Next-gen sequencing finds further clinical utility in oncology

William Check, PhD

January 2018—One of the plenary sessions at the 2017 meeting of the Association for Molecular Pathology—"High Impact Molecular Diagnostics for Cancer and Inherited Diseases"—was a virtual mini-course in the latest and most useful applications of next-generation sequencing to detect germline and somatic mutations in cancer. Both speakers zeroed in on the clinical utility of their innovative diagnostic techniques.

A. John Iafrate, MD, PhD, addressed the expanding use of NGS to guide targeted therapies in lung cancer. Dr. Iafrate, a pathologist at Massachusetts General Hospital, medical director of its Center for Integrated Diagnostics, and a professor at Harvard Medical School, focused on detecting the growing number of pathogenic gene fusions discovered in lung cancer. He and his colleagues several years ago devised a new method termed anchored multiplex PCR.

"In general, we can say there is growing evidence in solid tumors, especially in lung tumors, that gene fusions comprise the main genetic driver in those tumors," Dr. Iafrate said in an interview. "In those fusions, gene partners often include kinases, which are often very good drug targets." Effective targeted therapeutics have been developed for the variants. "At least three fusion-specific inhibitors have been approved and many more are currently in trials."

One of the barriers to detecting fusions is that the kinase segment can be fused with a variety of partners, which is where anchored multiplex PCR comes in.

Colin C. Pritchard, MD, PhD, co-presenter and head of the genetics division in the Department of Laboratory Medicine and co-director of the genetics and solid tumors laboratory at the University of Washington, says testing for germline or inherited cancer predisposition genes and testing tumors for those same genes is increasingly being done for new reasons. "Testing for these genes is done in two worlds. One is folks who do Mendelian inherited genetic testing in the setting of cancer, such as *BRCA* genes or genes for Lynch syndrome," to make recommendations about risk and screening for and preventing cancer. On the other hand are those who test tumors for mutations for many of the same genes to guide therapy and for cancer prognosis. "These two worlds are starting to merge, and we are looking at germline testing in new ways," said Dr. Pritchard, who is also an associate professor of laboratory medicine.

His laboratory offers a panel called ColoSeq Tumor that is aimed at guiding interpretation of variants in inherited syndromes. "We are using tumor data to inform interpretation of what is or is not happening in the germline. Just two years ago we in molecular pathology were not doing this," he said.

Dr. Iafrate

Dr. Iafrate began his survey of newer applications of NGS in oncology by recounting the format and early results of NCI-MATCH (Molecular Analysis for Therapy Choice), which he described as an "umbrella trial for 30

phase two targeted therapies." In keeping with the trend to incorporate a diagnostic into drug therapy trials, in NCI-MATCH more than 100 genes are being sequenced, allowing identification of genetic changes that if found will allow enrollment in one of the 30 phase two drug arms.

Dr. Iafrate's laboratory, as well as laboratories at Yale, MD Anderson, and the NCI, are applying this large cancer gene panel to biopsies of the more than 6,000 patients enrolled in the trial, with a turnaround time of 15 days.

Approximately 18 percent of patients are matched, or assigned to a trial arm, on the basis of sequencing. Dr. Iafrate said this was lower than hoped. Several of the arms focus on genetic alterations that are rare across tumor types, and it has been difficult to fill these arms.

While the more common genotype arms have been filled, clinical utility awaits analysis of the therapeutic outcomes data. But a good outcome for the laboratory component of the trial has already been verified. During the validation stage of the trial, analysis of split samples for the four laboratories revealed a high concordance rate with the panel. "We were able to harmonize the performance of the assay across four different labs," important evidence that NGS can be performed reproducibly and safely.

The discovery, analysis, and detection of gene fusions in solid tumors is an increasingly important area in oncology and has already led to the development of highly effective therapies, including for *ALK* and *ROS1* fusions in lung cancer, Dr. Iafrate said. "We have focused our research on gene fusions in lung cancers, where approximately 10 percent of tumors have a gene fusion as driver, but we now know driver fusions are found across many cancers but at a lower prevalence."

He shared a table of the 16 most prevalent actionable rearrangements in lung tumors. *ALK* and *MET* were present at three to five percent, but the other 14 were found at frequencies of less than one percent to 1.5 percent. Many of these gene fusions involve kinases and were discovered relatively recently. Dr. Iafrate also presented a table showing the prevalence of recurrent fusions involving 20 kinase genes across 20 different cancer types. These fusions were scattered across the cancers, each occurring in many types but in low numbers. For instance, kinase fusions containing *ALK* or *MET* were each found in five different types of cancer and *FGFR3* fusions in seven tumor types.

"These data surprised me," Dr. Iafrate said. "How will we detect so many rare types of fusions in so many tumor types?" It would be ineffective to analyze tumors for only one fusion at a time, he pointed out, since important clinically actionable fusions will be missed. Tissue would also be limiting, and the costs very high to screen for many fusions in that manner. "Now we have a totally different view of the landscape of gene fusions, and only NGS will be able to effectively detect so many fusions," he said.

To prove his point, he spoke about the pros and cons of IHC, FISH, and RT-PCR. FISH and RT-PCR have poor to limited scalability and poor multiplexing ability. "IHC is very robust for detecting certain fusions," he said of a comparison between IHC and FISH for detecting *ALK* in non-small cell lung cancer. To address the problems inherent in detecting a variety of novel gene fusions, Dr. Iafrate and his colleagues developed anchored multiplex PCR, or AMP (Zheng Z, et al. *Nat Med.* 2014;20:1479–1484). "This method allows us to find all possible gene fusions only knowing the common partner, such as *ALK* or *ROS1*," he said. In this method hundreds of targeted primers are pooled, allowing gene fusion libraries to be amplified simultaneously in one PCR reaction. The library is then sequenced by NGS. "Up-front library construction is a bit different than standard NGS," he tells CAP TODAY. In addition, "We built special bioinformatic pipelines to allow us to identify when we have an unexpected fusion partner and whether it creates an in-frame productive protein or RNA." Dr. Iafrate presented fusion analysis data using AMP from his clinical laboratory at MGH. In nearly 2,000 samples screened with a 50-gene RNA fusion panel, RNA rearrangements were drivers in about 10 percent overall. About 15 to 20 percent of these rearrangements involved novel partners, and that would not be detected with conventional PCR-based methods.

Finding these fusions is clinically important because they can have positive therapeutic consequences. Initially the kinase inhibitor crizotinib was shown to be effective against non-small cell lung cancers that have *ALK*

rearrangements as drivers, with response rates near 60 percent. After about 10 months, however, tumors relapsed owing to the development of resistance, often as a result of mutations in the kinase domain. Enter the second-generation ALK inhibitor alectinib, which has activity against some of the common resistant forms of the ALK fusion protein, but also penetrates the central nervous system better than crizotinib. “One sees some really incredible responses” with alectinib, Dr. Iafrate said. Progression-free survival was shown to be much improved over crizotinib, though overall survival was not. Alectinib was approved in November 2017 for first-line therapy for *ALK*-positive NSCLC tumors. It has now been shown that crizotinib also induces responses in ROS1-positive and *MET* exon 14 rearranged NSCLC tumors. Interestingly, demographic analysis of ALK- and *ROS1*-positive tumors showed that these patients are younger and more likely to be nonsmokers than the overall NSCLC cohort. For instance, among all patients the rate of never smoking was 22 percent, virtually the same as among ROS1-negative tumors.

Given the success of anti-ALK and ROS1 therapy, Dr. Iafrate explained, there is excitement to target other fusions including 1) RET fusions, which are present in two to three percent of NSCLC, 2) BRAF fusions, which are detected in many types of solid tumors (thyroid, lung, melanoma, astrocytoma), and 3) NTRK1, 2, or 3 gene fusions. Among 2,000 solid tumors screened for NTRK fusions, Dr. Iafrate said, its prevalence was very rare, less than 0.5 percent. Summarizing the status of these gene fusions, Dr. Iafrate said that testing and specific treatment for ALK and ROS1 fusions is now standard of care and that testing and specific treatment for NTRK and RET likely soon will be.

Sequencing with a large gene panel will turn up many possible novel fusions in any individual tumor specimen. This creates an issue: What are the appropriate criteria for reporting novel fusions? Dr. Iafrate’s group uses an algorithm that recognizes two groups: non-validated fusions that have been previously described in databases or in the literature and non-validated fusions with a never before seen partner. In the first case they will report the fusion without orthogonal confirmation if several characteristics are present: 1) the fusion is in-frame and at a canonical breakpoint, 2) it is the most prevalent intergenic fusion call, 3) the number of supporting reads >100, and 4) the “anchored” partner should be known in this tumor type (i.e. ALK in lung cancer or BRAF in melanoma). This scheme has exceptions. For instance, for FGFR2 almost any 3′ in-frame partner in cholangiocarcinoma would be reported. Completely novel fusions require orthogonal confirmation with FISH or RT-PCR.

“We had a hard time validating our first fusion panel,” Dr. Iafrate said. He and others are finding “in the fusion world” that there are several challenges to validating fusion assays. Many fusions are rare, many labs don’t routinely extract RNA so archives don’t exist, and orthogonal approaches can be time-consuming, such as the need to make new FISH probes. Dealing with the limit of detection issue is particularly challenging. Normal tissues have two copies of DNA per cell and can vary tremendously in their amount of RNA. So diluting into “normal RNA” can produce unpredictable results. It is important to be able to find a positive control with a low cell fraction. “Engineered cell lines or spiked artificial templates are a good start” to resolving this problem, Dr. Iafrate said.

In summary, Dr. Iafrate covered the technologies that can be used to detect gene fusions and emphasized the need to be able to find novel partners. He discussed several gene fusions and their clinical implications and treatment. He concluded with a practical discussion of laboratory issues, including how to report novel fusions and how to validate fusion assays.

Dr. Pritchard discussed two classes of genetic variants altered in cancer that have DNA repair functions: genes involved in homologous recombination repair, chiefly BRCA1 and BRCA2, and genes involved in mismatch repair, including MSH2 and MLH1. Both classes have treatment implications.

In targeted therapies, “ovarian cancer leads the way,” Dr. Pritchard said. Three drugs in a class called PARP inhibitors (PARPi) have been approved; use of two of the drugs is based on BRCA1 and 2 mutation status. “Germline and somatic testing for BRCA1 and 2 is now standard of care for guiding PARPi treatment in ovarian cancer,” Dr. Pritchard said.

Cancers with genetic alterations in mismatch repair genes are eligible for treatment with immuno-oncology drugs against another target, PD-1/PD-L1. The FDA approved in 2017 the antibody pembrolizumab, a PD-1/PD-L1

inhibitor, for any MMR-deficient cancer after all standard therapy options have been exhausted.

Dr. Pritchard showed published data demonstrating what he called “extraordinary” response rates to the PARPi olaparib in DNA repair-mutated prostate cancer (Mateo J, et al. *N Engl J Med.* 2015;373:1697–1708). Of 16 patients with biallelic DNA repair defects, 14 (88 percent) responded, while the response rate to olaparib was only six percent (2/33) in those without biallelic DNA repair defects.

Dr. Pritchard and colleagues have found that germline mutations in DNA repair genes, such as BRCA1 and 2 and ATM, are common in metastatic prostate cancer. Twelve percent (82/692) of the men with metastatic prostate cancer they tested had deleterious germline mutations in 16 DNA repair genes. Moreover, of the 61 men with deleterious germline mutations whose tumors were available, 36 (59 percent) had second allele loss-of-function mutations. “This is evidence that the germline mutation could have caused the cancer,” Dr. Pritchard said in an interview.

Dr. Pritchard

“This was a really surprising finding,” he added. “We thought until recently that germline mutations in these genes were rare in prostate cancer. While that’s true in prostate cancer overall, in the small subset of men with metastatic disease a much higher percentage have a somatic mutation in mismatch repair genes. Already in the NCCN [National Comprehensive Cancer Network] guidelines there is a recommendation to consider germline genetic testing in any man with metastatic prostate cancer.”

Because of the clinical value of detecting tumors with defective DNA repair genes, clinical testing in Dr. Pritchard’s laboratory includes up-front tumor and germline paired sequencing with a 60-gene DNA repair-focused NGS panel that includes full exon and intron coverage, copy number and structural variant analysis (introns are critical at the DNA level to detect rearrangements), microsatellite instability by NGS, and loss of heterozygosity analysis.

For the next application of NGS, Dr. Pritchard described tumor sequencing in a Lynch syndrome workup. He talked first about sequencing after a negative germline test and then about sequencing as first-line screening.

If a colorectal tumor is positive either for microsatellite instability or by IHC and negative for BRAF V600E/MLH1 methylation, germline testing is done. If germline testing is negative, “we can do a tumor-based confirmation test” to exclude Lynch, Dr. Pritchard said.

It is this context that provides a new clinical utility for NGS, which can sometimes explain a positive screening test with a negative germline result. About two to three percent of colorectal cancer with a positive initial screen but a negative germline result have double somatic mutations in one of the four mismatch repair genes. This is about the same frequency as true Lynch syndrome in screen-positive CRC, a fact that is increasingly clinically important with universal tumor-based Lynch screening.

These screen-positive, germline-negative cases are sometimes called “Lynch like,” a term Dr. Pritchard considers confusing. “I prefer to call them double somatic,” he tells CAP TODAY. “That’s really a new concept driven by universal Lynch screening.” Double somatic mismatch repair genetic alterations explain up to 75 percent of so-called Lynch-like cases.

“At the University of Washington we do a lot of clinical testing in this setting, almost always after a negative

germline test," Dr. Pritchard says. If germline testing at an outside laboratory comes up negative, the specimen is sent to Dr. Pritchard's laboratory for further workup, where paired tumor and germline sequencing is done with a panel they developed called ColoSeq Tumor. The test includes full sequencing of mismatch repair genes, including introns, to maximize the sensitivity for germline mutations and to improve detection of structural variation that is common in cancers.

To demonstrate the spectrum of results with samples that are positive on initial screening but negative on germline examination, Dr. Pritchard showed results from an NGS panel analysis of over 300 consecutive patients with Lynch-like syndrome referred to their laboratory for further workup. (Such patients make up only three percent of all screened CRC patients.) Double somatic mutations are found in about 75 percent. Missed Lynch syndrome is discovered in seven percent of cases. False IHC is uncovered in three percent of patients. In five percent the MLH1 mutation is found, while 10 percent are unexplained.
"The good news," he tells CAP TODAY, "is that this testing works to reassure up to three-fourths of these patients that they don't have Lynch syndrome but double somatic mutations that explain their positive screening results."

Can tumor NGS be used to replace IHC and MSI-PCR as first-line screening in a Lynch workup? Dr. Pritchard asks. "If we could, it would simplify testing." Traditional screening employs a complex algorithm, while NGS detects MSI, mismatch repair mutation status, as well as BRAF, KRAS, and NRAS mutations all in one test. If the test is positive, that provides the etiology of the tumor. If negative, there's no need for further testing.

To explore this possibility, Dr. Pritchard and collaborators in genetics and pathology at Ohio State University are analyzing CRC cases by a tumor-only NGS panel interpreted by blinded expert review.

Use of NGS as first-line screening is "more conceptual" at this time, Dr. Pritchard says, adding that the data are currently preliminary. Tumor NGS may be able to replace traditional Lynch screening in the future, he said in an interview. "We are increasingly doing NGS on colorectal cancers to guide therapy and to qualify patients for targeted therapy. This is a brand-new area and we are excited about it." He thinks it is going to be the future. "But first we need more data."

Cost may already be favorable for NGS, he says, given the total cost of the many individual assays. Turnaround time needs to be addressed.

The third new area in which somatic NGS can provide clinical utility is in helping with variant classification in patients with germline variants of unknown significance (VUS).

"We are seeing increasing tumor testing to clarify Lynch-like cases as standard of care," Dr. Pritchard said. The same tests are sometimes used in patients with germline VUS in mismatch repair genes that might explain IHC results. He is "extra cautious," he says, in using tumor sequencing results to reclassify VUS findings: "We don't yet use tumor sequencing data from a patient as a sole basis to reclassify a germline VUS from that same patient because there are not yet guidelines."

Consider a patient with a germline VUS in MSH2, a Lynch syndrome gene, whose tumor has loss of MSH2 protein by IHC and microsatellite instability, which supports that the MSH2 variant may be causative. Evidence may be seen of second allele inactivation at the MSH2 locus, either loss of heterozygosity or a second hit. "All of these clues add a lot of information that this germline variant may be pathogenic," Dr. Pritchard says. "Nonetheless, when we have this experience in practice, we have been very cautious how we handle it in clinical reporting." Reports do sometimes say tumor findings make it more probable that this germline alteration is pathogenic while emphasizing that the VUS is not formally reclassified.

Tumor testing might help with a germline VUS that is considered highly probably pathogenic, or, conversely, one that is close to being benign. Still, Dr. Pritchard cautions, "There are many pitfalls here. Proceed carefully." There could be a missed germline or somatic mutation or double somatic mutations in cis configuration.

Dr. Pritchard reported their experience in 40 patients who had a germline mismatch repair VUS. After tumor sequencing, four were reclassified to likely pathogenic and one to likely benign. Tumor sequencing provided only part of the basis for reclassification.

“Germline variant classification guidelines should incorporate somatic data,” he concluded. “Guideline committees are currently working on this.”

He shared a case to illustrate how somatic findings can inform germline variant classification: a woman in her 40s with CRC whose father was diagnosed with CRC in his 50s. She had a VUS germline variant—a very rare deep intronic variant in the MSH2 gene. That same MSH2 deep intronic variant was observed twice as a somatic mutation in colorectal cancer in other patients who had loss of MSH2 and MSH6 that could not be explained by any other mutation. Her final diagnosis was Lynch syndrome caused by an inherited pathogenic deep intronic mutation in MSH2 that results in the introduction of a cryptic exon and a premature frameshift.
[hr]

William Check is a writer in Ft. Lauderdale, Fla.