Mate pair sequencing yields rich new data

Anne Paxton

November 2019—The LUVOIR telescope proposed this year by NASA, when it is launched into orbit, will outperform the Hubble Telescope 40-fold in ability to detect and visualize deep space objects in detail. But while dazzling in concept, the LUVOIR is still in development. Interestingly, at the genomic level, a similarly impressive advance in detection called mate pair sequencing has already progressed from research to clinical use in diagnosing cancer.

With mate pair sequencing, a novel next-generation sequencing technique, Mayo Clinic is advancing the laboratory's current capabilities for visualizing genetic rearrangements, thus increasing the diagnostic yield of testing for a variety of neoplasms.

"Traditionally, as cytogeneticists, we have the ability to look on a single-cell level at chromosomes in a karyotype, but those tests are qualitative and depend on the quality of the metaphases. You are looking by eye," says Linda Baughn, PhD, co-director of Mayo's genomics laboratory and assistant professor of laboratory medicine and pathology. "Sometimes a rearrangement is cryptic, where you just cannot see it. Mate pair sequencing is an unbiased whole genome molecular test that allows us to precisely identify the rearrangement without relying on people to see it visually using a microscope."

Just as a high-powered telescope might do with details of distant space objects, mate pair sequencing, or MPseq, can more precisely characterize the genomic complexity, providing fresh insight beyond what is detectable by fluorescence in situ hybridization or chromosome studies. By allowing detection of structural rearrangements with higher resolution compared with FISH or conventional chromosome analysis, MPseq enables characterization of precise genomic rearrangement breakpoints.

"Almost all cancers have some kind of rearrangements," Robert Jenkins, MD, PhD, professor of laboratory medicine and pathology at Mayo, says. "They generate structural abnormalities that either disrupt the structure of the DNA or generate messenger RNA or fusion RNA or change the area of the genome where they are inactive to active. The chromosome studies just don't have the resolution to detect the mechanism of what genes are being involved in the rearrangements. They only have a resolution of approximately five to 10 megabases, and that is not good enough."

At Mayo Clinic (from left): Linda Baughn, PhD; Patricia Greipp, DO, consultant, Division of

Laboratory Genetics and Genomics; Nicole Hoppman, PhD; and Beth Pitel, MS, CG(ASCP). They and other Mayo researchers didn't realize the level of complexity associated with some hematologic neoplasms until they started looking at mate pair, Pitel says. [Photo: Joseph Kane]

For patients with acute myeloid leukemia, the characterization of precise genomic breakpoints can be critically important. Last year Mayo scientists reported results from a comparative study of 68 known abnormal and 20 karyotypically normal AML samples. MPseq confirmed all the recurrent primary AML-specific abnormalities that had been detected by chromosomes, FISH, or both. In addition, MPseq also provided clarification in eight cases with abnormalities that could not be resolved by conventional cytogenetic studies. Specifically, MPseq molecularly defined eight recurrent AML-fusion events. In addition, the methodology revealed two cryptic abnormalities that conventional cytogenetic studies missed. The overall improvement in diagnostic yield for detection of AML-specific structural rearrangements was 11 percent (Aypar U, et al. *Eur J Haematol.* 2019:102 [1]:87–96).

"Mate pair is a game-changer for the field of cytogenetics," says study coauthor Jess F. Peterson, MD, also a co-director of the Mayo genomics laboratory and associate professor of laboratory medicine and pathology.

Dr. Peterson began at Mayo in 2017 as the lab was poised to be the first to go live with the AML mate pair test for clinical use. As that milestone was reached, "I was amazed at the precision of this assay," he says. "We have heavily relied upon conventional chromosome analysis and FISH to detect recurrent abnormalities associated with hematologic neoplasms, including gains, losses, and chromosomal rearrangements. Utilizing mate pair sequencing, we can confidently detect copy number abnormalities, in addition to chromosomal rearrangement breakpoints at the gene exon/intron level."

Mayo researchers did not realize the level of complexity associated with some hematologic neoplasms until they started looking at mate pair, says study coauthor Beth Pitel, MS, CG(ASCP)CM, a development technologist at Mayo. "FISH and chromosomes are wonderful, but mate pair helps structurally characterize those cases that fall into the atypical realm." For a particular chromosome result, for example, if it is uncertain what genes are involved in the translocation, the laboratory can perform mate pair and resolve it.

While most samples Mayo processes have been heme-based, the laboratory also has live clinical assays for constitutional and solid tumor evaluations. "We have had a number of congenital cases where we want to fully characterize what is happening chromosomally and assess the genes that are at certain breakpoints," Pitel says.

Tumor lineage testing—analyzing two tumors from a patient to determine whether they are from a single primary tumor—is another potential application Mayo is exploring. And more uses likely lie ahead. "We have developed an assay for AML, but using the exact same technology, the exact same chemistry, the exact same tools, we have actually seen more cases where we are using mate pair sequencing as a reflex test. So we can be talking about any disease," she says.

The instrumentation needed to perform MPseq is not complicated or unusual for laboratories equipped to perform NGS. "Equipment-wise, you need a wet bench," Pitel says. Mayo uses the Illumina Nextera reagent kit and Covaris ultrasonicator for its library prep, which is currently done manually but could be performed using automated liquid handlers. "We sequence on an Illumina HiSeq platform, but we could move to more advanced sequencers like the NovaSeq, and that would drive our costs down. You also need a thermal cycler" to amplify DNA and RNA samples

by PCR, “but that is standard lab equipment these days.”

Hutton Kearney, PhD, above right (with Pitel), leads the software development team that created a dynamic visualization interface that supports the lab by providing an interactive experience for those who analyze and interpret MPseq data.

However, a significant amount of infrastructure and support has been necessary for this assay to be brought to the level of a clinical-grade test, Pitel says. A dynamic visualization tool, Mayo Clinic Ingenium, was a primary goal for the group prior to launch of the assay. The software development team (led by Hutton Kearney, PhD, and Stephanie Smoley) created a dynamic visualization interface that supports the laboratory in a number of ways by creating an interactive experience for those who analyze and interpret MPseq data. The software can, among other things, track each case through lab director signout, create publication-grade images depicting genomic rearrangements, and display historical data. “The development of Mayo Clinic Ingenium has been paramount to the success of this assay,” Pitel says.

Among its other advantages, mate pair resolves a practical limitation of FISH assays, Dr. Peterson says. “As more disease-specific abnormalities are discovered, the development and validation of new FISH probes is simply not feasible. We have to use a technology that will allow us to evaluate the entire genome of these neoplasms.” Replacing FISH is the eventual goal.

The ideal sample for analysis is a diagnostic specimen with at least 20 percent tumor burden, “below which we start to lose our sensitivity,” Pitel says. “Where we would normally pick up subclonal rearrangements, or copy number gains or losses with lower tumor burden, we start to see fewer sequencing fragments that would support a structural rearrangement. We can overcome these limitations, to a point, with deeper sequencing if we know about the low tumor burden ahead of time.”

Ideally, turnaround time for an MPseq case is about two weeks, which is typical of NGS. Higher test volumes and the ability to process more samples simultaneously in the future may reduce the TAT. “Right now we run a wet bench MPseq library prep once a week,” Pitel says. “If our volumes increase, then the clinical team will adapt by running multiple batches a week, decreasing the TAT. The more samples we have, the more adaptability the wet bench has, and the lower the TAT will be.”

This would not be the ideal assay for someone who, for example, has acute promyelocytic leukemia and for whom the laboratory needs to detect the *PML-RARA* fusion immediately, Dr. Peterson says. “For those kinds of cases we would run our FISH probe up front for quick results.” However, Dr. Peterson has seen cases of APL with *RARA* rearrangements that were not detected by *RARA* FISH probe sets (Peterson JF, et al. *Cancer Genet.* 2019;237:51–54). “Mate pair sequencing was performed and revealed either a cryptic *PML-RARA* fusion or a variant

RARA rearrangement," he says. "These are truly cryptic translocations that are undetectable using traditional cytogenetic techniques."

Could other laboratories adopt Mayo's mate pair sequencing application? Dr. Peterson is unsure. "It would be possible only if genetic laboratories heavily invest in the NGS platforms and the bioinformatics. A tremendous amount of support is required."

The point of mate pair is not only to make a diagnosis or change the prognosis for a patient but also to determine how a patient will be treated. "I can only speak to hematologic neoplasms," Dr. Peterson says. "But using mate pair we are able to detect gene fusions that may respond to targeted therapy, such as tyrosine kinase inhibitors." He knows of many patients who have been able to be treated but might not have been in the absence of mate pair sequencing analysis. In fact, MPseq is part of the Mayo Clinic algorithm for the evaluation of Ph-like ALL when a breakpoint detected by conventional analysis suggests a disruption of a known 3′ kinase gene but a FISH probe is not available. "The technique has afforded Mayo Clinic the ability to define novel 5′ gene partners," he says.

The Mayo Clinic team continues to explore the use of mate pair in other malignancies. "We are currently validating the B-ALL, T-ALL, and plasma cell myeloma assays," and results so far have been surprising, he reports. "We are finding abnormalities that we would have never suspected based on chromosomes and FISH results."

Dr. Peterson

Dr. Peterson believes mate pair could be applied to all areas of genetics to characterize any kind of translocation. "If you want to know specifics in greater detail of what is happening, mate pair is going to be the test."

Dr. Baughn points out, however, that FISH still has its benefits: "FISH is currently much less expensive than an NGS test, and the limit of detection is better compared to our current mate pair algorithm for classically described cytogenetic abnormalities."

For diagnostic samples, she says, MPseq works well. "But FISH is much more amenable to monitoring minimal residual disease, because the sensitivity for some of our probe strategies is 0.6 percent. Mate pair cannot go that low. We can only detect down to about 10 to 20 percent of rearrangements. So once a patient has been treated and they are expecting very low levels of that rearrangement, mate pair tends not to work very well."

But NGS is better, Dr. Baughn says, particularly for identifying *MYC* rearrangements. "The detection of *MYC* rearrangements is important for several B-cell neoplasms, including high-grade B-cell lymphoma and multiple myeloma. We've discovered from validation studies that a commonly used *MYC* break-apart FISH probe cannot identify approximately four percent of *MYC* rearrangements in high-grade B-cell lymphomas [King RL, et al. *Haematologica.* 2019;104(6):e248–e251] and about half of multiple myeloma cases that we are able to identify from the mate pair sequencing."

"So if we could ignore money completely—which is probably naïve to even think—characterizing abnormalities in newly diagnosed specimens through mate pair or any NGS test that can identify a single nucleotide variant, structural variations, and copy number changes would be the way to go," Dr. Baughn says.

Nicole Lynn Hoppman, PhD, started Mayo's cytogenetic next-generation sequencing work in 2012, collaborating with George Vasmatzis, PhD, to improve the MPseq algorithm for detection and develop early visualization software as part of the interpretive phase. The clinical launch was slow, says Dr. Hoppman, another co-director of the genomics laboratory, because the first version of the test involves looking for specific chromosome

abnormalities or signal patterns by FISH that would merit investigation by MPseq and there were only one or two a month of those test results to begin with. And there was a need, of course, for a "very robust validation of the algorithm."

To obtain a billing code, the laboratory had to first have the test live. "After being live with the test for several months," she says, "we went through the PLA application process with CMS and received PLA codes for the tests—0012U for blood, 0013U for bone marrow, and 0014U for solid tumors." These three tests are currently available as reflex tests, plus the AML panel developed by Dr. Baughn and colleagues, "which is basically a FISH replacement," Dr. Hoppman says. The next step is for B-ALL and T-ALL, "where we are taking all the FISH probes we have and adding several targets we don't have FISH probes for. Our plan is to do this for multiple malignancies." So across a range of abnormalities, "we can detect structural variations, copy number changes, and we are working on an algorithm to detect loss of heterozygosity."

While solid tumors can also be tested, Dr. Hoppman says, mate pair doesn't work well in FFPE samples. "So hematologic malignancies are definitely where we see the most volume for MPseq."

The number of patients who have benefited clinically from mate pair sequencing so far is small, Dr. Hoppman says, compared with the total number of patients diagnosed with hematologic malignancies. But for those patients individually, the benefit can be significant. "It can lead to a patient receiving a drug that they would not have been previously eligible to receive and that might put them into long-term remission."

One institution that uses Mayo Medical Laboratories as its reference lab for mate pair sequencing is Legacy Health in Portland, Ore. Yassmine Akkari, PhD, scientific director for cytogenetics and molecular pathology, says it is not likely that they would be able to perform mate pair sequencing at their institution. "I would absolutely love to. But a health system such as ours would not be able to bring something this technically and bioinformatically sophisticated in-house very soon."

Published reports from the Mayo team have demonstrated the value of MPseq in various clinical situations in which an accurate diagnosis provided value in the clinical management of cancer patients, Dr. Akkari says. "There are cases when we perform traditional tests such as conventional cytogenetics, FISH, and NGS and results come back normal. In these instances, we still cannot tell the oncologist or the referring clinician what the accurate diagnosis is and how the disease will behave in terms of prognosis. In essence, we haven't helped the patient yet." Having the option to perform mate pair sequencing, she says, provides an avenue to identify structural rearrangements that may have therapeutic consequences.

Although some NGS-based pipelines can detect a variety of genomic aberrations, "they are not as validated as MPseq," according to Dr. Akkari.

Genetic diseases can be the result of a variety of mechanisms, she notes. "There could be copy number changes, sequence variation, and/or structural abnormalities including translocations, inversions, or insertions. Many laboratories are equipped to offer several testing modalities, but the ones we have available can get to one of these mechanisms and not the others. FISH, chromosomes, arrays, and sequencing all have a lot of limitations."

Mate pair sequencing, on the other hand, is able to capture the majority of the mechanisms of disease that are known today, Dr. Akkari says. While it would be inefficient to use mate pair in all cases, in her view, "in cases where diagnosis and, more importantly, prognosis completely eludes us, mate pair sequencing is perfect."

Dr. Jenkins of Mayo says the bioinformatics pipeline is the most important piece. "The chemistry is pretty straightforward in most places that do NGS. The bioinformatics pipeline is the golden goose; it's the piece that is Mayo's intellectual property." The standard NGS that is done for any kind of whole genome or exome sequencing is called paired-end sequencing; DNA fragments are made and the end of the fragments are sequenced. "Mate pair is just a modification; it involves sequencing the ends of much larger fragments," he explains.

Most places could perform the mate pair sequencing, but they have not developed the interpretive part: mapping

the sequencing data back to the genome and then displaying the rearrangement in a way that makes sense, Dr. Jenkins says. “There are multiple pipelines that can do this. Mayo’s pipeline is just more efficient at mapping these DNA sequences and then visualizing the rearrangements.”

“It is incumbent upon us as laboratorians,” he continues, “to sift through the large amount of data and present back to clinicians a succinct report that describes the alterations that are predictive, meaning actionable, versus prognostic or diagnostic, meaning they have implications for the diagnosis but not necessarily for the treatment indications. Finding an actionable mutation is actually unusual. But that is not the only reason to do these tests. You can also find things to reclassify the tumor, and that is huge.”

The practical impact on patients has been evident at Mayo Clinic, Dr. Jenkins says. “Every week we find something that absolutely makes a diagnosis for a patient, or changes the diagnosis for a patient, or determines whether or not a patient will have a poor or a good prognosis.”

The jury is still out on mate pair sequencing’s usefulness with solid tumors, he says. “Mate pair needs to work in paraffin before we can start. It can work, but not at the same efficiency as some other technologies do. Mate pair is lovely in myeloma and leukemia because bone marrow is relatively easy to get, our blood is relatively easy to harvest, but it is a challenge in most institutions to freeze a piece of tumor for DNA.”

Another part of the reason mate pair has yet to catch on in solid tumors is the number of rearrangements that are present. “Your typical carcinoma has hundreds of rearrangements. So the real problem is how does one find the driver mutation in that pile of rearrangements?”

Dr. Jenkins views mate pair as part of the overall armamentarium of genetic testing. “I don’t think it is going to solve the world’s cancer problems, but it is a very useful test in some very specific situations. For hematologic cancers it has the potential to be a test to replace other more standard tests. The stretch goal is that mate pair could replace FISH and maybe even chromosomes for most of the standard run-of-the-mill leukemias and myeloma.” Whether it is generalizable, he says, depends on how the whole field evolves. “It has the potential, but there are competing technologies.”

The clinical laboratory needs to move past chromosome studies and FISH as a gold standard, Dr. Hoppman says, “because we know now from doing mate pair that we are missing things. Next-generation sequencing is advancing so rapidly, we also know that in the future we will be able to do this more cheaply. We just need to promote awareness of this technology and what it can do in the hope of guaranteeing reimbursement for these types of tests and changing guidelines so that our patients aren’t stuck behind our advancements in the laboratory.”

The genomic laboratory co-directors at Mayo are reaching out to raise awareness of the value of mate pair sequencing. “We try to publish a lot to raise awareness of our findings,” Dr. Baughn says. “We give talks, multiple posters, and we are planning to give a pre-symposium workshop at the American Society of Hematology meeting this year. We are trying to communicate the test to people.”

She and colleagues are “revolutionizing cytogenetic testing,” as she sees it, and bringing it to the molecular level to provide more clarity to rearrangements in hematologic malignancies. “But we are at the beginning stages where we need a lot more data. I am sold already, but the community probably needs more evidence that this is a better approach. We need to do a lot more sequencing and correlation with outcomes.”

Dr. Akkari is cautious about a universal prediction that mate pair sequencing will drive a revolution in diagnosis and treatment, mainly because clinical MPseq is currently performed only at Mayo. But “the more we know, the more powerful we can be against a certain disease. From that perspective, mate pair sequencing has shown a huge amount of value and has the potential to help patients. And I remain hopeful that it will revolutionize the diagnosis of hematologic malignancies.”

Anne Paxton is a writer and attorney in Seattle.