly isn't there—or maybe the negative result is a hint to use a different set of primers. Maybe a more sensitive test is in order. Maybe the patient has acquired a new, resistant mutation during treatment—or maybe noncompliance has become an issue.

Not all negative results are created equal when it comes to MPNs, in other words. "Negative results can be helpful to clinicians," says Todd Kelley, MD, medical director, molecular hematopathology, ARUP Laboratories, Salt Lake City. But, he adds, they need to know how to interpret a negative result and make the next testing decision.

"It's a complicated area," says David Czuchlewski, MD, associate professor of pathology, University of New Mexico, and associate director, molecular diagnostics, TriCore Reference Laboratories, Albuquerque. "Even for hematopathologists and others well-versed in it."

It's a topic familiar to Dr. Czuchlewski, who gave a talk on the subject at CAP '14 last fall. The subject has become even more engrossing, he says, with the discovery, in late 2013, of two new, highly relevant diagnostic mutations, *CSF3R* and *CALR* (calreticulin). "It was a big year," he says.

Far from upending everything, the new discoveries fit into the continuum of MPN molecular testing. If the first molecular test or two doesn't turn up a mutation, press on, like Sherman to the sea. If an initial test for *JAK2 V617F* is negative, for example, it might be smart to look for a less common mutation, such as *JAK2* exon 12. In *BCR-ABL1*-negative cases, *CSF3R* testing might provide an answer.

Nor are new mutations shutting the door on morphology. Despite its importance, molecular testing—not even next-generation sequencing—isn't particularly special, says Ayalew Tefferi, MD, professor of medicine and hematology, Mayo Clinic, Rochester, Minn. "The tools are different, but there are always new tools," says Dr. Tefferi. "It's like children: When there are new toys, they want new toys. But the concept is the same; the concepts don't change," a surprisingly low-key view from someone Dr. Czuchlewski calls "probably the world expert on new molecular approaches to MPNs."

In his CAP '14 talk, Dr. Czuchlewski listed several diagnoses that could mean a laboratory is on the wrong path, and perhaps relying too heavily on its molecular GPS, so to speak:

- Essential thrombocytopenia (ET) with *JAK2* exon 12 mutation (i.e. not V617F).
- Primary myelofibrosis (PMF) with *JAK2* exon 12 mutation (i.e. not V617F).
- Polycythemia vera (PV) with *MPL* mutation.
- PV with *CALR* mutation (typically, only rare patients now reported).

In essence, molecular testing of MPNs involves two roads, but unlike Robert Frost's traveler, pathologists can and need to take them both. One starts with clinical criteria, then moves through morphology and molecular testing; the other is the molecular testing itself.

One obvious fork in the road is the *BCR-ABL1* fusion gene. If it's present, it points to chronic myelogenous leukemia (though it's not specific to the disease); PV, ET, and PMF, on the other hand, are *BCR-ABL1*-negative MPNs. The fusion most often arises from a (9;22) translocation. The resulting Philadelphia chromosome was the first cytogenetic abnormality identified in the setting of MPNs, Dr. Czuchlewski notes. "Our approach to the newer tests can be somewhat similar to how we've approached the 9;22 translocation. The new stuff isn't replacing old testing and old information—it's adding to it." The result is a much more complete picture of the MPN molecular landscape, he says.

Dr. Czuchlewski presented three cases in his talk to help illustrate the point. The first involved a 79-year-old woman with persistent leukocytosis. One of the first questions to arise in such a case is: Could this be chronic myelogenous leukemia?

Certainly there are specific morphologic clues that can lead pathologists to suspect a CML diagnosis, though they were absent in this particular case. True, the patient had a very high white blood cell count (> 50 × 109/L), but it consisted predominantly of neutrophils and lacked a significant percentage of myelocytes. And there was no basophilia or eosinophilia.

Nevertheless, says Dr. Czuchlewski, in a case like this it's important to assess for a *BCR-ABL1* fusion. "It's a diagnosis that simply can't be missed, especially because the targeted therapy [imatinib] is so effective." Moreover, there are cases of CML that have unusual phenotypes, including ones that are neutrophil-predominant. Think of it as a stack of Eames chairs at Mount Vernon—puzzling but attention getting.

The lab can choose from several arrows in its quiver: karyotyping, FISH, and RT-PCR.

Karyotyping is a good option, says Dr. Czuchlewski, though it can miss a small number of cases involving cryptic rearrangements—the fusion occurs, but in an unusual form that cannot be detected on conventional cytogenetic analysis. "You could miss a true *BCR-ABL* fusion," he says. Another consideration: Karyotyping may be suboptimal in peripheral blood samples.

FISH is terrific for initial assessment of CML, Dr. Czuchlewski says. It's fast; it can detect cryptic rearrangements that elude karyotyping; and it will provide positive results even in cases that involve variant breakpoints.

RT-PCR has high analytic sensitivity. But it has a blind spot of sorts, Dr. Czuchlewski notes. Different forms of the transcript occur; the major one is found in the cluster region between exon 13 and exon 14 (corresponding to the p210 fusion protein) and is the one that's most associated with CML. In his lab, "We use an RT-PCR strategy to account for this heterogeneity at the breakpoints, essentially." But this requires using different primer sets to pick up the various transcript forms. "Because if it's a negative result, you want to make sure it's covered all the different possibilities that you're interested in," Dr. Czuchlewski says. There are rare cases of CML, for example, that have an alternative breakpoint in the *BCR-ABL1* gene, occurring after exon 19 (corresponding to the p230 fusion), which is called the micro-breakpoint.

Explains Dr. Kelley: "If FISH testing is positive for *BCR-ABL1* but then a quantitative RT-PCR test against p210 is negative, that's evidence that a rarer fusion form may be involved. But you have to know what you're looking for, and make sure you order a test that can detect that." There is indeed a needle in that haystack. You just have to know to look for it, and how.

In the case of the 79-year-old woman, there were two normal BCR signals on 22q11.2, and two normal ABL1 signals on 9q34. "So what does she have?" Dr. Czuchlewski asks. In this instance, it seemed reasonable to consider chronic neutrophilic leukemia (CNL), an MPN characterized by marked neutrophilia with minimal left shift.

Enter that 2013 breakthrough, CSF3R, which indeed identified CNL as the culprit here. For too many years, labs had no good molecular test for CNL. But two years ago, it emerged that the majority of these cases carry mutations in this gene (Maxson JE, et al. N Engl J Med. 2013;368:1781–1790). "This is very useful, because it gives us a clonal marker that allows us to clinch the diagnosis," says Dr. Czuchlewski. There's also some early suggestion—emphasis on early—that for some patients this may open the door to targeted therapy, especially with *JAK2* inhibitors.

More controversial is whether the CSF3R mutation is seen in atypical CML. In some early reports, including the New England Journal of Medicine paper, researchers have said yes. As so often happens in medicine (and with gubernatorial budgets), subsequent commentators have said no.

Given the evolving thoughts on this matter, pathologists might want to watch out for silly CSF3R test ordering. Clinicians, understandably, will be enthusiastic about the test, but, Dr. Czuchlewski says, "What we're talking about are cases where there's significant neutrophilia. And the vast majority of those cases are going to be patients who have an infectious or inflammatory etiology." Ideally, he says, this can be sorted out clinically without relying on mutation testing. "We don't want a situation where the reflex would be, 'This patient's neutrophils are increased. Let's get this mutational analysis to rule out a neoplasm'—when in fact all the morphologic and clinical clues indicate otherwise."

Mayo's Dr. Tefferi seconds that notion. Though he harbors no doubts that genetic information will become more influential, he, too, argues on behalf of morphology's strengths, even as the molecular trail grows longer. "Morphology can tell us so much," he says. "The problem is, other scientists and clinicians who are not trained as pathologists can't appreciate that. They think it's too subjective." He barely holds back a sigh.

Beyond *BCR-ABL1* testing, laboratories need to consider *JAK2* mutation testing. The most common mutation in the classic *BCR-ABL1*-negative MPNs is *JAK2* V617F.

Dr. Kelley

Here, again, it pays to keep looking. At ARUP, Dr. Kelley and his colleagues use allele-specific PCR for *JAK2* V617F, but they also use a qualitative test against another portion of the gene, *JAK2* exon 12. “The mutations that occur there tend to be insertions and deletion-type mutations,” says Dr. Kelley, who is also associate professor, Department of Pathology, University of Utah.

There’s some confusion about *JAK2* allele frequency, Dr. Kelley says. “This is getting way down in the weeds, but it is an issue,” he says. These mutations can be present at a very low frequency in a patient who has disease, making test sensitivities critical.

Say, for example, a lab is testing for a *JAK2* V617F mutation by Sanger sequencing. Since the method has a sensitivity of 10 to 15 percent, says Dr. Kelley, there needs to be a mutant allele burden in a sample of at least 10 to 15 percent for it to be detected. “So a negative result by that sort of a test has a different meaning than negative results by a test like allele-specific PCR, which typically has sensitivities well below one percent,” Dr. Kelley says. “We have to educate our test users about this issue, whether it’s other pathologists or hematologists. Test sensitivities can be quite variable, and they need to understand that to be able to interpret a result, especially a negative one.”

If a test result is negative, in the context of persistent clinical concern, “we would encourage the clinician to continue to investigate,” Dr. Kelley says. That could mean ordering a more sensitive test or a test directed against rare targets. In addition to *JAK2* exon 12 if PV is suspected, there are rare mutations in MPL if ET or PMF is suspected—Dr. Kelley points to two hotspots, codon 505 and codon 515. If a test is negative for 515, that doesn’t necessarily rule out a mutation in 505. “There’s a lot to understand in terms of the mechanics of the testing—and again, that’s important to interpreting a negative result,” says Dr. Kelley.

The second case Dr. Czuchlewski presented involved a 21-year-old male who appeared to have polycythemia vera. The search for a *JAK2* mutation, either V617F or exon 12, came up negative.

A diagnosis of PV can be made in the absence of *JAK2*, Dr. Czuchlewski says, but if initial tests fail to find it, “you might want to make sure you have all your ducks in a row in terms of the diagnosis.”

In this case, a JAK2 mutation made sense, so Dr. Czuchlewski and his colleagues kept up the hunt. They eventually found a far less common *JAK2* exon 12 mutation that was undetectable by standard allele-specific PCR. In PV, JAK2 V617F (exon 14) mutations account for 96 percent of mutations; three percent are mutations at *JAK2* exon 12. That leaves a small but worthy percent of cases that might require a different testing method.

The final case involved a 49-year-old man who had significant thrombocytosis. When the lab looked to the bone marrow biopsy to better characterize this and to make a diagnosis of an MPN, “The specimen was just terrible—basically uninterpretable from a morphologic standpoint,” Dr. Czuchlewski recalls. So they turned to molecular techniques.

The matter boiled down to whether this was an essential thrombocytopenia versus primary myelofibrosis. Until about 10 years ago, molecular testing in these situations started and stopped with JAK2 V617F. But as it turns out, a small percentage of these cases have mutations in the MPL gene.

Another big development occurred in 2013, as noted (Klampfl T, et al. N Engl J Med. 2013;369:2379–2390; Nangalia J, et al. N Engl J Med. 2013;369: 2391–2405), with the emergence of the CALR gene. In fact, the majority of ET and PMF cases with no previously known mutations share a mutation in the CALR gene. By putting all three together—JAK2 V617, MPL, and CALR—pathologists could assign a molecular marker to nearly 90 percent of ET/PMF cases.

“That was a very important finding,” says Dr. Kelley. “A lot of laboratories around the country, including our own, then scrambled to get tests up and running for those, because we essentially immediately had a demand for calreticulin testing for ET and primary myelofibrosis.” So important is calreticulin that the WHO plans to include it as part of its next MPN diagnostic criteria, says Dr. Tefferi.

Interestingly, says Dr. Czuchlewski, calreticulin mutations appear to lead to increased JAK2 signaling. CALR may also have prognostic considerations, as this mutation tends to occur in patients who are younger, who may have a lower risk of thrombosis, and who, in some cases, may have better survival.

In Dr. Czuchlewski’s lab, workup of these cases begins with JAK2 V617F testing, looking for CALR if that’s negative, and looking for MPL if both are negative. “We tend to do this as somewhat of a reflex process,” he says.
Turnaround time is an issue, naturally. Can the laboratory do this efficiently? What about cost?

That leads to another question, one posed by Dr. Tefferi: “When can you say, ‘It’s time to start doing next-generation sequencing?’”

Dr. Tefferi

As a national reference laboratory, ARUP receives plenty of requests for testing cascades or reflex panels, including for patients with MPNs. “That’s a more efficient way of doing it than just doing three or four different tests at the same time,” says Dr. Kelley.

“Of course, next-gen sequencing is not a cascade,” he continues. ARUP also uses a next-gen sequencing test that targets 53 mutations occurring across the spectrum of myeloid malignancies, including not only MPNs but also acute myeloid leukemia, myelodysplastic syndromes, and MDS/MPN overlap disorders like chronic myelomonocytic leukemia. “This can also be helpful in working up patients with the atypical presentations of a myeloproliferative neoplasm,” Dr. Kelley adds. “Some of this other single-gene testing is yielding unclear results.”

Given the lengthening list of culpable mutations, NGS looks powerful. “You don’t have to necessarily think about a very rare diagnosis; you can just look at the spectrum of mutations,” says Dr. Kelley.

As Dr. Czuchlewski noted in his talk, that could include ASXL1 (found in three percent of ETs and 13 percent of PMFs), TET2 (five percent ET, 17 percent PMF), DNMT3A (seven percent ET, six percent PMF), SRSF2 (three percent ET, 17 percent PMF), CHEK2 (two percent ET, three percent PMF)—the list continues, and could grow even longer. NGS might also help pathologists untangle the importance of the combined presence of mutations—CALR and ASXL1, for example.

Dr. Tefferi understands the allure. At Mayo, he has access to seemingly limitless MPN genetic data, and he's doing his best to put it to good use. "We've stored a lot of patient samples over the years," he says, "and there's complete follow-up of many of these patients." His goal is to provide rock-solid data to support the value of a mutation in diagnosis or prognosis. Only then, he says, will organizations such as WHO advocate a test's use. Only then will payers pony up. And only then will it make sense for a lab to add the test to its menu.

He realizes this is the slow-boat-to-China approach, and he hears the arguments to open up the throttle and add more passengers, so to speak. "We're finding one mutation after another, so why don't we just profile a patient for, say, 30 important mutations, or 230? Why don't we just use next-generation sequencing?"

"The problem with that is, you have to show me that approach actually helps patients," Dr. Tefferi says. Looking at the newer mutations, he says, it's clear that CALR is important; so is ASXL1, which is prognostically valuable not only in MPNs but also in myelodysplastic syndromes and other disorders. "But does the patient need 33 mutations? Why?"

He's familiar with the arguments that NGS yields more information with no additional costs. "So clinical laboratories are going ahead anyway. They're not even waiting for the data. Even our clinic is going to have its own platform that's going to go live soon." There may be no going back—but Dr. Tefferi persists with his questions. "Can you argue that the whole profile is important? Are you justified in ordering a 33-gene screen? I don't know how that's going to play itself out."

Dr. Tefferi makes an interesting advocate for traditional methodologies. Though poised on the cutting edge of molecular research, he says things like "Molecular testing is a risky business," and "You have to remember good clinical judgment goes hand in hand with the molecular test." Imagine Jeff Bezos urging Amazon customers to first check the library for a book before downloading it onto their Kindles.

It's not that Dr. Tefferi is rejecting NGS outright. In his own research, it's an invaluable method to help him sort through cases where multiple mutations may be in play. Preliminary indications are that in some patients, the more mutations present, the worse the prognosis; it also appears that some mutations have independent prognostic value. He's currently using NGS to explore the quantitative value—allele burden as well as the number of mutations—in PV, ET, and myelofibrosis.

Dr. Tefferi doesn't mince words. In his view, reliance on molecular testing involves a persistent amount of "magical thinking," and next-generation sequencing for diagnosis is, in his opinion, "overkill." It may be useful in a tiny fraction of patients, he says. "But we really don't have a problem with diagnosis right now"—or at least not a problem that needs to be solved by NGS. The method's real clinical value lies in prognosis and predicting response to treatment, he says.

Dr. Czuchlewski

Molecular testing in general is critical for monitoring, Dr. Kelley adds. “The laboratory has an important role throughout the treatment of these patients.”

Ideally, as a patient responds to treatment, a major molecular response will occur, with transcript levels falling in a somewhat predictable fashion. Monitoring treatment can also identify patients who respond initially, but then lose that response.

“Essentially we can adjust the effectiveness of treatment by looking, over a long period of time, at what’s happening with the transcript levels,” Dr. Czuchlewski says. This is no small matter, since tyrosine kinase inhibitors such as imatinib have transformed a disease like CML into a chronic medical condition, not a death sentence, says Dr. Kelley. That evolution, he says, has been made possible in part by laboratory testing. The goal, then, is to look for continued control of the disease at the molecular level, as opposed to, say, an acute leukemia, where monitoring would be used to identify a relapse.

Molecular monitoring of CML can present an “interesting wrinkle,” says Dr. Czuchlewski. Under the pressure of tyrosine kinase inhibitor treatment, mutations can arise in some cells of the CML clone, decreasing the effectiveness of the drug in those cells. Such selective pressure gives those cells a proliferative survival advantage, “almost Darwinian in process,” Dr. Czuchlewski says. When this happens, patients who previously had been responding to the therapy will exhibit a rise in transcript levels.

If that happens, the lab can resequence the ABL1 kinase domain, where mutations tend to occur, then suggest different therapeutic options. “We’re helping guide the clinician in a very sophisticated manner,” says Dr. Czuchlewski.

A common cause of increasing transcript levels is patient noncompliance. “There are side effects; the drugs are expensive; patients struggle with insurance,” explains Dr. Kelley. It’s important to know this, because it means a rising transcript level doesn’t always herald a problem with the clone.

In patients with *BCR-ABL1*-negative MPNs, there have been efforts to develop JAK2 inhibitor drugs. One, Jakafi (ruxolitinib), has been approved for patients with higher-risk forms of myelofibrosis, and for patients with PV who are intolerant of, or who have failed therapy with, hydroxyurea.

Given that the MPN story is unfolding with every new mutation, Dr. Kelley says, “It’s an exciting time to be involved in this kind of testing.”

More than ever, it would seem, it thus makes sense for pathologists to explore the negative with renewed zeal.

Says Dr. Tefferi, with almost Zen-like insight: “If a molecular result is negative, it doesn’t mean the mutation isn’t there.” How do we know if something exists? If a tree falls in the forest and no one is there to hear it, does it still

make a sound? “It just means I need to look at this a little more in depth. Be a good clinician. If we can get that into our heads, we’ll be OK.”

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Karen Titus is CAP TODAY contributing editor and co-managing editor.