Chromosomal rearrangements involving the \textit{NTRK1}, \textit{NTRK2}, and \textit{NTRK3} genes (\textit{NTRK} genes), which encode the high-affinity nerve growth factor receptor (TRKA), brain-derived neurotrophic factor/nerve growth factor-3 (BDNF/NT-3) growth factor receptor (TRKB), and neurotrophin-3 (NT-3) growth factor receptor (TRKC) tyrosine kinases (TRK proteins), act as oncogenic drivers in a broad range of pediatric and adult tumor types. \textit{NTRK} gene fusions have been shown to be actionable genomic events that are predictive of response to TRK kinase inhibitors, making their routine detection an evolving clinical priority. In certain exceedingly rare tumor types, \textit{NTRK} gene fusions may be seen in the overwhelming majority of cases, whereas in a range of common cancers, reported incidences are in the range of 0.1% to 2%. Herein, we review the structure of the three \textit{NTRK} genes and the nature and incidence of \textit{NTRK} gene fusions in different solid tumor types, and we summarize the clinical data showing the importance of identifying tumors harboring such genomic events. We also outline the laboratory techniques that can be used to diagnose \textit{NTRK} gene fusions in clinical samples. Finally, we propose a diagnostic algorithm for solid tumors to facilitate the identification of patients with TRK fusion cancer. This algorithm accounts for the widely varying frequencies by tumor histology and the underlying prevalence of TRK expression in the absence of \textit{NTRK} gene fusions and is based on a combination of fluorescence in situ hybridization, next-generation sequencing, and immunohistochemistry assays. (\textit{J Mol Diagn} 2019, 21: 553–571; https://doi.org/10.1016/j.jmoldx.2019.03.008)
encoded by different exon combinations currently recorded in the Ensembl genomic database.\textsuperscript{9} Given this complexity, the structure of these genes is summarized, as related to the exons encoding the canonical isoforms described in the UniProt Knowledgebase (Figure 1 and Table 1).\textsuperscript{10}

**NTRK Genes in Cancer**

**Molecular Characteristics of NTRK Gene Fusions**

The *NTRK1* gene was initially identified in a transfection assay designed to screen for transforming sequences in DNA isolated from a human colon carcinoma.\textsuperscript{11} This work led to the identification of an oncogenic fusion transcript comprising the 5′ exons of a tropomyosin gene (*TPM3*) and a sequence encoding an unknown protein tyrosine kinase,\textsuperscript{12} subsequently characterized as the 3′ exons of *NTRK1*. With both of these genes localized to the long arm of chromosome 1, the chromosomal rearrangement leading to this gene fusion would most likely have been a short inversion.\textsuperscript{13} Subsequently, the closely related *NTRK2* and *NTRK3* genes were characterized (Figure 1).\textsuperscript{14,15}

It is now apparent that somatic intrachromosomal or interchromosomal rearrangements involving *NTRK1*, DNA isolated from a human colon carcinoma.\textsuperscript{11} This work led to the identification of an oncogenic fusion transcript comprising the 5′ exons of a tropomyosin gene (*TPM3*) and a sequence encoding an unknown protein tyrosine kinase,\textsuperscript{12} subsequently characterized as the 3′ exons of *NTRK1*. With both of these genes localized to the long arm of chromosome 1, the chromosomal rearrangement leading to this gene fusion would most likely have been a short inversion.\textsuperscript{13} Subsequently, the closely related *NTRK2* and *NTRK3* genes were characterized (Figure 1).\textsuperscript{14,15}

It is now apparent that somatic intrachromosomal or interchromosomal rearrangements involving *NTRK1*,

**Figure 1** Genomic structure of *NTRK1*, *NTRK2*, and *NTRK3*, showing exons encoding the canonical isoforms described in the UniProt Knowledgebase. Regions of the corresponding mRNAs encoding function domains are marked. Introns are to scale, but for visualization purposes, exons of *NTRK2* and *NTRK3* are not shown to scale and intron spans are scaled 10-fold relative to *NTRK1*. Ig-like-1, Ig-like C2-type 1 region; Ig-like-2, Ig-like C2-type 2 region; LRR, leucine-rich repeat; LRRCT, leucine-rich repeat C-terminal domain; LRRNT, leucine-rich repeat N-terminal domain; TK, tyrosine kinase domain; TM, transmembrane domain.
NTRK2, or NTRK3 may be found as oncogenic drivers in a wide range of different pediatric and adult tumor types. In almost all such cases, the 5’ region of a gene that is expressed in the tumor is fused with the 3’ region of one of the NTRK genes. Driven by the promoter of the 5’ partner, the fusion transcript typically encodes an in-frame protein comprising the N-terminus of the 5’ fusion partner and the C-terminal tyrosine kinase domain of the TRK receptor. The fusion proteins lack the extracellular ligand binding domain of the full-length TRK protein and generally, but not universally, include one or more dimerization domains from the 5’ partner. This structure typically leads to ligand-independent dimerization and constitutive activation of the C-terminal TRK tyrosine kinase domain and associated downstream signaling processes.\(^{1,6,17}\) Currently, approximately 80 different 5’ NTRK gene fusion partners have been identified in a diverse range of human tumor types (Table 2).\(^1\) As tumors are increasingly profiled in research and routine health care settings using sensitive next-generation sequencing (NGS) approaches, it is likely that the number of characterized 5’ fusion partners will increase.

With coding exons located across a genomic region of 20.7 kb, NTRK1 (1q23.1) is the smallest of the three NTRK genes, with NTRK2 (9q21.33) and NTRK3 (15q25.3), which include several exceptionally large introns, covering genomic regions 17 to 18 times longer. Incomplete intron coverage in DNA NGS assays for NTRK2 and NTRK3 may have, therefore, historically resulted in a lower surveillance of fusion events in relation to these two genes, and may explain in part why a higher number of fusion partners have currently been identified for NTRK1 (n = 43) compared with NTRK2 (n = 24) and NTRK3 (n = 21). More specifically, the large size, high repetitive element content, and high GC content of certain NTRK2 and NTRK3 introns make a DNA hybridization capture design to achieve optimal sensitivity technically infeasible (see NGS section below). The introduction of RNA-based NGS assays into clinical practice has the potential to improve detection and provide a more accurate assessment of the true prevalence of fusions involving NTRK2 and NTRK3. Most NTRK1 gene fusion partners are localized to chromosome 1 [28 of 43 (65%)], consistent with intrachromosomal rearrangement being the primary molecular mechanism driving NTRK1 fusion events (Table 2). By contrast, for NTRK2 and NTRK3, the predominant molecular mechanism associated with fusion events appears to be interchromosomal rearrangements [17 of 24 (71%) and 16 of 21 (76%) characterized fusions, respectively].\(^1\)

The position of breakpoints within gene fusion partners and the exons consequently included in resultant fusion transcripts may both be variable.\(^7^0\) This is exemplified by an analysis by Farago et al\(^{17}\) of breakpoints in the tumors of 17 patients with TRK fusion non—small-cell lung cancer (NSCLC). In 16 of 17 patients (94%), the breakpoints revealed by NGS were within introns of the 5’ partner and 3’ NTRK gene, and in only one case, within exonic sequences of both. Although the exons included in fusions between the same two gene partners were somewhat variable, in each case the breakpoint in the NTRK gene was 5’ to the exons encoding the kinase domain (coding exons 13 to 17 of NTRK1 and NTRK2 and 13 to 18 of NTRK3 in relation to the canonical isoform transcripts), thus leaving the TRK kinase domains intact (Table 1).

Incidence of NTRK Gene Fusions in Cancer

Although in a small number of exceedingly rare pediatric and adult tumor types, NTRK gene fusions are common; they have also been identified in a wide range of more common cancers at lower frequencies. These different situations suggest that a diagnostic strategy driven by the known incidence of such fusions and the established biological TRK expression patterns in different tumor types may be the most effective approach for the identification of patients whose tumors harbor NTRK gene fusions. The incidence of NTRK gene fusions in different tumor types is summarized in the following section and in Figure 2.

Infantile Fibrosarcoma

This rare pediatric tumor is the most common non-rhabdomyosarcoma soft tissue tumor seen in the first year of life.\(^7^1\) Two studies have reported the incidence of ETV6-NTRK3 fusions in this tumor type to be 70% and 91%, with other spindle cell neoplasms being negative.\(^6^0,6^1\) However, more recently, LMNA-NTRK1 and EML4-NTRK3 fusions have also been described in infantile fibrosarcoma.\(^5^3,7^2,7^3\)

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**Table 1** Characteristics of NTRK Genes and Canonical Protein Isoform

| NTRK gene | Chromosomal location | Characterized transcripts* | Transcript\(^1\) | CCDS ID\(^1\) | Protein | UniProtKB identifier | Amino acids | Coding exons | Genomic span (coding exons), kb |
|-----------|---------------------|---------------------------|-----------------|-------------|---------|---------------------|-------------|-------------|-----------------------------|
| NTRK1     | 1q23.1              | 10                        | NM_002529.3     | CCDS1161.1  | TRKA    | P04629              | 796         | 17          | 20.7                        |
| NTRK2     | 9q21.33             | 8                         | NM_001018064.2  | CCDS5050.1  | TRKB    | Q16620              | 822         | 17          | 350.7                       |
| NTRK3     | 15q25.3             | 21                        | NM_001012338.2  | CCDS2322.1  | TRKC    | Q16288              | 839         | 18          | 379.2                       |

*As reported in the Ensembl database (https://www.ensembl.org/index.html, last accessed March 4, 2019).

\(^1\)As described in the UniProt Knowledgebase (https://www.uniprot.org, last accessed March 4, 2019).

\(^2\)National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov, last accessed March 4, 2019).

\(^3\)CCDS database (https://www.ncbi.nlm.nih.gov/projects/CCDS/CdsBrowse.cgi, last accessed March 4, 2019).

BDNF, brain-derived neurotrophic factor; CCDS, consensus coding sequence; ID, identification; NT-3, neurotrophin-3; TRKA, high-affinity nerve growth factor receptor; TRKB, BDNF/NT-3 growth factor receptor; TRKC, NT-3 growth factor receptor.
| NTRK gene | Fusion partner and chromosomal localization* | Tumor type |
|-----------|-----------------------------------------------|------------|
| NTRK1 (1q23.1) | **AFAP1** 4p16.1 | Glioblastoma<sup>8</sup> |
|          | **AMOTL2** 3q22.2 | Lung cancer<sup>19</sup> |
|          | **ARHGEF2** 1q22 | Glioblastoma<sup>20</sup> |
|          | **BCAN** 1q23.1 | Glioma,<sup>21,22</sup> glioneuronal tumor,<sup>23</sup> pilocytic astrocytoma<sup>24</sup> |
|          | **CEL** 9q34.13 | Pancreatic cancer<sup>25</sup> |
|          | **CD74** 5q33.1 | Lung cancer<sup>10</sup> |
|          | **CHTOP** 1q21.3 | Glioblastoma<sup>20</sup> |
|          | **CGN** 1q21.3 | Breast cancer<sup>27</sup> |
|          | **COP1** 1q25.1—q25.2 | Large-cell neuroendocrine cancer<sup>28</sup> |
|          | **CTRC** 1p36.21 | Pancreatic cancer<sup>2</sup> |
|          | **DDR2** 1q23.3 | Melanoma<sup>29</sup> |
|          | **DIAPH1** 5q31.3 | Thyroid cancer<sup>4</sup> |
|          | **EPHB2** 1p36.12 | Not specified<sup>10</sup> |
|          | **EPS15** 1p32.3 | Lung cancer<sup>11</sup> |
|          | **GATA2B** 1q21.3 | Breast cancer<sup>27</sup> |
|          | **GRIN4L** 1q22 | Melanoma<sup>2,29</sup> |
|          | **GONL** 1q22 | Melanoma<sup>2,29</sup> |
|          | **GPRAP1p** Xp11.23 | Lung cancer<sup>31</sup> |
|          | **GSN** 9q33.2 | Not specified<sup>10</sup> |
|          | **IRF2BP2** 1q42.3 | Lung cancer,<sup>2,32</sup> thyroid cancer,<sup>2,33,34</sup> prostate cancer<sup>19</sup> |
|          | **LMNA** 1q22 | Appendiceal cancer,<sup>2,32</sup> breast cancer,<sup>27</sup> cholangiocarcinoma,<sup>2</sup> colorectal cancer,<sup>2,23,32,35</sup> gallbladder carcinoma,<sup>32</sup> soft tissue sarcoma,<sup>2,32</sup> Spitzoid neoplasm,<sup>36</sup> uterine sarcoma<sup>37</sup> |
|          | **LRRC71** 1q23.1 | Uterine endometrial cancer<sup>31</sup> |
|          | **MDM4** 1q32.1 | Breast cancer<sup>27</sup> |
|          | **MEF2D** 1q22 | Glioma<sup>42</sup> |
|          | **MIR548F1** 10q21.1 | Pediatric mesenchymal tumor<sup>38</sup> |
|          | **MPRIP** 1p11.2 | Lung cancer<sup>17,26</sup> |
|          | **MRPL24** 1q23.1 | Lung cancer<sup>11</sup> |
|          | **NFASC** 1q23.1 | Glioblastoma<sup>21,33</sup> |
|          | **P2RY8** Xp22.33 and Yp11.3 | Lung cancer<sup>32</sup> |
|          | **PDE4DIP** 1q21.2 | Soft tissue sarcoma<sup>2</sup> |
|          | **PEAR1** 1q23.1 | Breast cancer<sup>27</sup> |
|          | **PIPSK1A** 1q21.3 | Neuroendocrine tumor<sup>19</sup> |
|          | **PLEKH9** 1q21.3 | Colon cancer<sup>19</sup> |
|          | **PPL** 1p31.3 | Thyroid carcinoma<sup>2,20</sup> |
|          | **PDX1** 1p34.1 | Lung cancer<sup>11</sup> |
|          | **RARGAP1L** 1q25.1 | Intrahepatic cholangiocarcinoma<sup>40</sup> |
|          | **SCYL3** 1q24.2 | Colorectal cancer<sup>41</sup> |
|          | **SOSTM1** 5q35.3 | Infantile fibrosarcoma,<sup>2,42</sup> lung cancer,<sup>23,43</sup> thyroid cancer<sup>33</sup> |
|          | **SSBP2** 5q14.1 | Thyroid cancer<sup>23</sup> |
|          | **TFG** 3q12.2 | Thyroid cancer<sup>32,44</sup> |
|          | **TP53** 17p13.1 | Spitzoid neoplasm<sup>36</sup> |
|          | **TPM1** 1q21.3 | Breast cancer,<sup>2,27</sup> cervical cancer,<sup>22</sup> cholangiocarcinoma,<sup>2</sup> colorectal cancer,<sup>2,19,22,32,45</sup> glioma,<sup>2,44</sup> infantile fibrosarcoma,<sup>22</sup> lung cancer,<sup>20,22,32</sup> soft tissue sarcoma,<sup>2,22,33</sup> thyroid cancer,<sup>32,47</sup> uterine sarcoma<sup>37</sup> |
|          | **TPR** 1q31.1 | Lung cancer,<sup>2</sup> thyroid cancer,<sup>44</sup> uterine sarcoma,<sup>37</sup> pediatric mesenchymal tumor<sup>18</sup> |
| NTRK2 (9q21.33) | **AFAP1** 4p16.1 | Melanoma<sup>2,29,32</sup> |
|          | **AGBL4** 1p33 | Glioma<sup>46</sup> |
|          | **BCR** 22q11.23 | Glioma<sup>22,32</sup> |
|          | **DAB2IP** 9q33.2 | Colorectal cancer<sup>11</sup> |
|          | **ETV6** 12p13.2 | Acute myeloid leukemia<sup>48</sup> |
|          | **GKAP1** 9q21.32 | Glioma<sup>22</sup> |
|          | **GNAQ** 9q21.2 | Bone sarcoma<sup>11</sup> |

*(table continues)*
Secretory Carcinomas

Secretory breast carcinoma is a rare distinct subtype of infiltrating ductal carcinoma. More than 90% of cases harbor ETV6-NTRK3 gene fusions. A morphologically and immunohistochemically related entity in the salivary gland was described by Skálová et al. and similarly harbors ETV6-NTRK3 fusions in >90% of cases. Indeed, NGS analysis suggested that secretory breast carcinomas are genetically

| NTRK gene | Fusion partner and chromosomal localization* | Tumor type |
|-----------|---------------------------------------------|------------|
| KCTD8     | 4p13                                       | Glioma     |
| NACC2     | 9q34.3                                     | Astrocytoma|
| NAV1      | 1q32.1                                     | Not specified|
| NOS1AP    | 1q23.3                                     | Anaplastic astrocytoma, glioma |
| PAN3      | 13q12.2                                    | Squamous cell cancer of the head and neck |
| PRKAR2A   | 3p21.31                                    | Glioma     |
| QKI       | 6q26                                       | Astrocytoma |
| RBPKMS    | 8p12                                       | Soft tissue sarcoma |
| SQSTM1    | 5q35.3                                     | Glioma, lung cancer |
| SLMAP     | 3p14.3                                     | Not specified |
| STRN      | 2p22.2                                     | Soft tissue sarcoma |
| TBC1D2    | 9q22.33                                    | Glioma     |
| TLE4      | 9q21.31                                    | Ganglioglioma |
| TRAF2     | 9q34.3                                     | Melanoma   |
| TRIM24    | 7q33-q34                                   | Lung cancer |
| VCL       | 10q22.2                                    | Glioma     |
| VCAN      | 5q14.2-14.3                                | Glioma     |
| EML4      | 2p21                                       | Congenital mesoblastic nephroma, glioma, infantile fibrosarcoma, thyroid cancer |
| ETV6      | 12p13.2                                    | Acute lymphoblastic leukemia, acute myeloid leukemia, breast cancer, colorectal cancer, congenital mesoblastic nephroma, gastrointestinal tract stromal tumor, glioma, fibrosarcoma, inflammatory myofibroblastic tumor, lung cancer, melanoma, neuroendocrine cancer, secretory breast cancer, sinonasal adenocarcinoma, soft tissue sarcoma, Spitzoid neoplasm, thyroid cancer |
| FAT1      | 4q35.2                                     | Not specified |
| HNRNPA2B1 | 7p15.2                                     | Multiple myeloma |
| LYN       | 8q12.1                                     | Squamous cell cancer of the head and neck |
| MYH9      | 22q12.3                                    | Spitzoid neoplasm |
| MYO5A     | 15q21.2                                    | Spitzoid neoplasm |
| RBPKMS    | 8p12                                       | Thyroid cancer, uterine sarcoma |
| SPECC1L   | 22q11.23                                   | Uterine sarcoma |
| SQSTM1    | 5q35.3                                     | Thyroid cancer |
| STRN      | 2p22.2                                     | Adult fibrosarcoma |
| STRN5     | 14q12                                      | Adult fibrosarcoma |
| TFG       | 3q12.2                                     | Fibrous tumor |
| TPM4      | 19p13.12-p13.11                            | Soft tissue sarcoma |
| UBE2R2    | 9p13.3                                     | Multiple myeloma |
| VIM       | 10p13                                      | Thyroid cancer |
| VPS18     | 15q15.1                                    | Not specified |
| ZNF710    | 15q26.1                                    | Glioblastoma |

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*Gene nomenclature and chromosomal localizations are described according to the Human Genome Nomenclature Committee database; 5' fusion partners associated with more than one NTRK gene are in bold; fusions in which the NTRK gene is listed as the 5' partner are not included.

1 A brief history of NTRK fusions (http://ntrkfusions.com/a-brief-history-of-ntrk-fusions, last accessed February 5, 2019).

Loxo Oncology, Inc., data on file.
more similar to secretory carcinomas of the salivary gland than they are to other primary breast cancers. Recently, it has been reported that a subset of salivary gland carcinomas harbor \( \text{ETV6-RET} \) gene fusions. Cases of secretory carcinoma of the skin and thyroid harboring \( \text{ETV6-NTRK3} \) fusions have also been described.

**Congenital Mesoblastic Nephroma**  
This rare spindle cell tumor of the kidney mainly occurs in newborns or young infants. \( \text{ETV6-NTRK3} \) fusions can be found in almost all cases with specific histologic subtypes (cellular and mixed) but have not been reported in the other dominant subtype (classic). The \( \text{ETV6-NTRK3} \) fusion is typically accompanied by trisomy 11. However, variant \( \text{NTRK} \) gene fusions may also be found in such tumors.

**Thyroid Carcinoma**  
Thyroid cancers in patients <20 years of age represent >2% of all thyroid malignancies diagnosed in the United States, with papillary thyroid carcinoma accounting for the vast majority (90%). One series of 27 cases demonstrated \( \text{NTRK} \) gene fusions in 26% of pediatric papillary thyroid carcinomas. \( \text{NTRK} \) gene fusions have also been found less commonly (<10%) in thyroid cancers in adults.

**Brain Tumors**  
In an analysis of 112 pediatric high-grade gliomas, Wu et al reported that eight tumors (7%) harbored one of five different \( \text{NTRK} \) gene fusions. Notably, of the 10 patients with non-brainstem high-grade gliomas who were younger than 3 years, such fusions were detected in four tumors (40%). In a large study of 390 predominantly adult [373 (98%)] gliomas, \( \text{NTRK} \) gene fusions were identified in eight tumors (2%), six of which involved \( \text{NTRK2} \).

**Common Cancers**  
\( \text{NTRK} \) gene fusions have been identified in several common cancers, at frequencies ranging from 0.1% to 2%, predominantly using NGS technologies. The tumor types in which \( \text{NTRK} \) gene fusions have been detected are diverse, and include breast cancer, non–small-cell lung cancer, colorectal cancer, and melanoma, with new entities added regularly. Among the most common tumor types likely to be encountered in routine pathology practice, \( \text{NTRK} \) gene fusion incidence is generally <5%. However, lacking widespread testing, these values are likely subject to numerous forms of bias, and as testing for \( \text{NTRK} \) gene fusions is increasingly adopted, a better appreciation of the true incidence across a wide spectrum of tumor types will likely emerge.

For example, after an NGS analysis of 140 Spitzoid neoplasms, melanocytic lesions predominantly encountered in children and adolescents, Wiesner et al reported that 23 (16%) harbored \( \text{NTRK1} \) gene fusions. \( \text{NTRK} \) gene fusions have also been reported in other tumor types, for which the frequency of occurrence has not yet been systematically characterized (Table 2). The relative rarity with which these events occur in the most common tumor types raises challenges for a routine screening approach.

**Other Somatic Changes in \( \text{NTRK} \) Genes in Tumors**  
In addition to gene fusions, other somatic genomic alterations involving \( \text{NTRK} \) genes have been identified in human cancers.
tumors, including point mutations, coding sequence deletions, and gene amplifications. Whether such changes are oncogenic and/or whether they may be predictive for the efficacy of TRK inhibitors has not been definitively demonstrated. Qian et al reported the identification of >12 unique mutations in NTRK2 and NTRK3 in primary leukemia samples. In model systems, four of these mutations were found to be transforming and sensitive to TRK inhibition at low nanomolar concentrations. Point mutations of unknown significance in NTRK genes were also identified by NGS in 5 of 55 patients (9%) with advanced thyroid cancer. Furthermore, in a large panel of 538 primary lung cancers, Marchetti et al identified mutations in the kinase domains of NTRK2 or NTRK3 in 9 of 29 large-cell neuroendocrine tumors (31%), 0 of 66 other pulmonary neuroendocrine tumors, and 0 of 443 NSCLCs. The significance of these point mutations is called into question in tumor types in which the wild-type biological expression of TRK proteins is limited. Recently, phase 1 data on larotrectinib, is highly selective, orally administered, ATP-competitive inhibitor of TRKA, TRKB, and TRKC, with half maximal inhibitory concentration (IC50) values in the low nanomolar range and minimal off-target activity against other kinases. Entrectinib is an orally administered small-molecule inhibitor of TRKA, TRKB, TRKC, proto-oncogene tyrosine-protein kinase reactive oxygen species (ROS), and the anaplastic lymphoma kinase (ALK) tyrosine kinase receptor, which also has IC50 values for the TRK kinases in the low nanomolar range.

The clinical impact of TRK inhibition was initially suggested in case reports of patients with TRK fusion cancer treated with larotrectinib and entrectinib. This potential impact was subsequently confirmed in an integrated analysis of the first 55 adult and pediatric patients with tumors of 17 different types harboring NTRK gene fusions. Larotrectinib demonstrated an objective response rate of 75%. In an updated analysis with a median follow-up of 17.6 months, the median duration of response had not been reached. Larotrectinib was generally well tolerated, with most of the adverse events (93%) being of grade 1 or 2. In the pediatric phase 1 study, the best response in seven patients without documented tumor NTRK gene fusions was progressive disease. Larotrectinib was US Food and Drug Administration approved in November 2018 for adult and pediatric patients with solid tumors who have an NTRK gene fusion and meet other specified criteria (VITRAKVI Prescribing Information, https://www.accessdata.fda.gov/drugsatfda_docs/label/2018/211710s000lbl.pdf, last accessed February 5, 2018). The activity of entrectinib in patients with tumors harboring NTRK gene fusions has also been confirmed in an integrated analysis of 54 adult patients with TRK fusion cancer of 10 different tumor types enrolled into one of three phase 1 and 2 clinical trials. After 15.5 months of follow-up, the objective response rate in this cohort was 57%. The median duration of response was 10.4 months. Entrectinib was also generally well tolerated, with most treatment-related adverse events being grade 1 or 2. In addition to these agents, outcome data are also beginning to emerge from ongoing phase 1/2 studies of TPX-0005 and DS-6051b, with both agents initially reported as being well tolerated.

Clinical Importance of Identifying Tumors Harboring NTRK Gene Fusions

There are several different techniques that may be used to detect or imply the presence of NTRK gene fusions in clinical samples and, thereby, facilitate the selection of patients for TRK inhibitor treatment (Table 3). In selecting the optimum approach, turnaround time, required expertise, and the cost of the test should be considered. Further complicating this is the expected incidence and particular characteristics of NTRK gene fusions within different indications. Ultimately, in situ methods, such as immunohistochemistry and fluorescence in situ hybridization (FISH), as well as highly targeted molecular approaches, such as RT-PCR, are
Table 3  Clinical Laboratory Techniques Used to Identify Tumors Harboring NTRK Gene Fusions

| Analytical technique | Sample requirements | Preanalytical considerations | Turnaround time | Advantages | Disadvantages |
|----------------------|---------------------|------------------------------|-----------------|------------|---------------|
| Pan-TRK IHC          | FFPE tissue         | Variability in fixation processes may impact the quality of staining | 1–2 days        | Rapid and inexpensive process Established approach, widely available within clinical laboratories | Indication-specific specificity for NTRK gene fusion prediction not well characterized Sensitivity with respect to TRKC fusion proteins may be low Assay not easily multiplexed for other biomarkers |
| FISH                 | FFPE tissue         | Must ensure adequate tumor cellularity | 1–2 days        | Established approach, widely available within clinical laboratories Probes are costly, but FISH is generally reimbursable | Requires expert interpretation Does not confirm detected fusion is expressed Not easily multiplexed with other biomarkers and may require more than one FISH assay to adequately cover all possible NTRK1 to NTRK3 fusions Limited scalability for high-volume testing |
| Fusion               |                     |                              |                 | High specificity Can detect alterations present in small subsets of cells Detects NTRK rearrangements without knowledge of 5’ partner | Individual assay limited to detection of specific 5’ partner and NTRK gene pair Sensitivity and specificity variable, depending on assay design and parameters Multiple or complex FISH assays may be required for complete coverage |
| NTRK break apart     |                     |                              |                 | Rapid and inexpensive test Well-established technique in molecular genetics laboratories | PCR primer pairs must be designed and validated for each specific fusion For FFPE tissue-based analyses, primers must closely flank breakpoints |
| RT-PCR               | FFPE, snap-frozen, or stabilized tissue | With variable intronic breakpoints, RT-PCR assays can be dependent on high-quality RNA from frozen/stabilized tissue | 5–10 days       | Rapid and inexpensive test Well-established technique in molecular genetics laboratories | PCR primer pairs must be designed and validated for each specific fusion For FFPE tissue-based analyses, primers must closely flank breakpoints |
| Defined gene partners |                    |                              |                 | High specificity because of PCR design Assays can be multiplexed, although limited | PCR primer pairs must be designed and validated for each specific fusion For FFPE tissue-based analyses, primers must closely flank breakpoints |
| 3’/5’ NTRK ratio     | May be challenging to optimize assay, especially if RNA quality is variable |                              |                 | Implies presence of NTRK gene fusion without knowledge of 5’ partner | Sensitivity depends on expression difference between wild-type gene and fusion, which is currently unvalidated/ unstudied Comprehensive coverage |

(table continues)
Table 3  (continued)

| Analytical technique | Sample requirements | Preanalytical considerations | Turnaround time | Advantages | Disadvantages |
|----------------------|---------------------|------------------------------|-----------------|------------|---------------|
| NGS                  | DNA-based NGS       | Data acquisition may be affected by tumor heterogeneity Sensitivity for fusions varies, according to enrichment method Fixation conditions may affect DNA quality | 2–3 weeks       | Ability to interrogate all clinically actionable genomic content Most tissue-sparing approach for broad genomic analysis Commercial kits available | May require high level of infrastructure investment Requires high-level bioinformatics capability Evolving reimbursement landscape Does not confirm that protein is generated Commercially available kits not configured to cover all NTRK introns involved in fusions Detected fusions may not be expressed or in frame Lower analytical sensitivity Slower to generate data, and requires more computational resources than targeted approaches |
| DNA-based NGS FFPE or frozen tissue | For FFPE tissue, sample age might affect DNA quality and sequencing read quality | Readily multiplexed across multiple biomarkers Commercially available kits available | | | |
| Whole genome         |                    | Covers most coding and noncoding regions, including large introns | | | |
| Hybridization capture|                    | Highly scalable Theoretically capable of detecting all classes of actionable mutations, including fusions with unknown partners | | Requires more input DNA than amplicon methods Complex library preparation processes Large introns of NTRK2 and NTRK3 can prove problematic | |
| Amplicon (target enrichment by PCR) | FFPE, snap-frozen, or stabilized tissue | RNA is more labile than DNA Only transcriptionally active fusions detected Allows in-frame vs out-of-frame confirmation for all fusions Commercially available kits are designed to cover all potentially oncogenic actionable fusions, without knowledge of 5’ partners or breakpoints Detection of transcripts expressed at low levels may be challenging | | Requires complex multiplex amplicon design For gene fusions, 5’ and 3’ partners must be defined and potential breakpoint regions must be covered by amplicons | |
| RNA-based NGS Hybridization capture | Highly scalable Can detect unknown | | | Requires more input RNA than amplicon methods |
predominantly single analyte methods, meaning they investigate only one biomarker at a time. Although these may be appropriate solutions for the short-term (and in selected clinical scenarios), they are likely to be eclipsed by broader testing methods. As treatment decisions in oncology are increasingly driven by consideration of a growing number of genomic biomarkers, comprehensive approaches that are inherently multiplexable and that can detect multiple types of genomic alterations (eg, NGS analysis of both DNA and RNA), are likely to become the dominant diagnostic modality in advanced cancers. This would thereby allow for the routine identification of patients with tumors harboring NTRK gene fusions without requiring the pathologist to specifically test for such rearrangements.

**Immunohistochemistry**

Immunohistochemistry to assess the level of protein expression in tumor cells is a fast, cost-effective, widely used technique in diagnostic pathology. Its utility is, however, restricted by the limitation that assays only address one clinical question at a time, with each assay also requiring the use of two tissue sections. The identification of tumors harboring particular gene fusions by immunohistochemistry rests on the premise that the chromosomal rearrangements result in an up-regulation of fusion gene expression in the tumor cell beyond that seen in cells not harboring such genomic changes. In this context, immunohistochemistry has been shown to be an effective approach with respect to identifying patients with NSCLC whose tumors harbor ALK gene rearrangements, and who are, therefore, suitable candidates for ALK tyrosine kinase receptor inhibitor therapy. Indeed, current NSCLC clinical guidelines indicate that immunohistochemistry is an equivalent alternative to FISH for ALK testing in relation to informing treatment selection.103

Regarding the identification of patients with TRK fusion cancer, recently published studies have shown that immunohistochemistry might be an effective diagnostic approach in certain indications that are not yet routinely subjected to molecular genomic profiling, and that have a low incidence of tumors harboring NTRK gene fusions, as a way of selecting patients for subsequent molecular testing. Ideally, the antibody used for such a test would

| Analytical technique | Sample requirements | Preanalytical considerations | Turnaround time | Advantages | Disadvantages |
|----------------------|---------------------|------------------------------|-----------------|------------|---------------|
| Amplicon (anchored multiplex RT-PCR) | DNA plus RNA NGS FFPE, snap-frozen, or stabilized tissue | For FFPE tissue, sample age might affect DNA quality and sequencing read quality | | | |

**Table 3 (continued)**

CNV, copy number variation; FFPE, formalin fixed, paraffin embedded; FISH, fluorescence in situ hybridization; IHC, immunohistochemistry; indel, small insertion and deletion; NGS, next-generation sequencing; NT-3, neurotrophin-3; SNV, single-nucleotide variant; TRK, tyrosine kinase; TRKC, NT-3 growth factor receptor.
detect all three TRK proteins (pan-TRK antibody) and bind a C-terminal epitope in the tyrosine kinase domain, such that all translated fusion transcripts may be detected with one assay.

Two studies have suggested that immunohistochemistry using the pan-TRK rabbit monoclonal antibody, EPR17341 (Abcam, Cambridge, UK), might be an effective way to identify tumors harboring NTRK gene fusions.32,38 This antibody recognizes an undisclosed epitope close to the C-terminus of the three TRK proteins, a region that would be expected to be present in functional TRK fusion proteins. Hechtman et al32 investigated 23 tumors in which NTRK gene fusions had been detected by a DNA-based NGS assay, MSK-IMPACT. Subsequent analysis using an RNA-based fusion assay (ArcherDx, Boulder, CO) did not identify a fusion transcript in two of these cases (five not tested). Of the remaining 21 tumors, 20 were found to be positive for TRK protein by pan-TRK immunohistochemistry. In all positive cases, staining was cytoplasmic, with certain tumors additionally staining in the plasma membrane, nuclear membrane, or nucleus. The one discordant case was a mismatch repair–deficient colorectal cancer harboring an ETV6-NTRK3 fusion, which was positive in the fusion assay but negative for protein expression by immunohistochemistry. An additional limited series of 20 consecutive cases, which included tumors that did not harbor NTRK gene fusions, according to the ArcherDx assay, were all negative by immunohistochemistry. This indicated a sensitivity and specificity for pan-TRK immunohistochemistry in this series of tumors of 95% and 100%, respectively. However, given the small number of NTRK fusion-negative cases examined, and the selection bias used, a degree of caution should be exercised in relation to the specificity estimate.

Rudzinski et al38 investigated the immunohistochemical performance of the pan-TRK antibody EPR17341 and the TRKA rabbit monoclonal antibody, EP1058Y (Abcam), in 30 pediatric mesenchymal tumors harboring an NTRK gene fusion and 48 not harboring, or not expected to harbor, an NTRK gene fusion. Of 30 tumors harboring NTRK gene rearrangements, 29 were positive by pan-TRK immunohistochemistry, giving a sensitivity of 97%. In most cases, staining was cytoplasmic, although in some it was cytoplasmic and/or nuclear. Of the 48 cases not harboring or not expected to harbor an NTRK gene fusion, all but one, which had weak cytoplasmic staining, were negative for TRK expression, giving a specificity of 98%. As various tissue types and tumors may express TRK proteins in the absence of NTRK gene fusions, this specificity estimate should only be considered as relating to the mesenchymal tumor types included in this study. In a parallel analysis with the TRKA antibody, EP1058Y, which recognizes an undisclosed sequence around tyrosine 791 of human TRKA, 26 of 26 tested cases (100%) harboring NTRK gene fusions, including tumors with NTRK2 and NTRK3 gene fusions, showed positive cytoplasmic and/or nuclear staining.
However, 14 of 49 negative control samples also had weak staining and 4 had moderate staining, giving a specificity for the TRKA antibody of only 63%. Positive staining with this antibody may, therefore, have limited potential as a biomarker in relation to the specific identification of tumors harboring NTRK gene fusions.

However, two further studies using the EPR17341 pan-TRK antibody show that the interpretation of immunohistochemistry data may be more challenging than previously appreciated. Gatalica et al. analyzed 11,502 formalin-fixed, paraffin-embedded tumor samples of various cancer types for the presence of gene fusions using an ArcherDx fusion assay. Thirty-one cases (0.27%) were deemed to harbor NTRK gene fusions, and of the 28 assessed by pan-TRK immunohistochemistry, 21 were scored positive (≥1% of tumor cells staining at any intensity above background), giving a sensitivity of only 75%. In particular, 45% of tumors with NTRK3 fusions were deemed negative in this analysis by immunohistochemistry. Of 4108 tumors not found to be harboring NTRK gene fusions, 3942 were also scored negative by immunohistochemistry, giving a specificity of 96%. Furthermore, a second large immunohistochemistry study of 3574 tumor samples, using the same antibody on an automated platform, showed that pan-TRK staining varied widely in both intensity and in the percentage of tumor cells staining across different tumor types.

Using a cutoff of >1% of tumor cells stained, 288 tumor (8%) were scored as positive for TRK expression, but only 12 of 139 of these tumors (9%) with tissue available for further testing were deemed to harbor NTRK gene fusions by dual in situ hybridization. It is likely that the remaining 127 tumor (91%) expressed TRK proteins either as part of the normal expression profile of the tumor cell progenitor or else as a consequence of other unidentified tumor-associated alterations. Breaking these data down according to tumor type suggested that, in relation to identifying TRK fusion cancer, pan-TRK immunohistochemistry may be a useful initial enrichment tool in certain indications, such as colorectal cancer, but not in others, such as neuroendocrine tumors, where approximately 50% of lesions appeared to express TRK proteins in the absence of NTRK gene fusions. Therefore, particularly in tumors with a low prevalence of NTRK gene fusion events or cell types with baseline expression of TRK proteins, immunohistochemistry positivity should be used with caution as a surrogate biomarker for NTRK gene fusions, and positive cases should be further investigated by reflex NGS or molecular cytogenetic testing. This highlights the ongoing need for more comprehensive studies to determine the sensitivity and, in particular, the indication-specific specificity of immunohistochemistry in this context. Such analyses would allow for a comprehensive assessment of the role of immunohistochemistry in routine diagnostic algorithms for the identification of tumors harboring NTRK gene fusions.

As additional data are accumulated regarding the performance of immunohistochemistry approaches to screening for NTRK gene fusions, it is important to pay careful attention in particular to assay sensitivity, as even small compromises in this parameter could result in a significant underdetection rate for this generally rare genomic event. The ability to comprehensively assess sensitivity is limited by the small number of cases available for comparative analyses. However, the previously established models of immunohistochemistry for ALK and ROS1 alterations rely heavily on excellent sensitivity, even if specificity (in the case of ROS1) is less than ideal.

**Fluorescence in Situ Hybridization**

Interphase FISH assays, which may be performed on formalin-fixed, paraffin-embedded tumor samples, have now become established as standard diagnostic tools in pathology laboratories in relation to the detection of chromosomal alterations of therapeutic significance. The use of dual-color FISH probes allows for the detection of gene fusion events in clinical samples by either signal fusion or break-apart assays. If a fusion between two particular genes is characteristic in a tumor type, then a high-specificity fusion design may be appropriate. In situations in which there are a range of possible 5' fusion partners, and perhaps in which all possible fusion partners have not yet been characterized, a break-apart FISH assay, based on the proto-oncogene partner, may be more appropriate. However, several factors can influence the sensitivity and specificity of break-apart assays, including how far apart the probes are located genomically on the target chromosome, whether common fusion partners are intrachromosomal, and the threshold chosen to determine positivity. In particular, the finding that intrachromosomal rearrangements of NTRK1 are common raises the strong possibility of a propensity for false-negative FISH results in events in which such rearrangement/inversion is concomitant with an interstitial deletion, as has been described for both ALK and ROS1. In addition, much like immunohistochemistry, FISH assays are essentially limited to single analytes and to comprehensively evaluate the fusion status of all three NTRK genes by FISH, three separate assays would be required, unless multicolor approaches were developed.

Both fusion and break-apart assays may be used to identify patients with TRK fusion cancer. In particular, suspected cases of infantile fibrosarcoma, congenital mesoblastic nephroma, and secretory carcinoma of the breast and salivary gland may be routinely assessed by FISH for the ETV6-NTRK3 fusion to support histopathological diagnosis. Given the prevalence of this fusion in these tumors, such assessments have in some instances been based on ETV6 break-apart FISH. However, the identification of variant fusions in these tumor types suggests that even in such restricted indications, this diagnostic approach would result in the failure to identify all patients with tumors harboring NTRK gene fusions.

**RT-PCR**

There are two main RT-PCR approaches that may be used to identify tumors with NTRK gene fusions. First, in tumor
types in which most fusions are between a particular 5′ partner and a particular NTRK gene, and in which the exons included in the gene fusion from the 5′ and 3′ partners tend to be highly recurrent, then an effective diagnostic/monitoring strategy can be provided by conventional RT-PCR, such as is common with evaluation of the BCR-ABL1 fusion in chronic myelogenous leukemia.\(^{107}\) In this case, the forward primer would be derived from an exon sequence of the 5′ partner upstream of the expected breakpoint and the reverse primer from an NTRK exon downstream of the expected breakpoint. If the target RNA is to be derived from formalin-fixed, paraffin-embedded tissue, then primers should be specific for exons immediately flanking the expected breakpoint, to allow for a minimal sized RT-PCR amplicon. Such RT-PCR approaches have been shown to be effective in detecting ETV6-NTRK3 fusion transcripts in infantile fibrosarcoma, secretory breast cancer, and congenital mesoblastic nephroma.\(^{58,60,61,64}\) However, as a general principal in this context for RT-PCR assays, optimization of preanalytical variables (ie, warm/cold ischemic time, fixation conditions, and RNA extraction)\(^{108}\) and inclusion of robust internal controls for the sample-specific quality of extracted RNA should be performed. Sensitivity of RT-PCR will be affected in samples with nonamplifiable or degraded RNA.\(^{109}\) In addition, this approach would miss variant fusions in these and other tumor types, which is of particular relevance given the large and growing number of possible 5′ fusion partners and the variability of breakpoints so far identified.

An alternative RT-PCR approach that may be used to infer the presence of an NTRK gene fusion in a clinical sample is based on examining the ratio of expression of 3′ and 5′ amplicons of NTRK gene transcripts. In such assays, RT-PCR primer pairs can be sited upstream (5′ amplicon) and downstream (3′ amplicon) of the expected position of a gene fusion breakpoint. In a situation in which the 3′ fusion partner gene is not expressed in the normal tissue, or is expressed only at low levels, and fusion transcript expression is driven at a higher level by the promoter of the 5′ partner, an imbalance favoring the 3′ over the 5′ NTRK RT-PCR amplicon can be detected at which a gene fusion event has occurred, as demonstrated for tumors harboring ALK gene fusions in NSCLC.\(^{110}\) This type of approach, albeit using an assay examining the ratio between a short 3′ RT-PCR amplicon downstream of the expected breakpoint region and a long 5′ to 3′ amplicon of NTRK1 spanning the expected breakpoint region, was used by Brzezińska et al\(^{11}\) to infer the presence of NTRK1 gene fusions in 4 of 33 papillary thyroid cancers (12%).

**Next-Generation Sequencing**

The massively parallel sequencing capability provided by NGS now allows for the sequence-based detection of somatic tumor alterations in routine clinical care in a highly multiplexed manner, on limited tissue. As new targeted therapy and immunotherapy agents emerge, the list of oncogenic alterations and biomarkers across tumor types is growing, requiring the generation of increasing amounts of information from the available clinical material. NGS assays can be based on either the analysis of DNA or RNA and can survey the entire genome, exome, or transcriptome, or can be tailored to panels of genes relevant to treatment selection decisions or prognosis.\(^{112}\) Targeted approaches are designed to enrich clinical samples for the selected genes, and thereby improve sequencing depth and sensitivity. Alternatively, the entire genome can be interrogated. Although whole genome sequencing assays might in theory detect all gene fusion events, they may have lower analytical sensitivity due to decreased sequencing depth compared with targeted panels and require more analytical and computational resources.

DNA-based NGS assays can be designed to cover a targeted panel of genes enriched by either a PCR amplicon— or hybridization capture—based approach. Although amplicon-based enrichment strategies generally require less tissue, for fusion detection, the 5′ partner gene and exact breakpoints must be known for successful application of this method. Hybridization capture approaches generally require more input DNA but allow for the detection of novel fusion partners. Furthermore, custom-designed capture probes can be designed to interrogate tumors for all relevant types of genomic alterations, with covered intronic regions providing the ability to call structural variants with high sensitivity.\(^{31,113}\) An important caveat to such an approach is the localization of common fusion breakpoints within large intronic regions containing high numbers of repetitive elements, which make capture and sequencing technically infeasible.\(^{114}\) This is the case for relevant intronic regions of NTRK2 and NTRK3 and may lead to decreased sensitivity of capture-based DNA NGS assays for the detection of fusions within these genes.

An alternative approach for the detection of gene fusions in clinical samples is provided by RNA-based NGS. The benefit of an RNA-based assay is that the intronic regions causing technical issues in DNA-based NGS have been removed by splicing, allowing for more straightforward capture and/or amplification of fused regions, particularly those involving NTRK genes. RNA sequencing of the whole transcriptome can provide an overview of all transcribed gene fusions and expressed variation (allele-specific expression). As for DNA-based assays, targeted RNA sequencing of cancer-relevant genes can be achieved using hybridization capture— or amplicon-based technologies, with the benefit of reducing the sequencing requirement and simplifying the bioinformatic analysis. One such targeted amplicon-based approach is based on anchored multiplex PCR,\(^ {20}\) whereby target enrichment is achieved through the use of nested, unidirectional gene-specific primers, allowing for the detection of gene fusion transcripts without prior knowledge of 5′ fusion partners and breakpoints.\(^ {117}\) As with targeted DNA panels, a hybridization capture— or amplicon-based approach can also be used in RNA panels with identical benefits/limitations to those discussed above. The major disadvantage of RNA-based approaches is the highly variable, and sometimes poor quality, of RNA extracted.
from formalin-fixed, paraffin-embedded tissues. This disadvantage is notable and requires stringent assay internal controls to evaluate when results from a sample should be considered uninformative, rather than negative.

These complementary strengths and weaknesses between DNA- and RNA-based NGS demonstrate the specific need to carefully consider a multilayered approach to testing, and further highlight the role of molecular professionals in understanding the limitations of testing as applied to an individual patient sample, and how this expertise can be used to guide additional testing. As decisions on cancer care become increasingly tied to specific genomic biomarker status, however, a multiplexed and comprehensive analytical tool, such as NGS, is likely to become the preferred diagnostic modality for tumor genomic testing to obtain the most biomarker information from the least amount of tissue. Such a comprehensive NGS analysis of genomic changes in tumors can perhaps most effectively be achieved by the use of an approach in which both DNA and RNA are analyzed. This should allow for the detection of single-nucleotide variants, small insertions or deletions, copy number variations, and chromosomal rearrangements. In addition, if the appropriate regions are included in the assay design, information on microsatellite instability and other developing biomarkers can also be derived from the DNA portion of the assay. The inclusion of RNA in such assays is particularly important for the detection of NTRK gene fusions as it mitigates the inherent difficulties involved in designing capture probes to cover the large repetitive element—rich introns of NTRK2 and NTRK3. A hybridization capture or anchored multiplexed PCR approach, based on RNA analysis, also allows for the detection of unknown or unexpected fusion partners and variable breakpoints. This is particularly important for NTRK gene fusions that have proved to be promiscuous in terms of 5' partners and unpredictable in terms of recurrent breakpoints. The combined approach of analyzing both DNA and RNA (using a method that can detect unknown fusions, such as hybridization capture or anchored multiplexed PCR), either in tandem or in a reflexive manner, can therefore be considered a broad-based NGS screen for the identification of actionable drivers.

Plasma-Based NGS

If invasive tissue biopsy procedures are infeasible for patients, recent studies have indicated that plasma-based cancer genotyping through NGS of circulating cell-free tumor DNA (cfDNA) is an accurate and clinically effective alternative. However, although cfDNA assays may have high diagnostic specificity, in general, they have a more modest diagnostic sensitivity. This is a result of heterogeneous cfDNA shedding in patients with solid tumors (ie, an inherent biological challenge) as well as incomplete intron coverage by the most widely available commercial assays, which target cfDNA and do not currently evaluate circulating (c)RNA (ie, an assay design decision). Similar to the identification of T790M mutations in the setting of epidermal growth factor receptor tyrosine kinase inhibitor–treated NSCLC, it may be that cfDNA assays have a possible role in the monitoring of acquired NTRK tyrosine kinase inhibitor resistance mutations. Finally, circulating RNA is another potential specimen type to use in diagnostic tests. Although RNA is less stable outside the cellular membrane than DNA, cell-free membrane-bound (exosome-associated) RNA can be extracted from whole blood and assays (eg, droplet digital RT-PCR can be used to identify fusion transcripts).

Diagnostic Algorithm for the Detection of NTRK Gene Fusions

Given the variability in the incidence of NTRK gene fusions in different indications as well as the pragmatic requirement to assess a wide variety of other potential drivers in tumors, it is unlikely that one single diagnostic strategy will be optimal in terms of the efficient identification of patients with TRK fusion cancer. Although testing all advanced solid tumors with a broad NGS assay inclusive of NTRK genes may be the ultimate ideal, workflow and reimbursement realities currently dictate a more nuanced approach to optimize resource use. We have, therefore, proposed a diagnostic algorithm for solid tumors that might be used for the routine identification of such patients (Figure 3). We propose to categorize tumors initially into three broad, biologically defined groups, with a different diagnostic approach applied to each: tumors with a high incidence of characteristic NTRK gene fusions, tumors with a high incidence of TRK expression and a low incidence of NTRK gene fusions, and tumors with a low incidence of TRK expression and a low incidence of NTRK gene fusions.

Tumors with a high incidence of specific NTRK gene fusions include infantile fibrosarcoma, secretory carcinomas of the breast and salivary gland, and cellular and mixed congenital mesoblastic nephroma. We propose that tumors in these indications should be routinely analyzed for NTRK gene fusions, as per standard practice at the institution, typically by NGS, break-apart FISH, dual-gene FISH, or immunohistochemistry. Minimally, this should be based on screening for ETV6-NTRK3 fusions. If tumors are negative in FISH assays, they should be subjected to additional testing, ideally broad-based NGS, which includes both a DNA and an RNA component.

Tumor types with a high incidence of TRK expression, as determined by pan-TRK immunohistochemistry, and a low incidence of NTRK gene fusions include neuroendocrine tumors, some soft tissue sarcomas, and gastrointestinal stromal tumors. In these tumors, an enrichment approach using pan-TRK immunohistochemistry would not be effective given that many tumors express TRK proteins in the absence of NTRK gene fusions. For neuroendocrine tumors and soft tissue sarcomas, we therefore propose immediate testing using broad-based NGS analysis, inclusive of RNA and DNA. For gastrointestinal stromal tumors, the
first step should be KIT/PDGFRα genotyping, with broad-based NGS analysis performed on tumors that do not harbor actionable mutations in these genes.

The final category, tumors with a low incidence of TRK expression, as determined by pan-TRK immunohistochemistry and a low incidence of NTRK gene fusions, will include most common malignancies, including NSCLC, breast cancer, glioma, melanoma, and colorectal cancer. In certain indications, and we would anticipate increasingly in the future, NGS will be routinely requested as part of the diagnostic workup (eg, NSCLC), in relation to either broad-based or targeted analyses. If such analyses are narrowly targeted, with the aim of identifying standard actionable mutations, such as EGFR, and BRAF point mutations or ALK gene fusions, tumors wild type in these assays should be referred for broad-based NGS. In indications for which NGS is not routinely ordered, pan-TRK immunohistochemistry might be performed as an enrichment strategy to select tumors for broad-based NGS. Tumors positive for TRK expression could then be subjected to broad-based NGS to identify the subset harboring NTRK gene fusions. Given the heretofore poorly defined sensitivity and indication-specific specificity of pan-TRK immunohistochemistry, its widespread use as a screening tool cannot currently be advocated. However, in indications in which no molecular testing of the tumor will generally be done (eg, cholangiocarcinoma, thyroid cancer, or pancreatic cancer), the use of pan-TRK immunohistochemistry may identify tumors appropriate for NGS, albeit not with perfect sensitivity.

**Summary**

NTRK gene fusions occur in many different tumor types. In certain rare tumors, they are present in most lesions, whereas in common cancers, the incidence may be 0.1% to 2% of tumors. The pan-TRK inhibitor larotrectinib is now approved as a tissue agnostic treatment for patients with TRK fusion cancer and entrectinib is in active clinical trials. Several other multikinase inhibitors, which target TRK proteins, are also in development. The marked and durable responses achieved with TRK tyrosine kinase inhibitors in patients with TRK fusion cancer, regardless of patient age and fusion type, highlight the clinical importance of the routine identification of tumors harboring NTRK gene fusions. We have, therefore, proposed a diagnostic algorithm to facilitate the identification of patients with TRK fusion cancer, which accounts for the widely varying frequencies by tumor histology and the underlying prevalence of TRK expression in the absence of NTRK gene fusions. Our proposal is based on a combination of FISH, NGS, and immunohistochemistry assays.

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