NTRK fusion testing: ups, downs of four methods

Sherrie Rice

November 2019—With two inhibitors approved by the FDA for the treatment of *NTRK*-fusion-positive solid tumors, the next step is to determine whom to test and how.

If the efficacy of the compounds—larotrectinib and entrectinib—were the only thing to consider in implementing a testing algorithm, knowing whom to test would be easy. “You would want to test everyone because the more of these patients we identify, the more we can help,” said Jeremy Segal, MD, PhD, director of the Division of Genomic and Molecular Pathology, University of Chicago Medical Center, in a recent CAP TODAY webinar made possible with the support of Genentech. “Unfortunately,” he added, “we also need to consider the incidence and prevalence of these anomalies, and that’s where a lot of the difficulty lies with *NTRKs*, clinically and for the laboratory.”

Some rare cancers have high rates and some common cancers have low rates, “and this has significant implications in how we approach testing for these tumors,” said webinar co-presenter Jyoti Patel, MD, director of thoracic oncology, University of Chicago Medical Center.

The cancers enriched for *TRK* fusions are secretory breast carcinoma, mammary analogue secretory carcinoma, and infantile fibrosarcoma (75 percent to more than 90 percent frequency). Harboring *TRK* fusions at lower frequencies (five to 25 percent) are pontine glioma, spitzoid melanoma, thyroid cancer, GIST, and congenital mesoblastic nephroma.

At the lowest frequencies (less than one percent to less than five percent): lung, colorectal, pancreatic, breast, and head and neck squamous cancers, in addition to other sarcomas, astrocytoma/gliobastoma, cholangiocarcinoma, and melanoma.

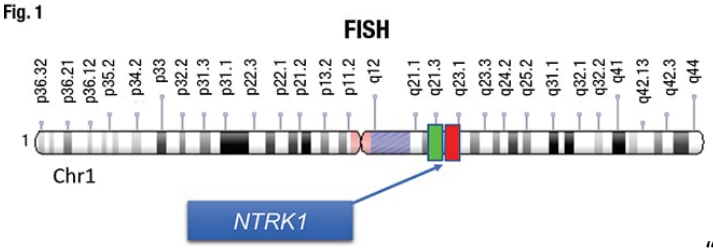
The neurotrophic tropomyosin kinase receptor encompasses three transmembrane proteins: TrkA, TrkB, and TrkC, encoded respectively by *NTRK1*, *2*, and *3*. It is estimated, Dr. Patel said, that 1,500 to 5,000 patients harbor *TRK*-fusion-positive cancers in the U.S. each year.

The low rates of *NTRK* fusions in common cancers forces more careful thinking about screening strategies, Dr. Segal said, and a consideration of the cost and use of available tissue. In the webinar, he reviewed the pros and cons of the four main N*TRK* testing methods, starting with immunohistochemistry.

“It’s easy to do. All of the *NTRKs* can be stained with a single antibody that targets their C terminal domains, which are homologous to each other,” he said. So a single slide and single stain. “And when you look at positive cases, you can see a large variety of staining patterns.” More worrisome, he added, are cases in which there is almost no staining, which is seen more frequently with *NTRK3* than with *1* and *2*. “This forces you to reduce your cutoff level for positivity to maintain your sensitivity.” But there will still be a sensitivity gap because a number of cases will be too low to pick up. “So a lot of labs use a cutoff of even one percent of cells positive to find these. Then you have to face the potential problem of specificity because any tissue that expresses a reasonable amount of *NTRKs* may show up as a false-positive.” This problem is worse in neural tissues that tend to express neural growth factor receptors.

The overall published sensitivity range is 75 percent to 100 percent; for specificity it’s 63 percent to 100 percent.

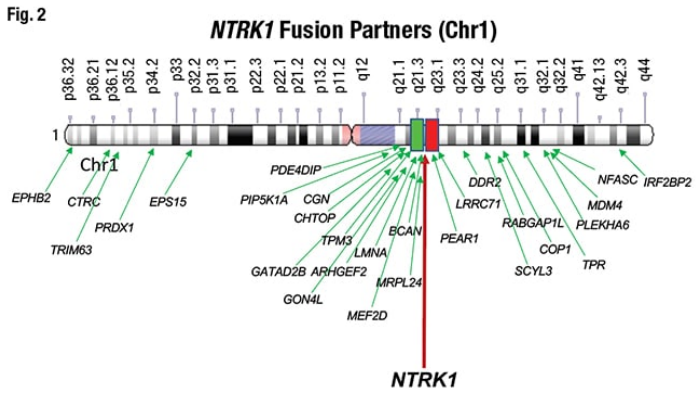
FISH is another technique, but here too sensitivity is a concern, Dr. Segal said, particularly for *NTRK1*.



"You're going to have two different color FISH probes upstream and downstream of the gene, and the regions are usually fairly big—hundreds of thousands of base pairs. In reality, these probes are so close to each other that when you have a normal or nonfusion interphase cell, these probes are going to stay effectively in the same place, so you'll have overlapping signals." Imagine there's another chromosome, in this case chromosome 12, and *ETV6*. A breakage and fusion event creates *ETV6-NTRK3*. "And these probes have separated now because they're disconnected and on different chromosomes; they can go wherever they want in the nucleus. That's going to lead to a potentially long distance separation," which is easy to detect and good for *NTRK3* and *NTRK2* because their fusion partners tend to be on different chromosomes. "They can move away as far as they like."

But the situation is a little different for *NTRK1*. "Let's imagine that what we had in this case is a short-range rearrangement within chromosome 1 (**Fig. 1**), an inversion to create some kind of fusion gene. But the red and green spots haven't moved far away from each other; they're still tethered."

They may not move far enough away to be able to tell they're separate, he said, "so you can potentially get false-negative results for a fusion like this, particularly if the tumor cell percentage is low, and not too many of the cells have it." This is a common problem with FISH for *EML4-ALK* fusion, Dr. Segal said. "But if you look into partners of *NTRK1*, unfortunately, the vast majority of the partners occur also on chromosome 1. A lot of them are very close to *NTRK1*, even overlapping with the probe binding regions (**Fig. 2**). That's going to be an issue, and I don't know which of these are going to be detectable or undetectable, but you have to be concerned about anything that's going on in this area." This raises concerns for sensitivity of FISH-based detection, particularly for *NTRK1*, he said.



RT-PCR is the third technique, one that some laboratories use to detect *ETV6-NTRK3* fusions in rare tumor types. "RT-PCR is not a partner agnostic methodology," Dr. Segal said, "so in order to detect a fusion, you need to design primers that will bind and hit on both sides of the fusion."

To look comprehensively for *NTRK* fusions, primers would have to be designed to cover many exons of numerous genes. "Once you did that, probably the only way to evaluate the data," he said, "would be to use a next-gen sequencing approach. But there's a bigger problem, which is that we're still learning about all the different possible *NTRK* partner genes. Even if you happen to design an RT-PCR approach optimally for the genes we know, you're still going to have a sensitivity gap and you won't know what kind of fusions you're missing."

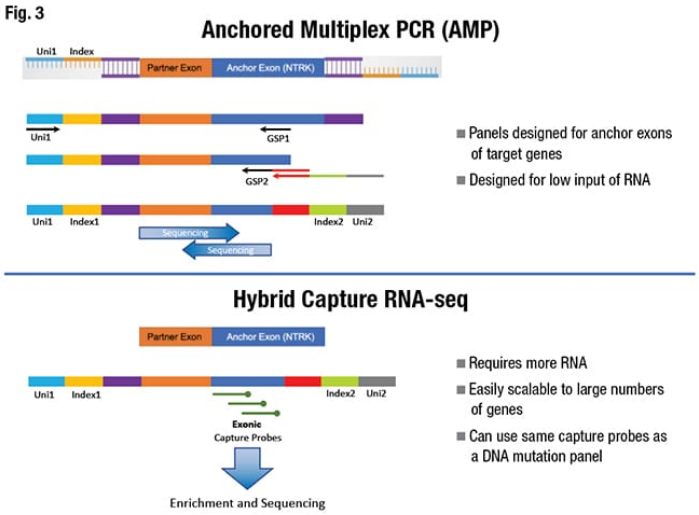
For proper sensitivity, partner agnostic methods are important. The only way to do a partner agnostic fusion detection and learn the identity of a partner gene is with a next-generation sequencing approach, Dr. Segal said. DNA or RNA can be used as a starting material, and each has pros and cons. "If you go the DNA route, then you're

in the position of always needing to pay careful attention to introns, within which a fusion event—breakage and recombination—will take place. But even if we know which intron is involved, we don't know where in the intron this is going to occur. So we're stuck." The only thing that's abnormal about that fusion gene, he said, is the exact breakpoint—"the place where you go from being a part of one gene to being a part of another gene." The gene sequence on either side is normal. To detect the fusion, that exact breakpoint must be found, and that means evaluating and sequencing the entire intron, he said. For that, his laboratory uses a hybrid capture sequencing approach. "We try to sequence all possible fragments from the intron by pulling down everything we can from the intron using specially designed hybrid capture probes." Decisions are still being made about which introns need to be covered for the *NTRKs*. "But for *NTRK1*, probably the bare minimum area you need to cover is intron seven through 11."

The problem of looking at introns gets worse as more introns are looked at, Dr. Segal said. "And that's unfortunate for *NTRK2* and *NTRK3* in particular, because the regions that need to be picked up are huge. For *NTRK2*, it's over 100,000 base pairs of intron coverage. For *NTRK3*, it's almost 200,000 base pairs, and these introns are littered with areas of bad, repetitive regions and bad homology. So a lot of labs don't even try to do *NTRK3* [intron] tiling, even if they can do *NTRK2*."

Many laboratories choose instead to focus on introns four and five of *ETV6*, hoping to pick up *ETV6-NTRK3*, he said. "But if you do that, you're going to miss all of the other fusion partners of *NTRK3*." Thus, sensitivity for detection via DNA-based approaches "may be a little questionable," he said.

With an RNA-based approach, all of the intron-associated problems are gone. "It's been spliced out." The gene will be transcribed into a pre-spliced RNA transcript, and that is going to be spliced down into a final spliced mRNA. Evaluation of post-spliced mRNA allows for straightforward partner agnostic NGS fusion detection, "and all we have to worry about now are direct exon to exon connections."



There are two main ways to engineer the library prep and sequencing to look for this properly. One is anchored multiplex PCR, for which the panels are designed for anchor exons of target genes (**Fig. 3**) and which is designed for low input of RNA. "You can design them for a variety of different genes, and it's easy to add additional primers to them."

His laboratory uses hybrid capture RNA-seq, which takes a little more RNA but is easily scalable to large gene numbers because any number of probes can be added. "Because we're using exon capture probes, we can use the same capture probes we use for our DNA mutation panel." The RNA-based NGS methods are much easier to multiplex compared with DNA-based NGS, he said, because there's no need to cover "intron after intron after intron" for a lot of genes, which can become costly.

The published sensitivity range for RNA-based fusion detection systems is 93 percent to 100 percent. "For our

laboratory, we've tested a variety of fusions, in many different genes, and our overall sensitivity is about 98 percent, including 100 percent for all the *NTRKs* that we've tested," he said.

Dr. Segal

But there are limitations, the largest of which is that the RNA quality from pathology tissue samples is poor. But quality control can be performed up front, "so at least you will figure out which samples won't work, and maybe that's around 10 percent of them. For the ones that do work, you can be quite confident about the data from them." The other limitation: The expression levels of fusion gene and normal transcripts may affect the sensitivity.

Overall, he said, the RNA-based approach is good (though it's the lesser used of the two) with the caveat that the lab will have some samples that don't work properly. "And you'll need to figure those out up front and what you're going to do with them."

If a laboratory is not already doing these types of RNA and DNA analyses routinely, adding one could mean a steep extra cost and use of tissue. "But if you're already doing this type of analysis in the lab, then it's trivial and cost free to add a new marker," Dr. Segal said. "So the big question is what are you already doing, and how do we move the field over time so that we're doing these analyses and getting reimbursed for them on a more routine basis."

More near term, he said, is the challenge of figuring out whom and how to test. For now, "it comes down to institutional-specific factors." At the University of Chicago Medical Center, RNA and DNA assessments are performed on every lung cancer case—but not for other tumors. "We need to figure out how we expand this over time and in a reasonably cost-affordable way," Dr. Segal said.

"We would all like to expand the screening that we do for these *NTRK* fusions," he added, "because if you can find the patients who have them, we now have a great intervention."

Sherrie Rice is editor of CAP TODAY. The full webinar is at www.captodayonline.com.