TRK Inhibition: A New Tumor-Agnostic Treatment Strategy

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Abstract
Oncogenic somatic chromosomal rearrangements involving the NTRK1, NTRK2 or NTRK3 genes (NTRK gene fusions) occur in up to 1% of all solid tumors, and have been reported across a wide range of tumor types. The fusion proteins encoded by such rearranged sequences have constitutively activated TRK tyrosine kinase domains, providing novel therapeutic anticancer targets. The potential clinical effectiveness of TRK inhibition in patients with tumors harboring NTRK gene fusions is being assessed in phase I and II trials of TRK inhibitors, such as larotrectinib and entrectinib. Clinical trial results have demonstrated that larotrectinib is generally well tolerated and has shown high response rates that are durable across tumor types. These data validate NTRK gene fusions as actionable genomic alterations. In this review, we present the clinical data, discuss the different approaches that might be used to routinely screen tumors to indicate the presence of NTRK gene fusions, explore the issue of acquired resistance to TRK inhibition, and reflect on the wider regulatory considerations for tumor site agnostic TRK inhibitor drug development.

Key Points
Oncogenic NTRK gene fusions resulting in an inappropriately and constitutively activated TRK tyrosine kinase occur in up to 1% of all solid tumors.

Treatment of TRK fusion cancer with TRK inhibitor larotrectinib has resulted in high durable objective response rates across tumor types.

Identification of patients with advanced solid tumors who are likely to benefit from TRK inhibitors will be contingent upon the development of effective pretreatment screening strategies.

1 Introduction
The neurotrophic tyrosine kinase receptors, TRKA, TRKB and TRKC, are single-pass transmembrane proteins that function as high affinity receptors for neurotrophins, a family of proteins that regulate many aspects of neuronal development and function [1]. TRKA, TRKB and TRKC receptors are encoded by the NTRK1, NTRK2, and NTRK3 genes, which are located on human chromosomes 1q23.1, 9q21.33, and 15q25.3, respectively. The first member of this gene family to be characterized was the NTRK1 gene, which was initially identified in a human colon carcinoma as the 3' component of a somatic gene fusion between the non-muscle tropomyosin gene and a gene encoding a previously unknown protein tyrosine kinase [2]. The closely related human NTRK2 and NTRK3 genes were subsequently identified and mapped, with the encoded TRK proteins showing 40% amino acid identity overall, conserved cysteine residues in the extracellular domains and conserved tyrosine residues in the intracellular domain [3–5]. The overall structure of the three TRK proteins is also conserved, with the extracellular domains of each having a cysteine-rich cluster, three leucine-rich repeats, another cysteine-rich cluster and two immunoglobulin-like domains, followed by a transmembrane domain and an intracellular tyrosine kinase [1].

In normal signaling processes, neurotrophin ligand binding to the TRK extracellular domain leads to receptor dimerization and autophosphorylation of specific tyrosine residues in the intracellular kinase domain, leading to elevated tyrosine kinase activity. Through the binding of various adaptor proteins, activated TRK receptors engage diverse signaling pathways (Fig. 1), including those downstream of RAS,
phosphatidylinositol 4,5-bisphosphate 3-kinase and, phospho-
lipase C-\(\gamma\), modifying such functions as neuronal differenti-
ation, including neurite outgrowth, cell survival, cell growth,
and synaptic plasticity [1, 6].

Following the initial observation in colon cancer, it is now
apparent that somatic fusions involving the \(NTRK1\), \(NTRK2\)
or \(NTRK3\) genes (\(NTRK\) gene fusions) can occur in a wide
range of human tumor types [6, 7]. While, such fusions in
common human cancers may occur at relatively low frequen-

cy [8–10], in certain rare tumors, \(NTRK\) gene fusions are
highly characteristic, and may be present in most cases [11,
12]. Typically, in such somatic chromosomal rearrangements,
the 5’ region of a gene that is expressed by the tumor cell
progenitor is joined with the 3’ region of one of the \(NTRK\)
genes. The resultant mRNA fusion transcript encodes an in-
frame protein comprising the N-terminus of the fusion partner
joined to the C-terminus of one of the \(NTRK\) genes. The resultant mRNA fusion transcript encodes an in-
frame protein comprising the N-terminus of the fusion partner
joined to the C-terminus of one of the \(NTRK\) proteins, including the tyrosine kinase domain. In the majority of characterized fu-
sions, the 5’ partner gene sequence encodes one or more iden-
tifiable dimerization domains, which would be predicted to
lead to constitutive activation of the \(NTRK\) tyrosine kinase do-
main [6]. In addition to gene fusion events, somatic point
mutations of \(NTRK\) genes have also been identified in human
tumors, [13, 14]. Whether such mutations might be of pre-
dictive significance in relation to \(NTRK\) inhibitor therapy is not
definitively known. The lack of response in patients with
\(NTRK\) point mutations in a larotrectinib phase I trial and an in silico analysis of these events suggest that
\(NTRK\) point mutations may not be useful biomarkers in
this context [15, 16].

There are several examples of chromosomal rearrange-
ments in tumor cells resulting in gene fusions between an
expressed 5’ partner and a 3’ partner encoding a tyrosine ki-
nase that have been exploited therapeutically. Tyrosine kinase
genes involved in such somatic fusions include \(ABL\), \(ALK\),
\(ROS1\) and \(RET\) [17, 18]. Small molecule inhibitors of these
tyrosine kinases have been developed and have been shown
clinically to be highly effective anticancer agents in patients
with fusion-positive tumors. The efficacy of these tyrosine
kinase inhibitors (TKIs) confirms that such gene fusions
may function as tumor drivers, which, when targeted, lead to
a reduction in the viability of the tumor cells, manifested as a
clinical response. Such data suggested that the development of
TKIs targeting \(NTRK\) might be a novel and effective therapeu-
tic approach for the treatment of patients with tumors harbor-

![Fig. 1 NTRK gene fusions. LBD ligand binding domain, TM transmembrane, TK tyrosine kinase](image)

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TRKB, TRKC, ROS1, ALK, JAK2, and ACK1 kinases, with IC50 values for the TRK kinases in the low nanomolar range [19]. Entrectinib has shown antiproliferative and antitumor activity in in vitro and in vivo models of TRK fusion cancer [20]. Such data provide a strong scientific rationale for clinical studies of these two agents in patients with solid tumors harboring NTRK gene fusions.

### 2 NTRK Gene Fusions

It has been estimated that NTRK gene fusions may occur in up to 1% of all solid tumors and may involve a range of different 5’ partners [6, 7]. A particularly high frequency of such events has been identified in certain rare pediatric and adult tumor types. While fusions may occur in any of the three NTRK genes, it appears to be the case that, with the possible exception of brain tumors, they more commonly arise in the NTRK1 and NTRK3 genes, with NTRK2 less frequently involved [6, 7, 9]. Depending on the tumor type, the 5’ partner and NTRK gene partner may be characteristic or may be variable [6]. Over 60 different 5’ partner genes have so far been reported in human TRK fusion tumors (Table 2), with the NTRK1 gene having the most fusion partners identified to date.

Pediatric tumor types in which NTRK gene fusions occur at high frequency include infantile fibrosarcoma, the most commonly occurring non-rhabdomyosarcoma soft tissue sarcoma in children under 1 year of age. It has been reported that the majority of such tumors harbor an ETV6-NTRK3 fusion [11, 50], although cases with EML4-NTRK3 fusions have been identified [49]. In addition, an LMNA-NTRK1 fusion in a congenital infantile fibrosarcoma, [62] and LMNA-NTRK1 and TMP3-NTRK1 fusions in ETV6-NTRK3 fusion-negative congenital/infantile soft tissue lesions with similar morphological features to congenital infantile fibrosarcoma have been identified [63]. Similarly, most cases of the cellular subtype of the rare pediatric renal tumor, congenital mesoblastic nephroma, have ETV6-NTRK3 fusions [51, 52], although again, the EML4-NTRK3 fusion may also be found [49]. The identification of variant NTRK gene fusions in these particular tumor types is of significance in relation to the screening strategies that might be used in these indications to select patients for TRK inhibitor therapy. NTRK gene fusions have also been identified in other pediatric tumor types at moderately high frequencies, including non-rhabdomyosarcoma soft tissue sarcoma [64], childhood high grade glioma [43], pediatric papillary thyroid cancer [65, 66], and spitzoid neoplasms [32]. Rare cancers that may occur in both adults and children which have a high frequency of NTRK gene fusions include mammary analogue secretnary carcinoma of the salivary gland and secretory carcinoma of the breast. In both tumor types, it has been reported that more than 90% of cases harbor an ETV6-NTRK3 fusion [12, 53].

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### Table 1 Active clinical trials of TRK inhibitors in patients with NTRK fusion tumors

| Agent      | Kinase targets | Phase | NTRK fusion tumor type | Start date  | Status          | Estimated participants |
|------------|----------------|-------|------------------------|-------------|-----------------|------------------------|
| Larotrectinib | TRKA, TRKB, TRKC | I     | Advanced solid tumors  | May 2014    | Recruiting      | 90                     |
|            |                | II    | Advanced solid tumors  | October 2015| Recruiting      | 151                    |
|            |                | I/II  | Advanced solid or primary CNS tumors (pediatric) | December 2015 | Recruiting | 92                     |
| Entrectinib | TRKA, TRKB, TRKC, ALK, ROS1 | I     | Locally advanced or metastatic solid tumorsa | June 2014  | Recruiting      | 125                    |
|            |                | II    | Locally advanced or metastatic solid tumorsa | October 2015 | Recruiting | 300                    |
|            |                | I/ib  | Recurrent or refractory solid tumors and primary CNS tumors (pediatric) | December 2015 | Recruiting | 190                    |
| DS-6051b   | TRKA, TRKB, TRKC, ROS1 | I     | Advanced solid tumorsb | September 2014 | Not recruiting | 70                     |
| TSR-011    | TRKA, TRKB, TRKC, ALK | I     | Advanced solid tumors (Japanese patients) | February 2016 | Not recruiting | 15                     |
|            |                | I/la  | Advanced solid tumors and lymphomasb | October 2012 | Unknown | 72                     |
| TPX-0005d  | TRKA, TRKB, TRKC, ALK, ROS1 | I/II  | Locally advanced or metastatic solid tumor (including non-Hodgkin lymphoma)a | February, 2017 | Recruiting | 450                    |
| LOXO-195d  | TRKA, TRKB, TRKC | I/II  | Advanced solid tumor progressing after prior TRK inhibitor treatment | July, 2017  | Recruiting      | 93                     |

As registered with ClinicalTrials.gov

CNS central nervous system

a Inclusion of patients with ROS1, or ALK gene rearrangements permitted

b Inclusion of patients with ROS1 gene rearrangements permitted

c Inclusion of patients with ALK gene rearrangements permitted

d Second generation TRK inhibitor with activity against TRK proteins with resistance mutations

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TRKB, TRKC, ROS1, ALK, JAK2, and ACK1 kinases, with IC50 values for the TRK kinases in the low nanomolar range [19]. Entrectinib has shown antiproliferative and antitumor activity in in vitro and in vivo models of TRK fusion cancer [20]. Such data provide a strong scientific rationale for clinical studies of these two agents in patients with solid tumors harboring NTRK gene fusions.
| NTRK gene | Fusion partner and chromosomal localization | Tumor type | Citations |
|-----------|---------------------------------------------|------------|-----------|
| NTRK1 (1q23.1) | ARHGEF2 1q22 | Glioblastoma | [21] |
|           | BCAN 1q23.1 | Glioblastoma; glioneuronal tumor; pilocytic astrocytoma | [22–24] |
|           | CEL 9q34.13 | Pancreatic cancer | [25] |
|           | CD74 5q33.1 | Lung cancer | [10] |
|           | CHTOP 1q21.3 | Glioblastoma | [21] |
|           | CGN 1q21.3 | Breast cancer | [26] |
|           | COP1 1q25.1–q25.2 | Large cell neuroendocrine cancer | [27] |
|           | CTRC 1p36.21 | Pancreatic cancer | [7] |
|           | DDR2 1q23.3 | Melanoma | [28] |
|           | GATAD2B 1q21.3 | Breast cancer | [26] |
|           | GON4L 1q22 | Melanoma | [7, 28] |
|           | GRIIPAP1 Xp11.23 | Lung cancer | [29] |
|           | IRF2BP2 1q42.3 | Lung cancer; thyroid cancer | [7, 9, 30, 31] |
|           | LMNA 1q22 | Appendiceal cancer; breast cancer; cholangiocarcinoma; colorectal cancer; soft tissue sarcoma; Spitzoid neoplasm; uterine sarcoma | [7, 23, 26, 30, 32–34] |
|           | LRRC71 1q23.1 | Uterine endometrial cancer | [29] |
|           | MDM4 1q32.1 | Breast cancer | [26] |
|           | MIR548F1 10q21.1 | Pediatric mesenchymal tumor | [35] |
|           | MPRIP 17p11.2 | Lung cancer | [10, 36] |
|           | MRPL24 1q23.1 | Lung cancer | [37] |
|           | NFASC 1q32.1 | Glioblastoma | [9, 22] |
|           | P2RY8 Xp22.33 and Yp11.3 | Lung cancer | [30] |
|           | PDE4DIP 1q21.2 | Soft tissue sarcoma | [7] |
|           | PEAR1 1q23.1 | Breast cancer | [26] |
|           | PLEKHA6 1q32.1 | Colon cancer | [7] |
|           | PPL 16p13.3 | Thyroid carcinoma | [7, 21] |
|           | RABGAP1L 1q25.1 | Intrahepatic cholangiocarcinoma | [38] |
|           | SCYL3 1q24.2 | Colorectal cancer | [39] |
|           | SSBP2 5q14.1 | Thyroid cancer | [9] |
|           | SQSTM1 5q35.3 | Infantile fibrosarcoma; lung cancer; thyroid cancer | [7, 9, 40, 41] |
|           | TFG 3q12.2 | Thyroid cancer | [9, 42] |
|           | TP53 17p13.1 | Spitzoid neoplasm | [32] |
|           | TPM3 1q21.3 | Breast cancer; cholangiocarcinoma; colorectal cancer; glioma; infantile fibrosarcoma; lung cancer; soft tissue sarcoma; thyroid cancer; uterine sarcoma | [7, 9, 21, 26, 30, 34, 43–45] |
|           | TPR 1q31.1 | Lung cancer; papillary thyroid cancer; uterine sarcoma; pediatric mesenchymal tumor | [7, 34, 35, 42] |
|           | TRIM63 1p36.11 | Melanoma | [7, 28, 30] |
| NTRK2 (9q21.33) | AFAP1 4p16.1 | Glioma | [9] |
|           | AGBL4 1p33 | Glioma | [43] |
|           | BCR 22q11.23 | Glioblastoma | [30] |
|           | DAB2IP 9q33.2 | Colorectal cancer | [37] |
|           | GKAP1 9q21.32 | Glioblastoma | [24] |
|           | KCTD8 4p13 | Glioblastoma | [24] |
|           | NACC2 9q34.3 | Astrocytoma | [46] |
|           | NOS1AP 1q23.3 | Anaplastic astrocytoma | [24] |
|           | PAN3 13q12.2 | Squamous cell cancer of the head and neck | [9] |
|           | QKI 6q26 | Astrocytoma | [46, 47] |
|           | SQSTM1 5q35.3 | Glioma | [9] |
The efficacy of both larotrectinib and entrectinib in the treatment of patients with TRK fusion cancer was initially reported in multiple published case studies [33, 40, 54, 67–70]. The safety and antitumor activity of larotrectinib have been further explored in an integrated analysis of treatment outcomes in the first 55 patients with tumors harboring NTRK gene fusions consecutively enrolled into one of three clinical studies: a phase I study in adults, a phase I/II study in children, and a phase II study in adolescents and adults [7]. The primary end-point for this combined analysis was the overall response rate, as assessed by an independent radiology review committee. Patients were aged from 4 months to 76 years and 17 different tumor types were represented. The overall response rate according to independent review was 75% (95% CI 61–85) with 71% of the responses ongoing at 1 year (Fig. 2). Responses appeared to be independent of the age of the patient, the tumor type and the particular gene fusion event. Larotrectinib was generally well tolerated, with most adverse events being of grade 1 or 2. The most common grade 3 or higher adverse events were anemia (11% of patients), increased alanine aminotransferase or aspartate aminotransferase levels (7%), increased body weight (7%), and decreased neutrophil count (7%). Clinically significant treatment-related adverse events were uncommon. In particular, there were no grade 4 or 5 treatment-related adverse events and no such grade 3 events that occurred in more than 5% of patients. In the small number of patients who had adverse events leading to dose reductions, best responses were maintained at the lower doses. In addition, no patients discontinued treatment due to drug-related adverse events. These data suggest that the long-term administration of larotrectinib will be feasible.

The integrated analysis included patients from the phase I component of a first-in-child phase I/II study which enrolled infants, children, and adolescents, aged 1 month to 21 years, with a locally advanced or metastatic solid tumor or central nervous system tumor [64]. This study began enrollment prior

| NTRK gene | Fusion partner and chromosomal localization | Tumor type | Citations |
|-----------|-------------------------------------------|------------|-----------|
| STRN      | 2p22.2                                    | Soft tissue sarcoma | [7]       |
| TBC1D2    | 9q22.33                                   | Glioblastoma | [24]      |
| TLE4      | 9q21.31                                   | Ganglioglioma | [48]      |
| TRAF2     | 9q34.3                                    | Melanoma    | [28, 30]  |
| TRIM24    | 7q33-q34                                  | Lung cancer  | [9]       |
| VCL       | 10q22.2                                   | Glioma      | [43]      |
| VCAN      | 5q14.2-q14.3                              | Glioma      | [24]      |
| NTRK3 (15q25.3) | AFAP1 4p16.1 | Glioblastoma | [30]      |
| AKAP13    | 15q25.3                                   | Glioma      | [18]      |
| BTBD1     | 15q25.2                                   | Glioma      | [43]      |
| EMLA      | 2p21                                      | Congenital mesoblastic nephroma; glioblastoma; infantile fibrosarcoma; thyroid cancer | [30, 31, 49] |
| ETV6      | 12p13.2                                   | Acute lymphoblastic leukemia; acute myeloid leukemia; breast cancer; colorectal cancer; congenital mesoblastic nephroma; gastrointestinal stromal tumor; glioma; infantile fibrosarcoma; lung cancer; mammary analog secretory carcinoma of salivary gland; melanoma; neuroendocrine cancer; secretory breast cancer; soft tissue sarcoma; Spitzoid neoplasm; thyroid cancer | [7, 11, 12, 23, 26, 30, 43, 50–59] |
| LYN       | 8q12.1                                    | Squamous cell cancer of the head and neck | [9]       |
| MYH9      | 22q12.3                                   | Spitzoid neoplasm | [55]     |
| MYO5A     | 15q21.2                                   | Spitzoid neoplasm | [55, 60] |
| RBPSM     | 8p12                                      | Thyroid cancer; uterine sarcoma; | [9, 34]   |
| SQSTM1    | 5q35.3                                    | Thyroid cancer | [31, 61] |
| TFG       | 3q12.2                                    | Fibrous tumor | [41]      |
| TPM4      | 19p13.12-p13.11                           | Soft tissue sarcoma | [7, 30] |
| ZNF710    | 15q26.1                                   | Glioblastoma | [30]      |

Gene nomenclature and chromosomal localizations are described according to the Human Genome Nomenclature Committee database.
to completion of the adult phase I larotrectinib study. With responses seen in 14 (93%) of 15 pediatric patients with Response Evaluation Criteria in Solid Tumors [71] (RECIST)-evaluable tumors harboring NTRK gene fusions, the demonstration of a favorable safety profile including minimal larotrectinib-associated neurological toxicity, and no evidence of an impact on patient quality of life, this accelerated clinical development program facilitated the early demonstration of the activity and feasibility of larotrectinib in pediatric as well as adult patients. It consequently serves as a paradigm to demonstrate how delays in pediatric anticancer drug development can be minimized [72].

Preliminary data on the safety and efficacy of entrectinib have also been reported from a combined analysis of two phase I studies, ALKA-372-001 and STARTRK-1 [23], which included four patients with tumors harboring NTRK gene fusions. A thorough analysis of the activity of entrectinib in patients with TRK fusion cancer is limited by this small sample size and the lack of other published clinical data. Of these four patients, three had partial responses to entrectinib treatment and one experienced stable disease by RECIST, with three-dimensional volumetric assessment nevertheless indicating a 60% reduction in total tumor burden in this patient. Entrectinib was generally well tolerated, with most treatment-related adverse events in the overall combined population of 119 patients with advanced solid tumors being grade 1 or 2 in severity, and reversible with dose modifications. The most common treatment-related adverse events of any grade were fatigue/asthenia (46% of patients), dysgeusia (42%), paresthesias (29%), nausea (28%), and myalgias (23%), with the most common at grade 3 or higher being fatigue/asthenia (4%) and weight increase (2%).

4 Clinical Detection of NTRK Gene Fusions

Consistent with the strong scientific rationale, the clinical data available to date suggest that larotrectinib is a highly effective antitumor agent in patients with tumors harboring NTRK gene fusions [7, 64]. While it is possible that certain patients whose tumors do not have such lesions might also benefit from TRK inhibition, currently available data suggest that the development of an effective diagnostic strategy to detect NTRK gene fusions in tumor samples, and thereby to guide the selection of TRK inhibitor treatment, will be optimal. There are currently several approaches that may be used to directly detect or indirectly imply the presence of gene fusion events in clinical
samples, including fluorescence in situ hybridization (FISH), immunohistochemistry, reverse-transcriptase polymerase chain reaction (RT-PCR) and next-generation sequencing (NGS) of DNA or RNA (cDNA). Each of these approaches has strengths and weaknesses. In selecting the most appropriate diagnostic strategy, it must also be borne in mind that certain rare tumors will have a very high incidence of NTRK gene fusion events, while for other common tumor types such as colorectal or lung cancers, the incidence may be around 1% of cases. It is therefore possible that different diagnostic strategies may be utilized for these different situations.

Break apart FISH has traditionally been viewed as a standard approach for the detection of clinically relevant gene fusion events [73, 74], with RT-PCR providing additional confirmation where 5' and 3' partners are non-variable [75]. For those tumor types where only one particular NTRK gene fusion may be expected, for example, the ETV6-NTRK3 fusion in secretory breast cancer, then a fusion FISH approach with ETV6 and NTRK3 probes can be used with some degree of confidence. However, given the variability of possible 5' NTRK gene fusion partners in most tumor types, an approach using break apart FISH probes for each of the three NTRK genes (and therefore three separate FISH assays per patient sample) would be required for comprehensive coverage. One possible disadvantage of using break apart FISH is that the 5' gene fusion partner would not be identified. However, this may not be an issue of particular importance, given that there is to our knowledge currently no indication of a differential response to larotrectinib according to fusion partner. As FISH assays are relatively costly, require considerable expertise in interpretation, and may occasionally produce equivocal hybridization results [39], their routine clinical use in this setting may be restricted to those rare tumor types where particular NTRK gene fusions are essentially pathognomonic.

An alternative and/or complimentary approach to the detection of NTRK gene fusion events might be provided by RT-PCR, using primers cited in the coding sequence of the 5' fusion partner and the NTRK kinase domain. However, the large number of potential 5' fusion partners in a screening context would most likely make the development of comprehensive multiplex RT-PCR assays technically challenging. A variation of this approach is to use multiplex RT-PCR reactions to assess the ratio of 5' and 3' amplicons of each of the NTRK genes, with an imbalance in this ratio for a particular gene indicating a possible fusion event [76, 77]. The clinical utility of this approach will rest on the validation of such assays, which would in part be related to NTRK gene expression levels in NTRK gene fusion-negative cells.

The massively parallel sequencing capability facilitated by NGS technology provides a new alternative approach to the routine detection of gene fusions in clinical samples. NGS methods may be based on the analysis of DNA or RNA, and can be selectively targeted to analyze panels of genes of particular interest or can cover the entire genome, exome or transcriptome [78, 79].

Two general approaches exist to targeted panel design: amplicon-based and hybrid capture-based methods. In amplicon-based methods, PCR primers are designed to amplify a predetermined set of target sequences, and amplification products are then sequenced by NGS. This approach is generally limited to smaller gene panels and is best utilized for the detection of single nucleotide variants, small insertions and deletions (indels) and known gene fusions. Amplicon-based panels are typically used for the detection of cancer hotspot point mutations in clinical samples. In capture-based methods, biotin-labeled oligonucleotide probes targeting specific genomic regions of interest (intronic regions where breakpoints are known to occur are of particular importance for fusion detection) are hybridized with patient DNA. The probes are then captured using streptavidin-coupled magnetic beads, and the hybridized patient-derived sequences amplified by PCR and sequenced by NGS. This design allows for the analysis of copy number variation and the detection of novel fusion partners, as long as one of the partners is known (e.g., an NTRK gene fusion with any 5' partner could theoretically be detected).

One advantage of using a broad-based NGS panel is that multiple oncogenic genomic events can be identified from one sample, thereby obviating the need for multiple tests and aiding in sample conservation. However, one of the possible drawbacks in using selective assays to detect NTRK gene fusions is that, currently, available gene panels may not be configured to permit the detection of all potential fusion events, even if a hybrid capture design is used. For example, the US Food and Drug Administration (FDA) approved FoundationOne CDx NGS in vitro diagnostic can detect only selected fusions in DNA samples, which by design does not include those involving NTRK3 [80], and has only partial intron coverage for NTRK1 and NTRK2. Of particular note, the large NTRK introns create inherent complexity and challenges for DNA-based NGS approaches. To use an alternative approach of whole genome sequencing would, however, add to the cost and complexity of analysis, would necessitate a reduction in analytical sensitivity due to decreased sequencing depth, and would increase the time required to generate results [79].

Though a circulating tumor (ct)DNA-based assay could theoretically simplify biopsy procedures, these assays suffer from the same inherent challenges as all tissue DNA-based NGS approaches, which are compounded by the specific detection issues inherent in all ctDNA assays. Additionally, as recently noted in an American Society of Clinical Oncology and College of American Pathologists joint review, although certain ctDNA assays have demonstrated clinical validity and utility in advanced cancer, there is currently insufficient evidence of such for the majority of assays, which would include those relating to gene fusions [81].
The detection of gene fusions through RNA-based NGS may provide an attractive alternative approach to DNA-based NGS assays [82]. RNA-based NGS allows for assay design without the need to cover the entirety of NTRK intronic regions. Additionally, when using such methods, gene fusions which do not result in an expressed kinase will not be detected. However, the lower stability of RNA compared with DNA poses challenges with regard to the collection of clinical samples for such diagnostic assessments. Nevertheless, in the future, it is likely that NGS diagnostics which rely on RNA for fusion detection will increasingly be used to facilitate the detection of NTRK gene fusions in routine clinical practice.

In comparative studies, NGS has demonstrated superior performance in terms of fusion detection relative to other genomic testing methods. Specifically, when samples scored negative for particular fusions by FISH have been re-screened using NGS, a considerable fraction have been found to harbor those previously tested for fusion [83–85]. Factors that might explain such discrepancies include the presence of complex chromosomal rearrangements, technical issues with the FISH process, and the higher sensitivity of NGS. One further factor that should be considered is that, with the exception of the US, patient access to tumor NGS in clinical diagnostic settings is currently extremely limited, although this situation is likely to change over coming years.

While approaches based on molecular analyses are generally seen as the most accurate methods for identifying samples harboring gene fusion events, standard immunohistochemistry may still prove an extremely cost-effective and useful screening approach in this context. This is evidenced by current clinical guidelines relating to the selection of patients with lung cancer for ALK TKI treatment, which draw an equivalence between immunohistochemistry and FISH testing in the identification of tumors harboring ALK gene fusions [86]. Although the evidence-based setting of appropriate thresholds for scoring positivity in different tumor types might be necessary for routine clinical use of an immunohistochemistry-based assay for TRK fusion proteins, it might be that a less rigorous scoring system could enable the use of a pan-TRK immunohistochemistry assay for screening purposes, followed by reflex testing using a molecular method.

The feasibility of developing such an assay has been suggested by two recent studies [30, 35]. The first of these compared the performance of the pan-TRK monoclonal antibody EPR17341 (Abcam) with MSK-IMPACT, a DNA-based NGS assay and an Archer Dx fusion assay, an RNA-based NGS assay. Of 23 tumors deemed to have NTRK gene fusions by MSK-IMPACT, NTRK fusion transcripts were not detected in two using the Archer Dx assay. Of the remaining 21 samples, 20 were found to be positive for TRK protein by pan-TRK immunohistochemistry. Twenty additional tumors scored negative for NTRK gene fusion transcripts by Archer Dx analysis were also scored negative for TRK protein by immunohistochemistry, giving a sensitivity and specificity for pan-TRK immunohistochemistry in relation to the detection of tumors with transcribed NTRK gene fusions of 95 and 100%, respectively, based on this specific set of samples. In the second study, the same pan-TRK monoclonal antibody was evaluated in pediatric mesenchymal tumors. Twenty-nine of 30 tumors with NTRK gene fusions confirmed by DNA-based NGS assays were positive for TRK expression by immunohistochemistry, and 47 of 48 tumors lacking an identified NTRK gene fusion by NGS were negative for TRK expression. The sensitivity and specificity for pan-TRK immunohistochemistry in this study in relation to the detection of NTRK gene fusions was therefore 97 and 98%, respectively. Although further development and broader testing of this approach across multiple tumor types is clearly required, these studies suggest that a strategy of routine screening for TRK fusion proteins in common cancers using immunohistochemistry might be feasible.

5 Resistance Mutations and Solutions

It is now well established that long-term treatment with TKIs leads to acquired resistance to such agents, typically arising through the occurrence of mutations that alter drug binding [17]. In line with such expectations, acquired resistance mutations have been reported in patients with TRK fusion cancer who had progressed while receiving the pan-TRK, ALK, ROS1 multikinase inhibitor, entrectinib [70, 87], or larotrectinib [7, 64]. This issue was specifically investigated in the recently published integrated analysis of 55 larotrectinib-treated patients with NGS-confirmed NTRK gene fusion cancers. At progression, tumor or plasma samples were obtained from nine patients who had an initial documented objective response, or stable disease for at least 6 months, and the NTRK gene involved in the fusion was sequenced. Samples from all nine patients showed one or more kinase domain mutations affecting this NTRK gene [7]. Mutations identified included those in solvent front (seven patients), gatekeeper (two) and xDFG (two) domains, locations within the kinase similar to those described for resistance mutations in other classes of kinase inhibitors.

For several TKIs, the problem of acquired resistance has been addressed by the subsequent development of next-generation inhibitors, which are clinically active in the presence of kinase domain mutations that render early generation agents ineffective. Exceptionally in this field, one such novel agent, LOXO-195, has been developed in parallel with the first agent, larotrectinib. LOXO-195 is a potent selective inhibitor of all three TRK tyrosine kinases with IC50 values of <5 nM. Using in vitro and in vivo model systems, it was initially demonstrated that this TRK inhibitor was active against TRK proteins with acquired solvent front and xDFG
domain mutations. In a subsequent first-in-human, proof of concept investigation, the first two patients with TRK fusion cancer who developed acquired resistance mutations while on larotrectinib were treated with LOXO-195 under FDA-allowed single-patient protocols. Despite the presence of TRK solvent front resistance mutations, rapid responses were seen in both patients [88]. This opens up the possibility of extending the period of disease control for patients with TRK fusion cancer through sequential TRK inhibitor therapy. A phase I/II study of LOXO-195 in patients with previously treated TRK fusion cancer is currently recruiting (NCT03215511).

### 6 Regulatory Considerations for TRK Inhibitor Drug Development

Historically, the FDA has granted marketing approval for novel agents for indications based on specific tumor histologies. The reason for this is that, until recently, the field has considered treatments as being histology context dependent. This was exemplified by the differential response to vemurafenib in BRAF V600E mutated melanoma versus colorectal cancer [89]. However, on May 23, 2017, the FDA approved an anticancer treatment for the first time, the PD-1 monoclonal antibody pembrolizumab, on the basis of tumors having a particular molecular characteristic, regardless of, or agnostic of, tumor site [90]. This approval was based on a combined analysis of 149 patients with metastatic, microsatellite instability-high (MSI-H) or mismatch-repair-deficient (dMMR) solid tumors; 90 patients had colorectal cancer, and 59 had 1 of 14 other types of tumor. Responses to pembrolizumab were seen in 40% of patients overall, with response rates similar for those with colorectal (36%) and other (46%) cancers. The accelerated approval granted by the FDA for the MSI-H/dMMR indication required the sponsor to conduct further nonrandomized trials to evaluate activity in additional patients [90].

The broad antitumor activity observed with TRK inhibitors in patients with TRK fusion cancer also lends itself to be considered as a tumor agnostic treatment strategy. Both entrectinib and larotrectinib received orphan drug designation under the Orphan Drug Act of 1983, since NTRK gene fusions are found in less than 1% of all solid tumors and have a combined US prevalence of <200,000, meeting the definition of a rare disease or condition. Receiving an orphan drug designation qualifies the sponsor for certain incentives, such as a waiver of the prescription drug application user fee, tax credits for qualified clinical testing, and, upon approval, potential 7-year marketing exclusivity (orphan drug exclusivity). The development of novel therapeutics such as TRK inhibitors has necessitated rethinking of the definitions of “disease”, “condition” and “indication” under the regulatory framework.

### 7 Conclusions

NTRK gene fusions occur across a wide range of different tumor types and have now been clinically validated as actionable genomic alterations. In certain rare tumors, the prevalence of such aberrations is typically very high, while in common cancers, it is thought to be around 1% of cases. The highly selective TRK inhibitor larotrectinib has been shown to be generally well tolerated and highly active in patients with TRK fusion cancer, regardless of tumor type, patient age, and fusion type. Several multikinase inhibitors with activity against TRK proteins are also in clinical development, including entrectinib. Given the striking differences in the prevalence of NTRK gene fusions between certain rare and other common tumor types, it is likely that future clinical diagnostic strategies to identify patients who might benefit from TRK inhibitor therapy will need to be varied accordingly, and will most likely include FISH, immunohistochemistry and NGS assays. The approval of pembrolizumab for MSI-H tumors has set the precedent for considering tissue agnostic approval for agents, such as TRK inhibitors, which demonstrate activity across different tumor types that carry a common genomic alteration.

### Compliance with Ethical Standards

**Conflict of Interest** Dr. Shivaani Kummar has received research funding for the clinical trial of larotrectinib. Dr. Ulrik Lassen declares that he has no conflicts of interest that might be relevant to the contents of this manuscript.

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