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REVIEW

Detection of Tumor *NTRK* Gene Fusions to Identify Patients Who May Benefit from Tyrosine Kinase (TRK) Inhibitor Therapy

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Chromosomal rearrangements involving the *NTRK1*, *NTRK2*, and *NTRK3* genes (*NTRK* genes), which encode the high-affinity nerve growth factor receptor (TRKA), brain-derived neurotrophic factor/neurotrophin-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. *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, *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 *NTRK* genes and the nature and incidence of *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 *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 *NTRK* gene fusions and is based on a combination of fluorescence *in situ* hybridization, next-generation sequencing, and immunohistochemistry assays. *(J Mol Diagn 2019, 21: 553—571; https://doi.org/10.1016/j.jmoldx.2019.03.008)*

Chromosomal rearrangements involving the neurotrophic receptor tyrosine kinase 1, 2, and 3 genes, *NTRK1*, *NTRK2*, and *NTRK3*, which lead to functional gene fusions, have been found to act as oncogenic drivers in a broad range of tumor types.[1] Furthermore, such *NTRK* gene fusions have now been shown to be actionable genomic events, predicting response to therapy directed against TRK kinases, making their routine detection an evolving clinical priority.[2]

The *NTRK1*, *NTRK2*, and *NTRK3* genes encode related single-pass transmembrane receptor tyrosine kinases, high-affinity nerve growth factor receptor (TRKA), BDNF/NT-3 growth factor receptor (TRKB), and NT-3 growth factor receptor (TRKC), respectively, that function as high-affinity receptors for neurotrophins. Together, these proteins regulate the development, maintenance, and function of neural tissues.[3] There is a high degree of homology between the TRK proteins, with each having an extracellular region, including leucine-rich repeats, Ig-like C2-type 1 and Ig-like C2-type 2 domains, a transmembrane region, and an intracellular region, including 10 evolutionarily conserved tyrosines and a tyrosine kinase domain.[4]

The *NTRK* genes show complex alternative splicing patterns in normal and tumor tissues,[5–8] with multiple transcripts

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encoded by different exon combinations currently recorded in the Ensembl genomic database.[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).[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.[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,[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.[13] Subsequently, the closely related *NTRK2* and *NTRK3* genes were characterized (Figure 1).[14,15]

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

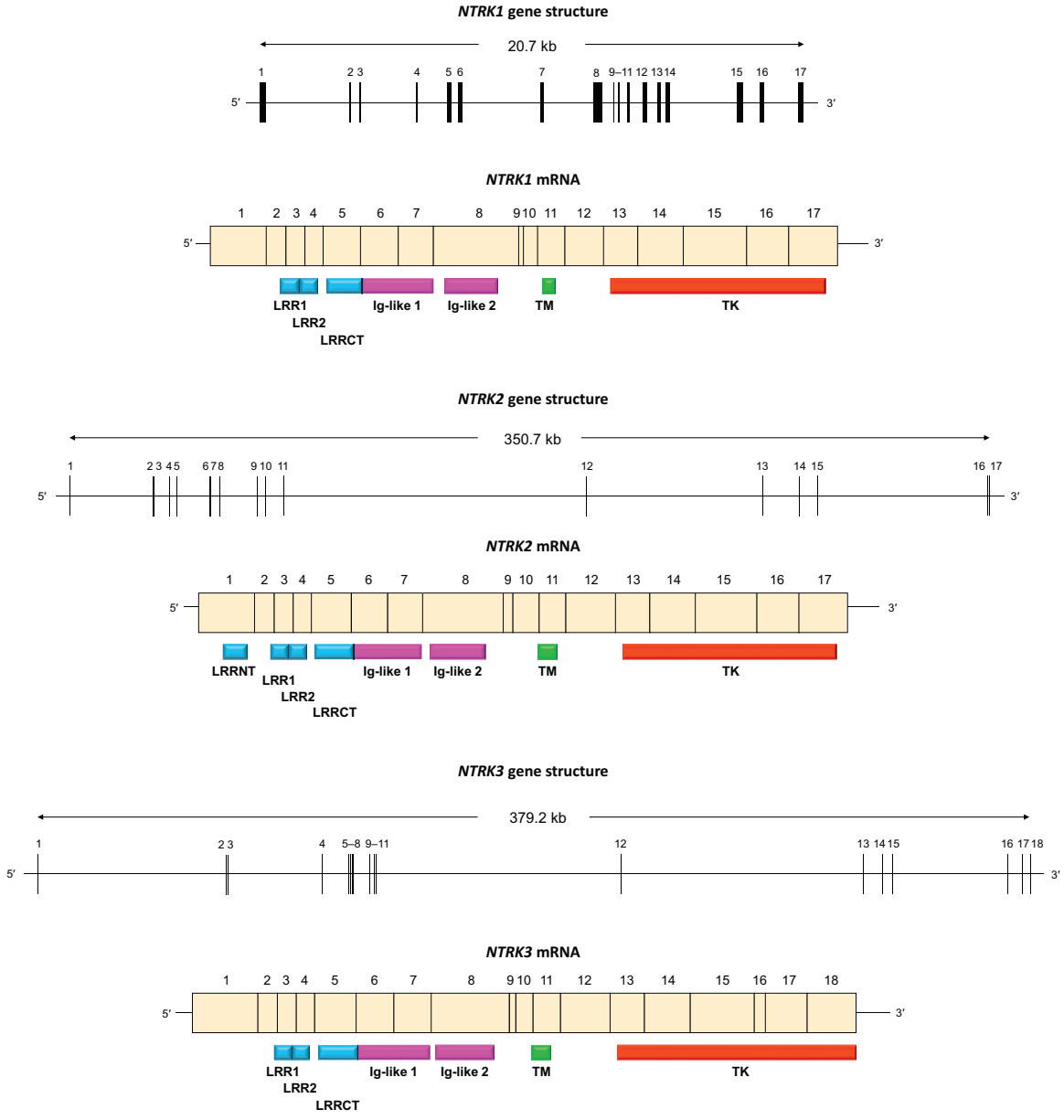

Figure 1 Genomic structure of *NTRK1*, *NTRK2*, and *NTRK3*, showing exons encoding the canonical isoforms described in the UniProt Knowledgebase. Regions of the corresponding mRNAs encoding function domains are marked. Introns are to scale, but for visualization purposes, exons of *NTRK2* and *NTRK3* 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.

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

NTRK gene	Chromosomal location	Characterized transcripts*	Canonical protein isoform†						
			Transcript‡	CCDS ID§	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	CCDS35050.1	TRKB	Q16620	822	17	350.7
NTRK3	15q25.3	21	NM_001012338.2	CCDS32322.1	TRKC	Q16288	839	18	379.2

*As reported in the Ensembl database (*https://www.ensembl.org/index.html*, last accessed March 4, 2019).
†As described in the UniProt Knowledgebase (*https://www.uniprot.org*, last accessed March 4, 2019).
‡National Center for Biotechnology Information (*https://www.ncbi.nlm.nih.gov*, last accessed March 4, 2019).
§CCDS database (*https://www.ncbi.nlm.nih.gov/projects/CCDS/CcdsBrowse.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.

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.[16,17] 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 give 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.[70] 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.[71] 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.[60,61] However, more recently, *LMNA-NTRK1* and *EML4-NTRK3* fusions have also been described in infantile fibrosarcoma.[53,72,73]

Table 2 *NTRK* Gene Fusions Identified in Human Tumors

NTRK gene	Fusion partner and chromosomal localization*		Tumor type
NTRK1 (1q23.1)	***AFAP1***	4p16.1	Glioblastoma[18]
	AMOTL2	3q22.2	Lung cancer[19]
	ARHGEF2	1q22	Glioblastoma[20]
	BCAN	1q23.1	Glioma,[21,22] glioneuronal tumor,[23] pilocytic astrocytoma[24]
	CEL	9q34.13	Pancreatic cancer[25]
	CD74	5q33.1	Lung cancer[26]
	CHTOP	1q21.3	Glioblastoma[20]
	CGN	1q21.3	Breast cancer[27]
	COP1	1q25.1—q25.2	Large-cell neuroendocrine cancer[28]
	CTRC	1p36.21	Pancreatic cancer[2]
	DDR2	1q23.3	Melanoma[29]
	DIAPH1	5q31.3	Thyroid cancer‡
	EPHB2	1p36.12	Not specified[30]
	EPS15	1p32.3	Lung cancer‡
	GATAD2B	1q21.3	Breast cancer[27]
	GON4L	1q22	Melanoma[2,29]
	GRIPAP1	Xp11.23	Lung cancer[31]
	GSN	9q33.2	Not specified[30]
	IRF2BP2	1q42.3	Lung cancer,[2,32] thyroid cancer,[2,33,34] prostate cancer[19]
	LMNA	1q22	Appendiceal cancer,[2,32] breast cancer,[27] cholangiocarcinoma,[2] colorectal cancer,[2,23,32,35] gallbladder carcinoma,[32] soft tissue sarcoma,[2,32] Spitzoid neoplasm,[36] uterine sarcoma[37]
	LRRC71	1q23.1	Uterine endometrial cancer[31]
	MDM4	1q32.1	Breast cancer[27]
	MEF2D	1q22	Glioma[22]
	MIR548F1	10q21.1	Pediatric mesenchymal tumor[38]
	MPRIP	17p11.2	Lung cancer[17,26]
	MRPL24	1q23.1	Lung cancer†
	NFASC	1q32.1	Glioblastoma[21,33]
	P2RY8	Xp22.33 and Yp11.3	Lung cancer[32]
	PDE4DIP	1q21.2	Soft tissue sarcoma[2]
	PEAR1	1q23.1	Breast cancer[27]
	PIP5K1A	1q21.3	Neuroendocrine tumor[39]
	PLEKHA6	1q32.1	Colon cancer[2]
	PPL	16p13.3	Thyroid carcinoma[2,20]
	PRDX1	1p34.1	Lung cancer[19]
	RABGAP1L	1q25.1	Intrahepatic cholangiocarcinoma[40]
	SCYL3	1q24.2	Colorectal cancer[41]
	SQSTM1	5q35.3	Infantile fibrosarcoma,[2,42] lung cancer,[23,43] thyroid cancer[33]
	SSBP2	5q14.1	Thyroid cancer[33]
	TFG	3q12.2	Thyroid cancer[33,44]
	TP53	17p13.1	Spitzoid neoplasm[36]
	TPM3	1q21.3	Breast cancer,[2,27] cervical cancer,[22] cholangiocarcinoma,[2] colorectal cancer,[2,19,22,32,45] glioma,[22,46] infantile fibrosarcoma,[2] lung cancer,[20,22,32] soft tissue sarcoma,[2,22,33] thyroid cancer,[33,47] uterine sarcoma[37]
	TPR	1q31.1	Lung cancer,[2] thyroid cancer,[44] uterine sarcoma,[37] pediatric mesenchymal tumor[38]
	TRIM63	1p36.11	Melanoma[2,29,32]
NTRK2 (9q21.33)	***AFAP1***	4p16.1	Glioma[33]
	AGBL4	1p33	Glioma[46]
	BCR	22q11.23	Glioma[22,32]
	DAB2IP	9q33.2	Colorectal cancer†
	ETV6	12p13.2	Acute myeloid leukemia[48]
	GKAP1	9q21.32	Glioma[22]
	GNAQ	9q21.2	Bone sarcoma‡

(*table continues*)

Table 2 (*continued*)

NTRK gene	Fusion partner and chromosomal localization*		Tumor type
	KCTD8	4p13	Glioma[22,24]
	NACC2	9q34.3	Astrocytoma[49]
	NAV1	1q32.1	Not specified[30]
	NOS1AP	1q23.3	Anaplastic astrocytoma,[24] glioma[22]
	PAN3	13q12.2	Squamous cell cancer of the head and neck[33]
	PRKAR2A	3p21.31	Glioma[22]
	QKI	6q26	Astrocytoma[49,50]
	RBPMS	8p12	Soft tissue sarcoma‡
	SQSTM1	5q35.3	Glioma,[22,24,33] lung cancer[22]
	SLMAP	3p14.3	Not specified[30]
	STRN	2p22.2	Soft tissue sarcoma[2]
	TBC1D2	9q22.33	Glioma[22,24]
	TLE4	9q21.31	Ganglioglioma[51]
	TRAF2	9q34.3	Melanoma[29,32]
	TRIM24	7q33-q34	Lung cancer[33]
	VCL	10q22.2	Glioma[46]
	VCAN	5q14.2-q14.3	Glioma[22,24]
NTRK3 (15q25.3)	***AFAP1***	4p16.1	Glioblastoma[32]
	AKAP13	15q25.3	Glioma,[52] lung cancer[19]
	BTBD1	15q25.2	Glioma[46]
	EML4	2p21	Congenital mesoblastic nephroma,[53] glioma,[22,24,32] infantile fibrosarcoma,[53] thyroid cancer[34]
	ETV6	12p13.2	Acute lymphoblastic leukemia,[54] acute myeloid leukemia,[55] breast cancer,[27,33] colorectal cancer,[33] congenital mesoblastic nephroma,[56–58] gastrointestinal tract stromal tumor,[2,59] glioma,[22,46] infantile fibrosarcoma,[2,60,61] inflammatory myofibroblastic tumor,[62] lung cancer,[2,19,22] melanoma,[2,33] neuroendocrine cancer,[63] secretory breast cancer,[22,32,64] secretory carcinoma of salivary gland,[2,23,32,65] sinonasal adenocarcinoma,[22] soft tissue sarcoma,[2] Spitzoid neoplasm,[66] thyroid cancer[2,22,33]
	FAT1	4q35.2	Not specified[30]
	HNRNPA2B1	7p15.2	Multiple myeloma[48]
	LYN	8q12.1	Squamous cell cancer of the head and neck[33]
	MYH9	22q12.3	Spitzoid neoplasm[66]
	MYO5A	15q21.2	Spitzoid neoplasm[66,67]
	RBPMS	8p12	Thyroid cancer,[33] uterine sarcoma[37]
	SPECC1L	22q11.23	Uterine sarcoma[22]
	SQSTM1	5q35.3	Thyroid cancer[34,68]
	STRN	2p22.2	Adult fibrosarcoma[69]
	STRN3	14q12	Adult fibrosarcoma[69]
	TFG	3q12.2	Fibrous tumor[42]
	TPM4	19p13.12-p13.11	Soft tissue sarcoma[2,32]
	UBE2R2	9p13.3	Multiple myeloma[48]
	VIM	10p13	Thyroid cancer[22]
	VPS18	15q15.1	Not specified[30]
	ZNF710	15q26.1	Glioblastoma[32]

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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.[39]

†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.

Secretory Carcinomas

Secretory breast carcinoma is a rare distinct subtype of infiltrating ductal carcinoma.[74] More than 90% of cases harbor *ETV6-NTRK3* gene fusions.[64,75,76] A morphologically and immunohistochemically related entity in the salivary gland was described by Skálová et al,[65] and similarly harbors *ETV6-NTRK3* fusions in >90% of cases. Indeed, NGS analysis suggested that secretory breast carcinomas are genetically

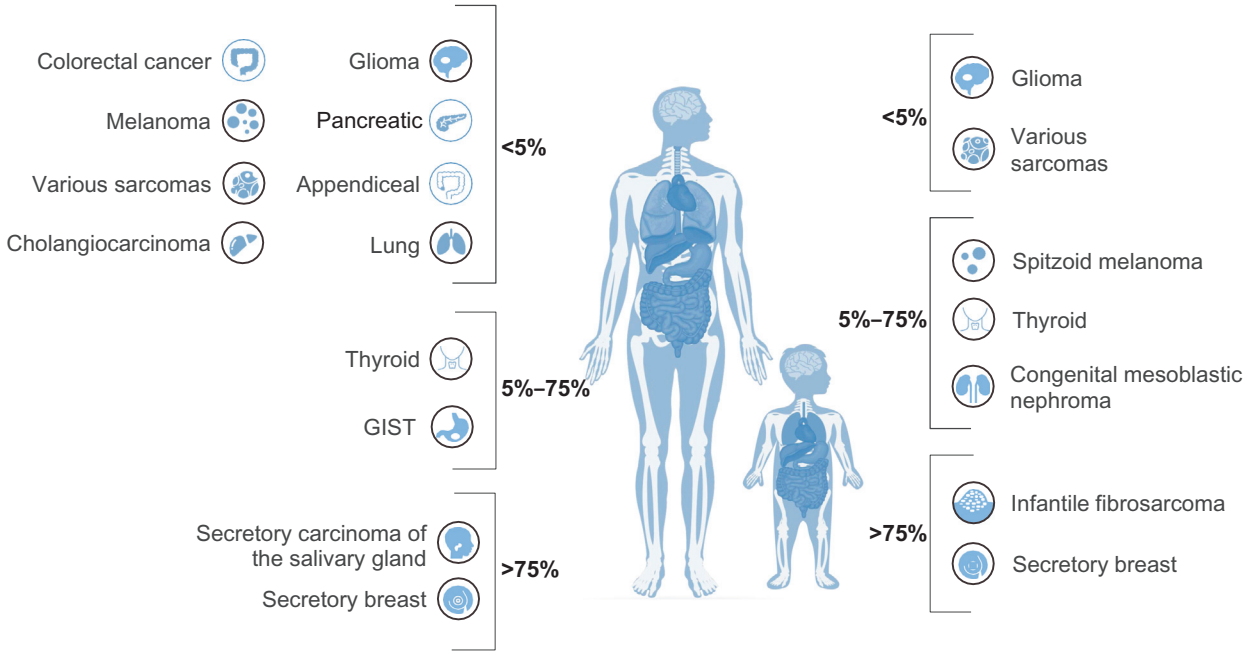

Figure 2 Spectrum of adult and pediatric tumors harboring *NTRK* gene fusions. GIST, gastrointestinal stromal tumor.

more similar to secretory carcinomas of the salivary gland than they are to other primary breast cancers.[75] Recently, it has been reported that a subset of salivary gland carcinomas harbor *ETV6-RET* gene fusions.[77] Cases of secretory carcinoma of the skin and thyroid harboring *ETV6-NTRK3* fusions have also been described.[78,79]

Congenital Mesoblastic Nephroma

This rare spindle cell tumor of the kidney mainly occurs in newborns or young infants. *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).[56,58] The *ETV6-NTRK3* fusion is typically accompanied by trisomy 11.[58,80] However, variant *NTRK* gene fusions may also be found in such tumors.[53]

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%).[81] One series of 27 cases demonstrated *NTRK* gene fusions in 26% of pediatric papillary thyroid carcinomas.[82] *NTRK* gene fusions have also been found less commonly (<10%) in thyroid cancers in adults.[22]

Brain Tumors

In an analysis of 112 pediatric high-grade gliomas, Wu et al[46] reported that eight tumors (7%) harbored one of five different *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%).[46] In a large study of 390 predominantly adult [373 (98%)] gliomas, *NTRK* gene fusions were identified in eight tumors (2%), six of which involved *NTRK2*.[24]

Common Cancers

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 *NTRK* gene fusions have been detected are diverse, and include breast cancer,[27] non–small-cell lung cancer,[17] colorectal cancer,[83,84] and melanoma,[29] with new entities added regularly. Among the most common tumor types likely to be encountered in routine pathology practice, *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 *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[36] reported that 23 (16%) harbored *NTRK1* gene fusions. *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 *NTRK* Genes in Tumors

In addition to gene fusions, other somatic genomic alterations involving *NTRK* genes have been identified in human

tumors, including point mutations, coding sequence deletions, and gene amplifications.[85–90] 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[89] 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.[89] Point mutations of unknown significance in *NTRK* genes were also identified by NGS in 5 of 55 patients (9%) with advanced thyroid cancer.[85] Furthermore, in a large panel of 538 primary lung cancers, Marchetti et al[87] 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.[91] Recently, phase 1 data on larotrectinib have been published showing lack of response to the drug in seven patients lacking tumor *NTRK* gene fusions, with the best response noted being progressive disease.[92] Point mutations can also occur as mechanisms of acquired resistance to TRK inhibition in patients with tumors harboring *NTRK* gene fusions.[2,93]

Gene amplification is a further mechanism that can drive oncogenesis for certain proto-oncogenes. Lee et al[86] examined 1250 tumor specimens for *NTRK* gene amplification by NGS. Twenty-eight tumors (2%) were deemed to have *NTRK* gene amplification (≥4.0 copies/cell), but only 4 of 27 cases analyzed (15%) were positive to any degree for TRK expression by pan-TRK immunohistochemistry.[86] Furthermore, using array comparative genomic hybridization data from 31 malignant melanomas, Pasini et al[88] reported that an amplified region of 1q23.1 encoding *NTRK1* was a candidate hotspot implicated in tumor progression. Subsequent quantitative PCR analysis of additional tumor samples suggested that *NTRK1* was amplified in 50% of primary malignant melanomas and that amplification correlated with worse clinical outcome.[88] More important, however, when considering such data, there is only limited published evidence for a clinical benefit of TRK inhibition in the absence of *NTRK* gene fusions, and further exploration of the association of nonfusion *NTRK* alterations with therapeutic response is needed.[94]

Clinical Importance of Identifying Tumors Harboring *NTRK* Gene Fusions

There are several small-molecule inhibitors of TRK tyrosine kinases at various stages of clinical development and regulatory approval, some of which target multiple kinases, such as entrectinib, TPX-0005, and DS-6051b, and one of which, larotrectinib, is specific for TRK kinases. Published data from phase 1 and 2 trials of larotrectinib and entrectinib have suggested the potential effectiveness of such agents in the treatment of patients with TRK fusion cancer. Larotrectinib is a highly selective, orally administered, ATP-competitive inhibitor of TRKA, TRKB, and TRKC, with half maximal inhibitory concentration (IC_{50}) 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 IC_{50} 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[95–98] and entrectinib.[35,43,63,93] 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.[2] 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.[99] 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.[92] 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%.[100] 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[101] and DS-6051b,[102] with both agents initially reported as being well tolerated.

Clinical Laboratory Techniques for 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	Individual assay limited to detection of specific 5′ partner and *NTRK* gene pair
NTRK break apart				Detects *NTRK* rearrangements without knowledge of 5′ partner	Sensitivity and specificity variable, depending on assay design and parameters Multiple or complex FISH assays may be required for complete coverage
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	Does not confirm that protein is generated Might miss fusion because of breakpoint variability
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
					may require different/ complex primer designs to allow for variable alternative splicing
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
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	Commercially available kits not configured to cover all *NTRK* introns involved in fusions Detected fusions may not be expressed or in frame
Whole genome				Covers most coding and noncoding regions, including large introns	Lower analytical sensitivity Slower to generate data, and requires more computational resources than targeted approaches
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)				Deep sequence coverage using low DNA input Most suitable for analysis of SNVs, indels, and defined gene fusions	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	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
Hybridization capture				Highly scalable Can detect unknown	Requires more input RNA than amplicon methods

(*table continues*)

Table 3 (*continued*)

Analytical technique	Sample requirements	Preanalytical considerations	Turnaround time	Advantages	Disadvantages
				fusion partners Is not impacted by large intronic regions of *NTRK* genes	Complex library preparation processes
Amplicon (anchored multiplex RT-PCR)				Highly scalable Allows analysis of low-input and/or degraded RNA Simple design based on use of unidirectional gene-specific primers, allowing for detection of unknown partners	Limited published data on sensitivity
DNA plus RNA NGS	FFPE, snap-frozen, or stabilized tissue	For FFPE tissue, sample age might affect DNA quality and sequencing read quality		Broad-based screen, allowing for the efficient detection of all classes of relevant genomic alterations in cancer, including gene fusions, SNVs, indels, and CNVs Sample libraries can be prepared without the physical separation of DNA and RNA Inclusion of RNA sequencing provides robust fusion detection	

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.

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

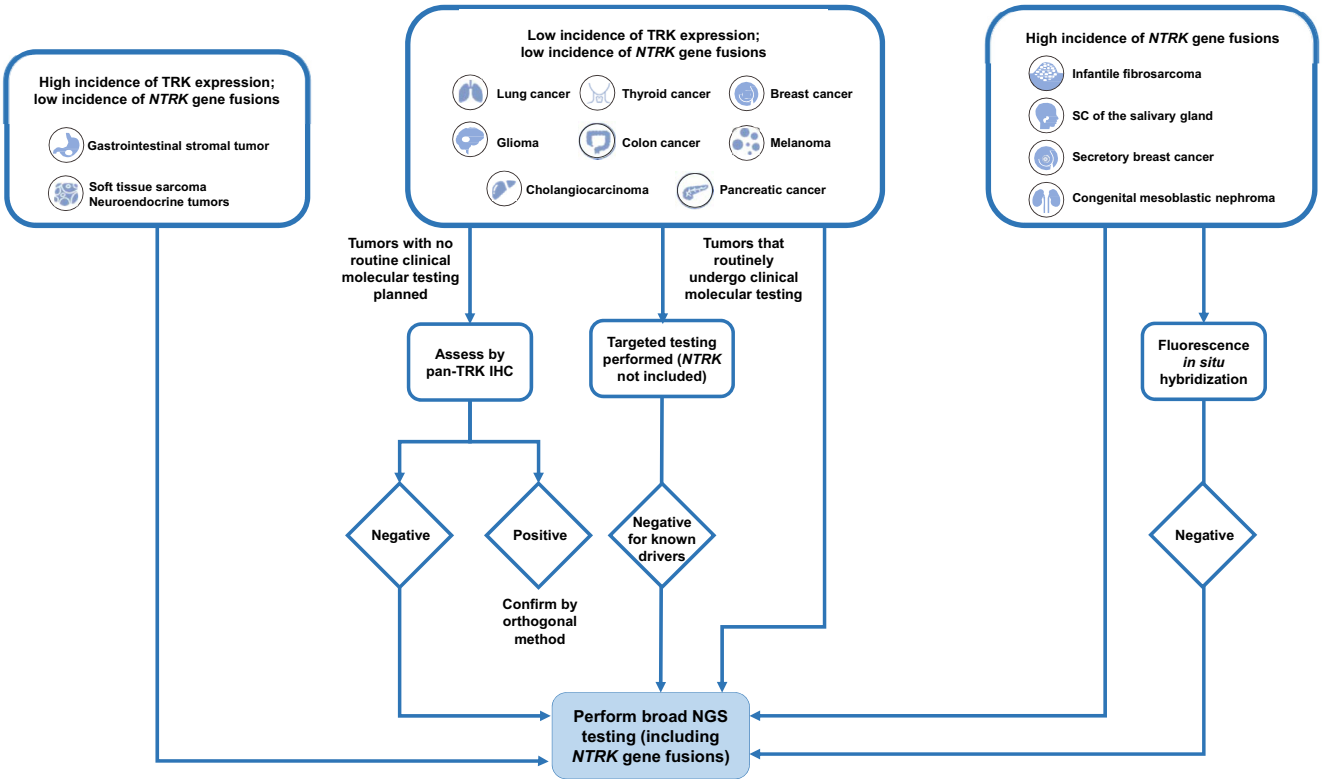

Figure 3 Diagnostic algorithm for the identification of tyrosine kinase (TRK) fusion cancer. IHC, immunohistochemistry; NGS, next-generation sequencing; SC, secretory carcinoma.

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 al[32] 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 al[38] 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[22] 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 scored 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.[91] 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.[104]

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.[105] 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.[106] 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.[53] 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.[53,72,73]

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 Brzeziańska et al[111] 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.[115] 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.[116,117] However, although cfDNA assays may have high diagnostic specificity, in general, they have a more modest diagnostic sensitivity.[118] 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,[119] it may be that cfDNA assays have a possible role in the monitoring of acquired *NTRK* tyrosine kinase inhibitor resistance mutations.[120] 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).[121]

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.[122] 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/PDGFRA* 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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