ABSTRACT

The neurotrophic tyrosine receptor kinase (NTRK) gene fusions encode three tropomyosin receptor kinases (TRKA, TRKB, TRKC) that contribute to central and peripheral nervous system development and function. NTRK gene fusions are oncogenic drivers of various adult and paediatric tumours. Several methods have been used to detect NTRK gene fusions including immunohistochemistry, fluorescence in situ hybridisation, reverse transcriptase polymerase chain reaction, and DNA- or RNA-based next-generation sequencing. For patients with TRK fusion cancer, TRK inhibition is an important therapeutic target. Following the FDA approval of the selective TRK inhibitor, larotrectinib, as well as the ongoing development of multi-kinase inhibitors with activity in TRK fusion cancer, testing for NTRK gene fusions should become part of the standard diagnostic process. In this review we discuss the biology of NTRK gene fusions, and we present a testing algorithm to aid detection of these gene fusions in clinical practice and guide treatment decisions.

INTRODUCTION

Fusions involving neurotrophic tyrosine receptor kinases (NTRK) were among the first gene translocations described in cancer. Selective inhibition of the resulting tropomyosin receptor kinase (TRK) fusion proteins offers a precision medicine approach to the treatment of a range of tumour types.

NTRK structure and function

Tropomyosin receptor kinase A, B and C (TRKA, TRKB and TRKC) encoded by the NTRK1, NTRK2 and NTRK3 genes located on human chromosomes 1q23.1, 9q21.33 and 15q25.3, respectively, are receptor tyrosine kinases expressed in human neuronal tissue.

All three TRK receptors comprise an extracellular ligand-binding domain, a transmembrane region and an intracellular adenosine triphosphate-binding domain. TRK receptors are activated when neurotrophins ligands bind to the extracellular domain of the receptor (figure 1A).

NTRK gene fusions

NTRK gene fusions result from intra-chromosomal or inter-chromosomal rearrangements that juxtapose the 3' region of the NTRK gene with the 5' sequence of a fusion partner gene expressed by the tumour cell progenitor (figure 1B). The NTRK gene fusion transcript encodes a protein composed of the N-terminus of the fusion partner with the TRK partner tyrosine kinase domain. In most characterised fusions, the 5' partner gene sequence encodes one or more dimersisation domains, resulting in a constitutively active fusion protein. This constitutive activation results in uninterrupted downstream signalling messages, thereby acting as a true oncogenic driver. Although fusions may occur in any of the three NTRK genes, most of those identified to date involve either NTRK3 or NTRK1.

TRK fusion cancer

Fusions involving the NTRK1, 2 and 3 genes have been identified as oncogenic drivers and diagnostic markers in various cancer types (table 1). TRK fusion proteins are often mutually exclusive of other known fusion proteins involving kinases. Specific NTRK gene fusions are associated with certain tumours, for example, the ETV6-NTRK3 gene fusion is exhibited by 90%-100% of mammary analogue secretory carcinomas, >90% of secretory breast cancers, and is present in most cases of infantile fibrosarcoma and congenital mesoblastic nephroma. In contrast some cancers have many different fusion partners. In lung cancer, seven different gene fusions involving the NTRK1 gene leading to constitutive TRKA tyrosine kinase domain activation have been described (table 1), for example, rearrangement of the 5' portion of the
Figure 1  Schematic figure showing the TRK receptor tyrosine kinases, activating neurotrophins and the major signal transduction pathways (A) and the genomic structures of NTRK1, NTRK2, and NTRK3, with the size of each gene in parentheses (B). The ETV6 and NTRK3 gene fusion and the resultant constitutively active TRK fusion protein is a typical example. GSK3β, glycogen synthase kinase 3 beta; Ig, immunoglobulin; mRNA, messenger ribonucleic acid; NTRK, neurotrophic tyrosine receptor kinase; PI3K, phosphoinositide-3-kinase; SAM, sterile alpha motif; TRK, tropomyosin receptor kinase.
Table 1  
NTRK gene fusions identified in adult and paediatric cancers by relative frequency of NTRK gene fusions

| Tumour                                      | NTRK1 | NTRK2 | NTRK3 |
|---------------------------------------------|-------|-------|-------|
| **Adult cancers**                           |       |       |       |
| High frequency (>80%)                       |       |       |       |
| Mammary analogue secretory carcinomas       |       | ETV6 |       |
| Secretory breast carcinoma                  |       |       | ETV6 |
| Intermediate frequency (5%–25%)             |       |       |       |
| Papillary thyroid cancer                    |       |       | ETV6 |
| Appendiceal cancer                          |       |       |       |
| Glioma/glioblastoma                        |       |       |       |
| Astrocytoma                                 |       |       |       |
| Gastrointestinal stromal tumour             |       |       | ETV6 |
| Head and neck cancer                        |       | PAN3 |       |
| Lung cancer                                 |       |       | TRIM24 |
| Sarcoma                                     |       |       |       |
| Breast cancer                               |       |       |       |
| Acute lymphoblastic leukaemia, acute myeloid leukaemia, histiocytosis, multiple myeloma, dendritic cell neoplasms | | | ETV6 |
| Uterine sarcoma                             |       | RBPM5 |       |
| Cholangiocarcinoma                          |       |       |       |
| Pancreatic cancer                           |       |       |       |
| Melanoma                                    |       |       |       |
| Colorectal cancer                           |       |       |       |
| Paediatric cancers                          |       |       |       |
| High frequency (>80%)                       |       |       |       |
| Secretory breast carcinoma                  |       | ETV6 |       |
| Infantile fibrosarcoma and other mesenchymal tumours |       | EMLA6 | ETV6 |
| Cellular and mixed congenital mesoblastic nephroma |       | | |
| Intermediate frequency (5%–25%)             |       |       |       |
| Papillary thyroid cancer                    |       |       | ETV6 |
| Spitz tumours                               |       |       | ETV6 |
| Paediatric high-grade gliomas               |       |       | ETV6 |
| Low frequency (<5%)                         |       |       |       |
| Ganglioglioma                               |       |       |       |
| Astrocytoma                                 |       |       |       |

Epidemiology of TRK fusion cancer

NTRK gene fusions may occur in as many as 1% of all solid tumours. They are found in numerous tumour types in both adult and paediatric patients (table 1). Two main categories of tumours are identified: rare cancers with a high frequency (>80%) of NTRK gene fusions and more common cancers with a lower frequency of NTRK gene fusions (either 5%–25% or <5%; table 1). A high frequency of NTRK gene fusions have been identified in mammary analogue secretory carcinomas (90%–100%) and secretory breast carcinomas (>90%) in adult patients, and in infantile fibrosarcomas (91%–100%) and other mesenchymal tumours (100%) and congenital mesoblastic nephromas (83%) in paediatric patients. NTRK gene fusions are found at a lower frequency in radiation-associated papillary thyroid cancer (14.5%) and adult patients and papillary thyroid cancer (26%) and Spitzoid tumours (16%) in paediatric or adolescent patients. The reported frequency of NTRK gene fusions in common cancer types is generally <5%, including head and neck cancer (0.2%), lung cancer (0.2%–3.3%), colorectal cancer (0.7%–1.5%), skin cutaneous melanoma (0.3%), and sarcoma (1%).

Treatments targeting NTRK gene fusions

A number of TRK inhibitors are emerging which can be subdivided into those that are selective inhibitors for TRK and those that are multi-kinase inhibitors active against a range of targets including TRK. Larotrectinib is currently the only selective TRK inhibitor and was approved by the Food and Drug Administration (FDA) in November 2018. Data on 55 larotrectinib–treated paediatric and adult patients with TRK fusion-positive advanced solid tumours, representing 17 unique cancer types, have been evaluated. Objective tumour responses,
based on independent radiologic review, were seen in 75% of patients. At 1 year, 71% of the responses were ongoing and 55% of patients remained progression-free. The median duration of response had not been reached after a median follow-up of 8.3 months. The same was true for median progression-free survival after a median follow-up of 9.9 months. Larotrectinib was well tolerated. Adverse events were predominantly of grade 1 or no patient discontinued larotrectinib due to drug-related adverse events. Furthermore, no adverse event of grade 3 or 4 that was considered by the investigators to be related to larotrectinib occurred in more than 5% of patients. Among infants, children and adolescents (n=24), larotrectinib was well tolerated and showed a high response rate in those with advanced, TRK fusion-positive solid tumours (n=17). Five of these children (median age, 2 years; range, 0.4–12 years) with locally advanced soft tissue tumours achieved a partial response to larotrectinib (RECIST v1.1) and underwent surgical resection after a median of six cycles (range, 4–9 cycles) of treatment. Similar findings were reported by Drilon et al for two children with locally advanced infantile fibrosarcoma. Larotrectinib treatment resulted in sufficient tumour shrinkage to allow for limb-sparing surgery with pathologic assessment confirming negative margins (R0 surgery). Both patients were progression-free without larotrectinib treatment after 4.8 months and 6.0 months of follow-up.

Favourable preliminary results were seen with entrectinib in two Phase I clinical trials of paediatric and adult patients with NTRK, ROS1 or ALK fusions leading to further investigations in patients with NTRK gene fusions. TRK inhibitors developed to overcome acquired resistance to first-generation TRK inhibitors are already in development. LOXO-195 (BAY 2731954) has demonstrated efficacy against treatment-resistant alleles of NTRK gene fusions in patients with TRK fusion-positive cancers. Repotrectinib, a TRK, ROS1 and ALK inhibitor, has demonstrated confirmed responses in patients with ROS1 or NTRK3 fusion-positive cancers who had relapsed on earlier-generation inhibitors.

### Testing Methods for TRK Fusion Cancers

For optimal clinical efficacy of TRK inhibitors, an effective diagnostic strategy to detect NTRK gene fusions in tumour samples is essential to guide treatment selection. Approaches that may be used to directly or indirectly detect the presence of a gene fusion in clinical tissue samples include immunohistochemistry (IHC), fluorescence in situ hybridisation (FISH), reverse transcriptase polymerase chain reaction (RT-PCR) and next-generation sequencing (NGS) using DNA or RNA.

### Immunohistochemistry

IHC enables detection of TRK overexpression as a surrogate for the presence of an NTRK gene fusion and provides a time-efficient and tissue-efficient technique that may be used for routine screening. Studies employing pan-TRK monoclonal antibody cocktails have demonstrated positive TRK expression in tumour samples. However, some studies indicate that interpretation of IHC data may be more challenging than initially ascertained. In an analysis of 11,502 formalin-fixed paraffin-embedded (FFPE) tumour samples of various cancer types for the presence of gene fusions, 31 cases (0.27%) with NTRK gene fusions were identified by NGS. Of the 28 cases that were assessed by pan-TRK IHC, 21 scored positive (>1% of tumour cells staining at any intensity above background), giving a sensitivity of only 75%, and 45% of tumours with NTRK3 fusions scored negative by IHC. False negative cases could be related to sample preparation, for example, fixation. Therefore, it is important to check if internal controls such as endothelial cells are positive, or to use external controls such as positive cell lines. Similarly, positive IHC results must be followed with confirmatory testing using a molecular method to verify the presence of a fusion, as overexpression of wildtype TRK proteins may also be detected.

### Fluorescence in Situ Hybridisation

Break-apart FISH is a well-established method for detecting clinically relevant gene fusion events and is of value in tumours

### Table 2 Overview of testing methods currently available for NTRK gene fusions

| Assay       | Advantages                                      | Disadvantages                                      |
|-------------|-------------------------------------------------|---------------------------------------------------|
| IHC         | Low cost<sup>23</sup> 53, Readsible<sup>34</sup> | May not be specific for NTRK gene fusion as it detects both wild-type and fusion protein<sup>18</sup>|
|             | Detects TRKA, B and C<sup>16</sup>               | Possible false positives<sup>34</sup>              |
|             | Turnaround time 1–2 days<sup>13</sup>           | Possible false negatives for fusions involving TRKC<sup>60</sup>|
| FISH        | The location of the target within the cell is visible<sup>54</sup>55 | The target sequence must be known for conventional FISH otherwise three separate tests are required for NTRK1, NTRK2 and NTRK<sup>36</sup> |
|             | Several targets can be detected in one sample using several fluorophores<sup>34</sup> | Complex chromosomal translocations can result in false positive signals<sup>56</sup>|
|             | Requires knowledge of only one of the two fusion partners when using break-apart probes | False negative results may be above 30%<sup>13</sup> |
|             | NTRK gene fusions with unknown partners can be detected using break-apart FISH | |
|             | FISH is readily available in most laboratories and institutes | |
| RT-PCR      | High sensitivity and specificity<sup>34</sup>  | Target sequences must be known (i.e., cannot readily detect novel fusion partners)<sup>32</sup>|
|             | Low cost per assay<sup>32</sup>                 | A comprehensive multiplex RT-PCR assay might be challenging because of the potentially large number of possible<sup>5</sup> fusion partners<sup>32</sup> |
| NGS         | May detect novel fusion partners (depending on the assay used)<sup>12</sup> | Commercially available DNA-based NGS platforms may not be capable of identifying all NTRK gene fusions, especially those involving NTRK2 and NTRK3, which have large intronic regions<sup>48</sup>|
|             | Can be used to evaluate multiple actionable targets simultaneously while preserving limited tissue<sup>32</sup> | DNA-NGS is limited by intron size<sup>56</sup> |
|             | Currently used for NTRK testing<sup>10</sup>    | RNA-NGS is limited by RNA quality<sup>56</sup> |
|             | RNA-NGS is preferred over DNA-NGS as sequencing for RNA-based testing is focused on coding sequences not introns<sup>51</sup> | |
with a high prevalence of NTRK gene fusions involving recurrent fusions\(^5\) (figure 2B). The ETV6-NTRK3 gene fusion was one of the first NTRK gene fusions reported and has been identified in numerous cancer types: \(^5\)it is amenable for detection using break-apart FISH (figure 2B). As FISH is largely limited to the detection of a single gene fusion, a separate break-apart FISH probe is required for each of the three NTRK genes. \(^5\) Furthermore, the 5’ gene fusion partner will not be identified. \(^5\) False negatives may result if the deletion is small enough to leave enough of the complementary regions for hybridisation of both FISH probes or if there is a complex FISH pattern with numerous nuclei showing atypical doublet fusion signals and only a few nuclei with split signals. \(^5\) Indeed, in one study ETV6 was associated with a 36% false negative rate. \(^6\)

**REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION**

RT-PCR provides an alternative or complementary approach to FISH, detecting NTRK gene fusions using primers in the coding sequence of the 5’ fusion partner and the NTRK kinase domain. \(^4\)\(^5\)\(^7\) A disadvantage of RT-PCR is that the large number of possible 5’ fusion partners may make a comprehensive multiplex RT-PCR assay challenging. \(^4\)\(^5\)\(^7\) An alternative approach could be to assess the ratio of 5’ and 3’ amplicons of each of the NTRK genes by multiplex RT-PCR reactions, with an imbalance in the ratio for a specific gene suggesting a possible fusion event. \(^7\)

**NEXT-GENERATION SEQUENCING**

NGS provides a precise method to detect NTRK gene fusions, with high sensitivity and specificity compared with other testing methods. \(^5\)\(^7\) An advantage of NGS is that multiple oncogenic events in addition to NTRK gene fusions can be identified from a single tumour sample. \(^5\)\(^7\) A wide variety of NGS-based approaches are available for fusion testing with the primary distinguishing factor being whether they are RNA- or DNA-based. \(^5\)\(^6\) Access to NGS in a clinical setting may be limited as availability of this technique varies between regions and countries.

**DNA-based next-generation sequencing**

Although DNA-based NGS panels may detect multiple oncogenic genomic events from one sample, not all DNA-based NGS platforms can identify all NTRK gene fusions, especially those involving NTRK2 and NTRK3 where detection of gene fusions is complicated by the presence of large introns that are typically inadequately sequenced and difficult to analyse. \(^5\)\(^6\) (figure 1B).

**RNA-based next-generation sequencing**

The advantage of RNA-based NGS over DNA-based NGS is that sequencing is focused on the mature mRNA hence is not affected by intron size. \(^5\)\(^6\) A disadvantage is the high reliance on RNA quality, which can be poor if obtained from FFPE samples. \(^5\)\(^6\) Many NGS assays now include RNA fusions in their gene panels, and it is likely that NGS diagnostics that depend on RNA for fusion detection will increasingly be used in clinical practice to test for NTRK gene fusions.

**NTRK gene fusion testing algorithm**

A proposed screening algorithm for identifying patients with TRK fusion cancer is presented (figure 3). The algorithm incorporates the strengths and availability of each diagnostic technique. The algorithm is based on the categorisation of tumours into two groups based on the incidence of NTRK gene fusion.

In tumours with a high frequency of NTRK gene fusion events, FISH is recommended, with pan-TRK IHC as an alternative if FISH is unavailable. Confirmation by targeted NGS in those cases with positive pan-TRK IHC can be conducted concurrently with treatment considerations. The pattern of TRK staining by IHC may also inform selection of a confirmatory test, as tumours harbouring NTRK1 rearrangements typically show strong, diffuse cytoplasmic staining. In contrast, tumours harbouring NTRK3 rearrangements may have weaker expression but often have at least focal nuclear staining. Negative results from FISH or pan-TRK IHC should be confirmed by NGS, although selection of a broader panel including other receptor tyrosine kinases is warranted as these tumours have a high likelihood of harbouring other diagnostic and/or therapeutic alterations.

In solid tumours where gene fusions are common, but the frequency of NTRK gene fusions is lower (5%–25%), an NGS panel that includes NTRK fusions is recommended as the preferred test for patients. For tumours with a very low frequency of NTRK gene fusions (<5%), but where molecular screening is common, inclusion of NTRK genes in routine NGS analysis is recommended. For tumours with a low frequency of NTRK fusions, where NGS is not available or is not routinely performed for a histotype, pan-TRK IHC should be performed for screening with NGS confirmation of positive IHC results.

In all cases where NGS is recommended, and particularly for those cases in which an NTRK3 rearrangement is favoured by IHC, RNA-based NGS is the ideal testing assay for NTRK gene fusions. Note that this algorithm is not intended to replace the independent medical judgement of the physician in the context of individual clinical circumstances to determine a patient’s care.

**Conclusions and future directions**

NTRK gene fusions have been identified across a range of tumour types and occur at a high frequency in certain rare cancers. \(^2\)\(^3\)\(^4\)\(^5\)\(^6\)\(^7\)\(^8\)\(^9\)\(^10\)\(^11\)\(^12\) More common cancers have a low but significant frequency of NTRK gene fusions\(^2\)\(^3\)\(^4\)\(^5\)\(^6\)\(^7\)\(^8\)\(^9\)\(^10\)\(^11\)\(^12\) and thus represent a sizeable at-risk patient population. With the recent FDA approval of the selective TRK inhibitor, larotrectinib (Vitrakvi), along with the continued development of multi-kinase inhibitors with activity in TRK fusion cancer, testing for NTRK gene fusions should become part of the standard diagnostic process. Marked differences in the prevalence of NTRK gene fusions across tumour types mean that clinical diagnostic strategies will vary accordingly but will rely on IHC, FISH and NGS assays. The
**Take home messages**

- The \( NTRK \) genes (\( NTRK1 \), \( NTRK2 \) and \( NTRK3 \)) encode for TRKA, TRKB and TRKC receptors, three transmembrane proteins, and are normally expressed in neuronal tissue during development.
- Fusions involving \( NTRK \) genes are oncogenic drivers across a wide range of tumour types and are either highly enriched in select tumour types or infrequently found in other cancers, including common tumours.
- \( NTRK \) gene fusions should be treated as tumour-agnostic biomarkers.
- Specific TRK inhibitors have shown histology-agnostic activity in adult and paediatric patients harbouring \( NTRK \) gene fusions providing high durable response rates with a low incidence of adverse events.
- IHC, FISH, RT-PCR and NGS are effective screening techniques for identification of TRK fusion cancer. Implementation of these methods can be tailored to individual patients based on histological and clinical presentation.

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**Figure 3** Testing algorithm for TRK fusion cancer. CMN, congenital mesoblastic nephroma; FISH, fluorescence in situ hybridisation; IHC, immunohistochemistry; MASC, mammary analogue secretory carcinoma; NGS, next-generation sequencing; NSCLC, non-small cell lung cancer; \( NTRK \), neurotrophic tyrosine receptor kinase; SBC, secretory breast carcinoma; TRK, tropomyosin receptor kinase.

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suggested testing algorithm for TRK fusion cancer considers the aetiology of tumours as well as the availability of testing methods to guide detection of these fusions in the clinic. The optimal use of tumour tissue, especially from small biopsies or cytology specimens, and optimisation of multiplexed approaches, remains an area of active research and development.

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