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Detection of *NTRK* fusions: Merits and Limitations of Current Diagnostic Platforms

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Abstract

Oncogenic fusions involving NTRK1, NTRK2, and NTRK3 with various partners are diagnostic of infantile fibrosarcoma and secretory carcinoma yet also occur in lower frequencies across many types of malignancies. Recently, targeted small molecular inhibitor therapy has been shown to induce a durable response in a high percentage of patients with NTRK fusion-positive cancers, which has made the detection of NTRK fusions critical. Several techniques for NTRK fusion diagnosis exist, including pan-Trk immunohistochemistry, fluorescence in situ hybridization, reverse transcription PCR, DNA-based next-generation sequencing (NGS), and RNA-based NGS. Each of these assays has unique features, advantages, and limitations, and familiarity with these assays is critical to appropriately screen for NTRK fusions. Here, we review the details of each existing methodology.

Keywords

NTRK fusion; Immunohistochemistry; Fluorescence in situ hybridization; Next-generation Sequencing

INTRODUCTION

The neurotrophic tyrosine receptor kinase genes (*NTRK1*, *NTRK2*, and *NTRK3*) encode a family of receptor tyrosine kinases (TrkA, TrkB, and TrkC, respectively) that serve important roles in cell survival, proliferation, and cellular differentiation in healthy human cells.[1] The Trk proteins are physiologically expressed predominantly in the central and peripheral nervous system as well as smooth muscle.[2, 3] Physiologic activation of the receptors is initiated by neutrotrophin binding to the extracellular domain, causing receptor dimerization and phosphorylation, and subsequent downstream activation of signaling pathways including phospholipase C, Ras/MAPK/ERK, and PI3K cascades.[1, 4, 5]

In-frame fusions involving any of various partners in the 5’ position, several of which are detailed by Cocco et al in a recent paper,[5] and the kinase domain of one of the three *NTRK* genes in the 3’ position are transcribed and translated into a fusion protein, resulting in aberrant expression and ligand-independent activation, and hence continuous, unregulated increased signaling of Trk and activation of its downstream targets. While the *ETV6*-

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NTRK3 fusion was originally described in infantile fibrosarcoma[6, 7] and secretory carcinoma of the breast and salivary gland,[8–10] other fusions involving the Trk proteins have been demonstrated in a vast array of tumor types, including other sarcomas,[11] melanocytic neoplasms,[12, 13] inflammatory myofibroblastic tumors,[14] gliomas,[15, 16] and carcinomas of the lung,[17] colon,[18] and thyroid.[19–21]

These fusions were discovered approximately twenty years ago, but the very recent development of Trk inhibitors and their approval by the Food and Drug Administration (FDA) has revitalized the interest of the oncology community. Approved in 2017, entrectinib exhibits activity against Trk as well as *ROS1* and *ALK* oncogenic fusions. More recently, in November of 2018, the FDA granted accelerated approval for Larotrectinib (Bayer and Loxo Oncology), a potent small molecule inhibitor with high selectivity for Trk.[22] Both have shown great promise in recent clinical trials. Entrectinib has shown efficacy in many tumor types exhibiting Trk fusions.[23–25] Similarly, Drilon et al. recently reported the results of a phase I histology-agnostic clinical trial of larotrectinib in adult and pediatric patients with locally advanced or metastatic solid tumors harboring an *NTRK* fusion. A dramatic response rate was seen, with 75% of patients responding and 55% of patients remaining progression free at 1 year.[26] Clinical responses were seen regardless of patient age, fusion partner, *NTRK* gene, and tumor type.

NTRK fusions occur in over 90% of infantile fibrosarcomas and secretory carcinomas yet are exceedingly rare in more common malignancies: 0.23% of a cohort of patients with non-small cell lung cancer (NCSLC),[17] 0.35% of a cohort of patients with colorectal carcinomas,[27] and 0.27% of a cohort of 11,500 patients with various solid tumors harbored *NTRK* fusions.[28] Given their only recently recognized therapeutic relevance, their rarity in common malignancies, and the challenge of accurately detecting the variety of *NTRK* fusions with different partners and genomic breakpoints, there has emerged a need in the pathology and oncology communities for detailed knowledge regarding assays for the detection of *NTRK* fusions. Here, we review the advantages and limitations of currently available testing modalities including immunohistochemistry (IHC), fluorescence in situ hybridization (FISH), reverse transcription polymerase chain reaction (RT-PCR), DNA-based next-generation sequencing (NGS), and RNA-based NGS. These findings are summarized in Table 1. It should be noted that *NTRK* fusions, which are both rare and diverse, are still being investigated, and recent studies assessing the diagnostic sensitivity and specificity for *NTRK* fusion detection assays have shown variable results. The true clinical validity and clinical utility of these assays may require years of additional study and more thorough evaluation of patient outcomes.

IMMUNOHISTOCHEMISTRY

Widely available to most clinical labs, IHC has the advantages of being inexpensive, having a rapid turnaround time of approximately 1 day, requiring as little as 1 unstained slide, and working independent of tumor purity. Antibody clone EPR17341, commercially available from Abcam (Cambridge, MA) and Roche/Ventana (San Francisco, CA), is the most studied clone available and is reactive with a conserved proprietary peptide sequence from the C-terminus of TrkA, TrkB, and TrkC. The Abcam antibody has demonstrated a sensitivity for

the detection of *NTRK* fusions ranging from 75% to 96.7% and a specificity ranging from 92 to 100%.[28–31] Cytoplasmic staining appears to be universal in *NTRK* fusion positive tumors, but fusion partner-specific staining patterns have also been observed (Figure 1A–C).[29] The 3 predominant patterns that have been described reflect localization of the fusion protein to the subcellular site of the fusion partner. For example, *ETV6-NTRK3* fusion positive samples demonstrate nuclear Trk expression, while *LMNA-NTRK1* fusion positive samples demonstrated peri-nuclear Trk expression as *LMNA* encodes nuclear lamin, and *NTRK* fusions with tropomyosin (*TPM*) partners show membranous staining as *TPM* encodes proteins that localize to the cytoskeleton.

Decreased sensitivity has been observed, however, for *NTRK3* fusions. In perhaps the largest study of pan-TRK IHC to date, 4138 cases, including 28 confirmed *NTRK* fusion-positive cancers, were examined. While sensitivity was 88% and 89% for *NTRK1* and *NTRK2* fusions, respectively, only 6 of 11 cases with *NTRK3* fusions were positive with clone EPR17341.[28] Thus, in patients with histology suggestive of secretory carcinoma or infantile fibrosarcoma, diagnostic testing for *ETV6-NTRK3* fusion with other assays (*NTRK3* FISH, RNA-based NGS, or RT-PCR) is of higher clinical yield. For patients with tumor histologies highly enriched in *ETV6-NTRK3* fusions, it may be sufficient to verify the presence of *NTRK3* rearrangement with *NTRK3* FISH.

Another limitation of pan-TRK IHC is the physiologic expression of Trk in neural as well as smooth muscle tissue (Figure 1D–F).[2, 3] IHC expression has been observed in fusion-negative tumors with neural or smooth muscle differentiation such as gastrointestinal stromal tumor, neuroblastoma, glioblastoma, leiomyosarcoma, primitive myxoid mesenchymal tumor of infancy, and fibrous hamartoma of infancy.[11, 29, 31, 32] Thus, tumors with neural and smooth muscle differentiation should not be screened via pan-Trk IHC for *NTRK* fusions.

FLUORESCENT IN SITU HYBRIDIZATION

FISH is a DNA-based assay performed with either fusion probes or break-apart probes and is used to assess DNA-level structural variants in formalin-fixed paraffin-embedded tissue (FFPE). One of the most commonly used commercial probes is an *ETV6* break-apart probe that has shown efficacy in confirming *ETV6-NTRK3* rearrangements in secretory carcinoma and infantile fibrosarcoma.[33] These break-apart probe sets often include a green-labeled probe at the 3’ end of ETV6 and an orange-labeled probe that overlaps the 5’ end of *ETV6*.[33] A positive result is a split or isolated 5’ signal, with thresholds varying from 5% of cells [14] to 15% of tumor cells.[34] Other laboratories have used *NTRK1, NTRK2*, and *NTRK3* break-apart probes to identify *NTRK* fusions.[11, 35] While a positive FISH result with a break-apart probe means that there is a structural variant involving the tested gene, neither the functional significance (whether the DNA-level structural variant results in a translated fusion) nor the partner is known. *ETV6* or *NTRK3* FISH is useful to support a histologic diagnosis of secretory carcinoma or infantile fibrosarcoma.[36] In theory, FISH has good sensitivity and specificity, and is often used as the gold standard for assessing for the presence of chromosomal abnormalities. However, one caveat is that if the breakpoints involve noncanonical sites or novel genes, the test will be reported as falsely negative.[36,

37] FISH testing generally has a quick turnaround time of less than a week, only uses 1–2 slides, and works well on low tumor purity samples.

REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION

RT-PCR is an RNA-based method for assessing *NTRK* fusion transcripts that can be performed either as a qualitative assay or as real-time quantitative PCR. This assay requires knowledge of both fusion partners and their exon breakpoints. In many cases, the canonical *ETV6* exon5-*NTRK3* exon15 fusion can be detected. However, a recent study examined a cohort of 25 salivary gland secretory carcinomas that lacked the canonical fusion by conventional RT-PCR. In four cases, the canonical fusion could be detected using highly sensitive nested RT-PCR techniques, and in five cases, an atypical *ETV6* exon 4-*NTRK3* exon 14 fusion was detected.[35] Overall, while RT-PCR has demonstrated great clinical utility in diagnosis and monitoring of other fusion-driven malignancies such as chronic myelogenous leukemia and acute promyelocytic leukemia, the diversity of *NTRK* fusion partners, variability of breakpoints and exons involved, and lability of RNA in archival FFPE tissue limits this technique's utility.

DNA-BASED NEXT-GENERATION SEQUENCING

DNA-based NGS assays examine genomic DNA from tumors to assess somatic mutational status of many genes simultaneously. These assays range from targeted assays that focus on a small or large panel of cancer-related genes, to whole exome and even whole genome sequencing. The platforms, chemistry and bioinformatic pipelines used in these assays can be highly variable. For instance, the assay used at Memorial Sloan Kettering Cancer Center, MSK-IMPACT, is hybridization capture-based and covers the entire coding region of 468 cancer-related genes as well as select introns including those in *ETV6*, *NTRK1*, and *NTRK2*.[29, 38] Similarly, the FoundationOne CDx™ assay covers 324 cancer-related genes and can detect rearrangements involving *ETV6*, *NTRK1*, and *NTRK2*. The sensitivity of DNA-based assays that include such cancer gene panels as well as whole exome sequencing depends on whether the genomic breakpoints of a defined fusion are covered by the panel, and how much coverage is present at that breakpoint. For both the FoundationOne CDx™ and MSK-IMPACT assays, only the exonic regions of *NTRK3* are covered, and because fusion breakpoints usually occur within introns, inadequate coverage of these introns can result in false negatives. Not only is coverage of the intronic regions impractical due to size limitations---the intronic regions, especially in the area of the exons coding for the *NTRK3* kinase domain, span up to 200 kilobases in length---but the intronic regions also contain highly repetitive regions that are impossible to tile in a hybridization-capture assay. Therefore, due to these considerations, sensitivity of detection of *NTRK3* fusions is limited.

One distinct advantage of DNA-based NGS testing is its ability to simultaneously assess mutations, amplifications, deletions, microsatellite instability status, and tumor mutation burden, as well as fusions.[38, 39] The knowledge of other oncogenic MAPK DNA-based alterations, such as *BRAF* p.V600E, *RAS* mutations, and other kinase fusions, is of particular value when triaging cases for follow-up *NTRK* fusion testing with other assays. For example, in a study of 21 colorectal carcinomas harboring kinase fusions, none had a

BRAF, *KRAS*, *NRAS* mutation.[27] In contrast, however, one study demonstrated *KRAS* alterations co-occurring with *NTRK* fusions in two cases of neuroendocrine carcinoma.[40] For the most part, since *NTRK* fusions and alterations in *BRAF/RAS* appear to be mutually exclusive, it may be possible to narrow down the cohort of common tumors that require fusion screening based on *BRAF/RAS* status.

In terms of specificity, the detection of a structural variant involving one of the *NTRK* genes shares the same problem as DNA-level rearrangements detected by FISH: they may not result in an expressed in-frame fusion protein, and therefore further assessment using alternative methods is often required for more information on the *NTRK* event. Other considerations for DNA-based NGS assays are that they require adequate tumor purity, adequate tissue from unstained slides or FFPE tissue curls, and turnaround time is generally at least two weeks.

RNA-BASED NEXT-GENERATION SEQUENCING

RNA-based NGS involves extraction of RNA from FFPE followed by preparation of cDNA and sequencing. One study examined the use of multiplexed amplicon-based sequencing to assess for fusion transcripts involving 19 driver genes and 94 possible partners.[41] The authors demonstrated 86% sensitivity for cases with at least 10 normalized reads when comparing against other methodologies including FISH, RT-PCR, Sanger sequencing and NGS panels. Sensitivity was improved by assessing for kinase domain overexpression by identifying differences in expression of the 3’ and 5’ aspects of the potential driver genes, a finding associated with fusion protein forming translocations.[42] Including 3’/5’ read ratios and decreasing the normalized fusion read requirement resulted in 100% sensitivity.[41]

An alternative method, anchored multiplex PCR, for example with the Archer FusionPlex® platform, has a benefit over amplicon based methods: gene fusions can be detected even if only one of the fusion partners is known.[43] In this method, a gene specific primer hybridizes to the *NTRK* (or other kinase gene) while a “universal” primer hybridizes to an adapter sequence downstream of the fusion partner. After cleanup, a second round of amplification is performed, again using a gene specific primer 3’ downstream to the first and a second universal primer again complementary to the adapter sequence. Once the PCR steps have been performed to create the library, sequencing and analysis is performed to quantify the processed transcripts.[43–45]

The main advantages of RNA-based NGS are that evidence of transcription is positively identified and the exact genes and exons involved in the transcript are characterized. In contrast to RT-PCR methods, as described above, RNA-based NGS methods can assess for the presence of fusions involving multiple genes and exons simultaneously.

The limiting factor for RNA-based sequencing methods is RNA quality. RNA is more labile than DNA due to the presence of hydroxyl groups and subsequent hydrolysis, and it is often degraded in FFPE tissues, especially with increasing storage time and age of tissue. Although many recent advances have improved the efficiency of RNA library preparation, laboratory handling of RNA samples requires highly specialized reagents, equipment, and

expertise.[46] Adequate quality control measures are therefore important to assess both the amount and quality of the RNA obtained. Metrics can include distribution of RNA fragment sizes, proportion of sequencing reads that are RNA versus DNA, and average sequencing coverage and depth.[47]

HYBRID DNA/RNA PANELS

Recently, platforms able to assess both DNA and RNA extracted from the same FFPE sample have been developed. After separate DNA and RNA library preparation, the libraries are pooled for interrogation in a single sequencing run. Covering 170 genes commonly altered in solid tumors, the TruSight Tumor 170, one such assay developed by Illumina, can assess fusions, splice variants, indels, point mutations, and copy number variants simultaneously. This assay uses hybridization capture to enrich the library for genes of interest prior to sequencing,[48] and it can thereby capture transcribed fusions, including those involving *NTRK1*, *NTRK2*, and *NTRK3*. Similarly, the Oncomine™ Comprehensive Assay by ThermoFisher covers 161 cancer associated genes and simultaneously interrogates DNA and RNA using Ion Torrent technology.[49] Since the Oncomine™ assay relies on amplicon-based technology, knowledge of both fusion partners must be known. Although all currently known *NTRK* fusion partners are included and additional revisions to the assay are constantly in development, one consideration with this platform is that it may miss novel or previously unreported fusions.

CONSIDERATIONS FOR DISEASE MONITORING: TREATMENT AND RESISTANCE MECHANISMS

As seen in treatment with other tyrosine kinase inhibitors, acquired resistance has been shown to develop for patient with *NTRK* fusions who receive small molecular inhibitor therapy. Some recent studies have observed the development of point mutations in the kinase domains, p.G667C in TrkA/*NTRK1* and p.G696A in TrkC/*NTRK3*.[5, 50, 51] The efficacy of larotrectinib is reduced in tumors that harbor these *NTRK* mutations or amplification of the fusion gene.[52] Sequencing of lesions demonstrating progression after Trk inhibitor therapy should therefore include the aforementioned regions within the kinase domain, as second generation Trk inhibitors are currently in clinical trials to try to extend the treatment response.[53]

Assessing circulating tumor DNA (ctDNA) has been shown to be an effective non-invasive method for monitoring for tumor recurrence and progression, and studies have demonstrated its use for monitoring tumors with oncogenic fusions.[50, 54, 55] Such methods may be used to monitor patients with solid tumors with *NTRK* fusions who are either progressing on therapy or do not have sufficient tumor material for initial testing. One caveat, however, is that some sensitivity issues have been identified when only monitoring ctDNA. In two recent meta-analyses, for example, ctDNA analysis was only 67% sensitive for detecting *KRAS* alterations in a colon cancer cohort, and 67% sensitive for detecting *EGFR* p.T790M point mutations in a lung cancer cohort.[56, 57] Detection of gene fusions may even be more difficult, as a recent study showed only 54% sensitivity for detecting *ALK* fusions in ctDNA from patients with lung cancer.[58] Finally, it should also be noted that the currently most

widely used platforms for ctDNA sequencing may not be effective for monitoring patients with *NTRK* fusions. The Guardant360® assay can identify point mutations in *NTRK1* and *NTRK3*, but only fusions involving *NTRK1* can be detected,[59] while the FoundationOne Liquid assay does not interrogate any of the *NTRK* genes or *ETV6*.[60]

CONCLUSIONS

Oncogenic *NTRK* fusions are seen in many cancer types. They are common in select rare tumor types while rare in common tumors. Identification of these fusions may provide important therapeutic opportunities for patients with advanced or unresectable cancers. Appropriate screening and/or confirmation of *NTRK* fusions depends on the tumor type and available material.

Developing an appropriate algorithm for testing patient samples will be dependent on the resources available as well as specific patient scenarios. Close communication between oncologists and pathologists is key. At Memorial Sloan Kettering Cancer Center, we use a combination of IHC, FISH, DNA-based sequencing, and RNA-based sequencing depending on the clinical situation and histologic findings. If a rare tumor type that commonly exhibits an *NTRK* fusion is suspected, such as infantile fibrosarcoma or secretory carcinoma, then *NTRK3* FISH is performed for diagnostic confirmation and eligibility for Trk inhibitor therapy. In advanced-stage patients with common malignancies that rarely exhibit *NTRK* fusions (e.g., lung adenocarcinoma, colon adenocarcinoma, etc.), DNA-based sequencing with MSK-IMPACT is performed to simultaneously screen for MAPK pathway alterations such as *RAS/BRAF* mutations and fusions, microsatellite instability, and copy number changes such as *HER2* amplification. Cases with structural variants of uncertain significance and *RAS/BRAF* wild-type cases involving tumor types that are often driven by MAPK pathway activation, such as colon or lung adenocarcinoma, are reflexed to an RNA-based NGS assay for further fusion analysis. Pan-Trk IHC is often also used when *NTRK* rearrangements of uncertain significance are detected by MSK-IMPACT or when MSK-IMPACT testing is not an option due to insufficient material or low tumor content.

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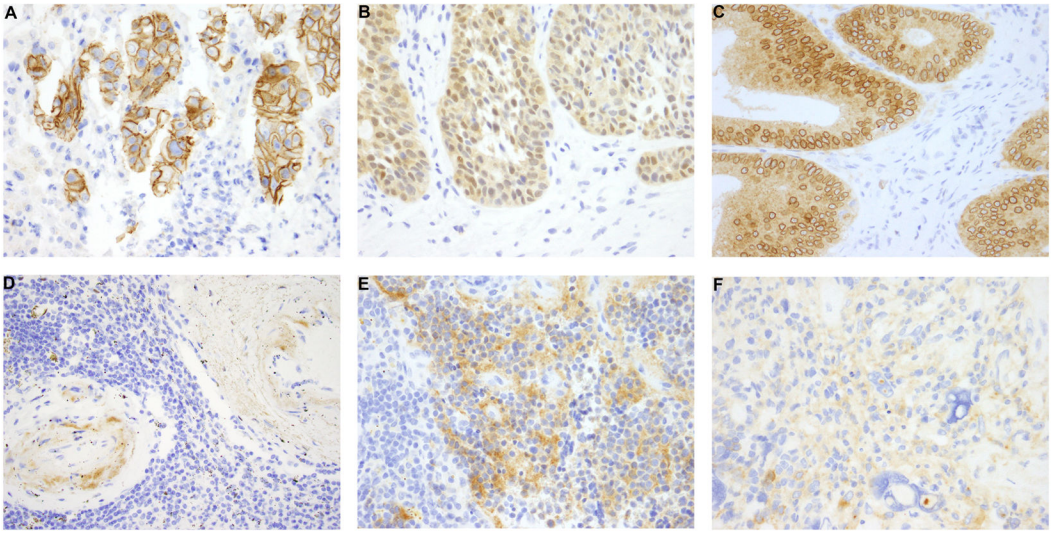

Figure 1.

Immunohistochemical staining with pan-Trk antibody (clone EPR 17341, Abcam) demonstrates a variety of staining patterns in malignancies with *NTRK* fusions, and the staining patterns correlate with the fusion partner. **(A)** A membranous staining pattern is seen in this case of intrahepatic cholangiocarcinoma with a *PLEKHA6-NTRK1* fusion. **(B)** A nuclear and cytoplasmic staining pattern is seen in this case of secretory carcinoma of the salivary gland with the canonical *ETV6-NTRK3* fusion. **(C)** Colonic adenocarcinoma with an *LMNA-NTRK1* fusions exhibits a cytoplasmic and perinuclear staining pattern. **(D)** Physiologic staining can be seen in smooth muscle, as seen in arterial walls. **(E-F)** Physiologic staining can also be seen in tumors of neural differentiation, such as neuroblastoma **(E)** and glioblastoma **(F)**, making interpretation difficult.

Table 1.

Features, advantages, and limitation of various assays used to identify *NTRK* fusions.

Testing method	Sensitivity	Specificity	Material required	Turn-around time	Cost	Additional notes
Immunohistochemistry	75–96%. Higher for *NTRK1* & *NTRK2* fusions, but approximately 50–70% for *NTRK3* fusions. [28–31]	92–100%. False positives seen in tumors with neural or smooth muscle differentiation	At least 1 unstained slide. Additional may be required for controls	1 day	$	Interpretation should take histologic tumor type into account
Fluorescent in situ hybridization	High sensitivity if canonical breakpoints	High specificity	At least 3 unstained slides. (1 for each NTRK gene tested)	1–3 days	$	Useful when high suspicion of *ETV6-NTRK3* fusions and supporting histology
Reverse transcription PCR	Variable. Both involved genes and exons must be known) RNA must be of sufficient quality	Variable. Dependent upon whether structural variant results in transcribed fusion	1 F06Dg of RNA (approximately 50,000 cells).	1 week	$	Can be quantitative
DNA-based Next-Generation Sequencing	Variable. Depends on extent and depth of *NTRK1–3* introns covered as well as tumor purity	Variable. Dependent upon whether structural variant results in transcribed fusion	250 ng of DNA (approximately 50,000 cells). We cut 20 unstained slides at 5 μm for biopsies; 15 unstained slides for resections	2–4 weeks	$ $ $	Also assesses point mutations and potentially other fusions so that *RAS/BRAF* wild type tumors can be further tested if needed
RNA-based Next-Generation Sequencing	Very high if RNA quality is sufficient	Very high	200 ng of RNA, (approximately 10,000 cells). We cut 10 unstained slides at 5 μm.	2–4 weeks	$ $ $	Assesses fusions across multiple genes
DNA/RNA hybrid sequencing assays	98–100% [48, 49]	96–100% [48, 49]	10–40 ng of RNA at greater than 20% tumor content	2–4 weeks	$ $ $	Can assess fusions, splice variants, indels, point mutations, and copy number variants simultaneously