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Testing algorithm for identification of patients with TRK fusion cancer

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ABSTRACT

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

INTRODUCTION

Fusions involving neurotrophic tyrosine receptor kinases (*NTRK*) were among the first gene translocations described in cancer.[1] Selective inhibition of the resulting tropomyosin receptor kinase (TRK) fusion proteins offers a precision medicine approach to the treatment of a range of tumour types.[2]

NTRK structure and function

Tropomyosin receptor kinase A, B and C (TRKA, TRKB and TRKC) encoded by the *NTRK1*, *NTRK2* and *NTRK3* genes located on human chromosomes 1q23.1, 9q21.33 and 15q25.3, respectively, are receptor tyrosine kinases expressed in human neuronal tissue.[3–5]

All three TRK receptors comprise an extracellular ligand-binding domain, a transmembrane region and an intracellular adenosine triphosphate-binding domain.[2 6] TRK receptors are activated when neurotrophin ligands bind to the extracellular domain of the receptor (figure 1A). The neurotrophins are specific to each receptor: nerve growth factor (NGF) activates TRKA, brain-derived neurotrophic growth factor (BDNF) and neurotrophin 4/5 activate TRKB and neurotrophin 3 activates TRKC.[2] Ligand–receptor interaction triggers receptor homodimerisation, phosphorylation of the kinase domain and activation of downstream signalling pathways that play pivotal roles in the development and function of the central and peripheral nervous systems.[2]

Binding of NGF to TRKA results in activation of the RAS mitogen-activated protein kinase pathway leading to increased proliferation and cellular growth mediated by extracellular signal-related kinase (ERK) signalling.[2] Phospholipase C-γ (PLCγ) and phosphoinositide-3-kinase (PI3K) are also activated.[2] BDNF binding to TRKB activates the RAS-ERK, PI3K and PLC-γ pathways resulting in neuronal differentiation and survival, and NT3 binding to TRKC preferentially activates the PI3K/AKT pathway, which prevents apoptosis and increases cell survival.[2] The proper regulation of TRK receptor levels and their activation is critical to normal cell function. Upregulation of TRK receptors has been reported in a number of central nervous system-related disorders, for example, TRKB in epilepsy, neuropathic pain or depression.[2]

NTRK gene fusions

NTRK gene fusions result from intra-chromosomal or inter-chromosomal rearrangements that juxtapose the 3' region of the *NTRK* gene with the 5' sequence of a fusion partner gene expressed by the tumour cell progenitor (figure 1B).[2] The *NTRK* gene fusion transcript encodes a protein composed of the N-terminus of the fusion partner with the TRK partner tyrosine kinase domain.[2] In most characterised fusions, the 5' partner gene sequence encodes one or more dimerisation domains,[7] resulting in a constitutively active fusion protein.[7] This constitutive activation results in uninterrupted downstream signalling messages,[7 8] thereby acting as a true oncogenic driver. Although fusions may occur in any of the three *NTRK* genes,[9] most of those identified to date involve either *NTRK3* or *NTRK1*.[7 9 10]

TRK fusion cancer

Fusions involving the *NTRK1*, *2* and *3* genes have been identified as oncogenic drivers and diagnostic markers in various cancer types (table 1).[7 9–38] TRK fusion proteins are often mutually exclusive of other known fusion proteins involving kinases.[39] Specific *NTRK* gene fusions are associated with certain tumours,[9] for example, the *ETV6-NTRK3* gene fusion is exhibited by 90%–100% of mammary analogue secretory carcinomas,[11] >90% of secretory breast cancers,[12] and is present in most cases of infantile fibrosarcoma[34] and congenital mesoblastic nephroma.[40] In contrast some cancers have many different fusion partners.[7] In lung cancer, seven different gene fusions involving the *NTRK1* gene leading to constitutive TRKA tyrosine kinase domain activation have been described (table 1), for example, rearrangement of the 5' portion of the

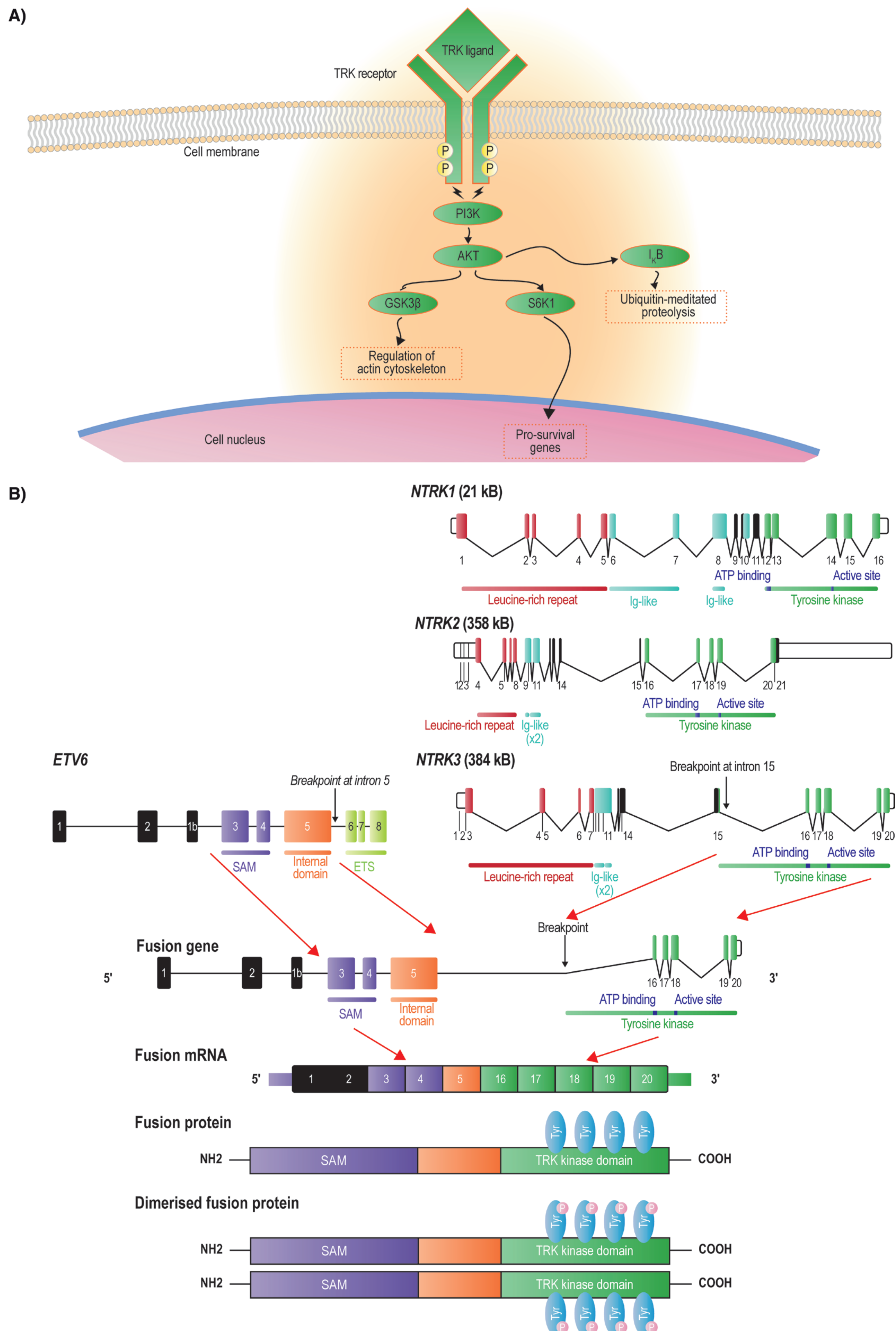

Figure 1 Schematic figure showing the TRK receptor tyrosine kinases, activating neurotrophins and the major signal transduction pathways (A) and the genomic structures of *NTRK1*, *NTRK2*, and *NTRK3*, with the size of each gene in parentheses (B). The *ETV6* and *NTRK3* gene fusion and the resultant constitutively active TRK fusion protein is a typical example. GSK3ß, glycogen synthase kinase 3 beta; Ig, immunoglobulin; mRNA, messenger ribonucleic acid; *NTRK*, neurotrophic tyrosine receptor kinase; PI3K, phosphoinositide-3-kinase; SAM, sterile alpha motif; TRK, tropomyosin receptor kinase.

Table 1 *NTRK* gene fusions identified in adult and paediatric cancers by relative frequency of *NTRK* gene fusions

	Fusion partner		
Tumour	***NTRK1***	***NTRK2***	***NTRK3***
Adult cancers			
High frequency (>80%)			
Mammary analogue secretory carcinomas			*ETV6*[11]
Secretory breast carcinoma			*ETV6*[12]
Intermediate frequency (5%–25%)			
Papillary thyroid cancer	*TFG*,[13] *SSBP2*,[9] *SQSTM1*,[9] *TPR*,[7] *PPL*[7]		*ETV6*,[9 43] *RBPMS*[9]
Low frequency (<5%)			
Appendiceal cancer	*LMNA*[18]		
Glioma/glioblastoma	*ARHGEF2*,[19] *BCAN*,[20 21] *CHTOP*,[19] *NFASC*[20]	*BCR*,[18] *AFAP1*,[9] *SQSTM1*[9]	*AFAP1*,[18] *ZNF710*,[18] *EML4*[18]
Astrocytoma		*QK1*,[7] *NACC2*[7]	
Gastrointestinal stromal tumour			*ETV6*[15]
Head and neck cancer		*PAN3*[9]	*LYN*[9]
Lung cancer	*CD74*,[7] *GRPAP1*,[23] *IRF2BP2*,[18] *MPRIP*,[7] *P2RY8*,[18] *SQSTM1*,[24] *TPM3*[18]	*TRIM24*[9]	
Sarcoma	*TPM3*,[9] *LMNA*[18]		*TPM4*[10]
Breast cancer	*CGN*,[25] *GATAD2B*,[25] *LMNA*,[25] *MDM4*,[25] *PEAR1*,[25] *TPM3*,[10 25]		*ETV6*[25]
Acute lymphoblastic leukaemia, acute myeloid leukaemia, histiocytosis, multiple myeloma, dendritic cell neoplasms			*ETV6*[26]
Uterine sarcoma	*LMNA*,[27] *TPM3*,[27] *TPR*[27]		*RBPMS*[27]
Cholangiocarcinoma	*LMNA*,[10] *RABGAP1L*[28]		
Pancreatic cancer	*CTRC*[10]		
Melanoma	*DDR2*,[29] *GON4L*,[29] *TRIM63*[29]	*TRAF2*[29]	*ETV6*[9]
Colorectal cancer	*LMNA*,[10] *TPM3*,[10] *SCYL3*[30]		*ETV6*[18]
Paediatric cancers			
High frequency (>80%)			
Secretory breast carcinoma			*ETV6*[12]
Infantile fibrosarcoma and other mesenchymal tumours	*SQSTM1*,[31] *TPM3*,[41] *LMNA*[41]		*EML4*,[32 41] *ETV6*[34 63]
Cellular and mixed congenital mesoblastic nephroma	*TPR*,[40] *LMNA*[40]		EML4,[32 40] ETV6[33 40]
Intermediate frequency (5%–25%)			
Papillary thyroid cancer	*TPR*,[35] *IRF2BP2*,[10] *TPM3*[14]		*ETV6*[35]
Spitz tumours	*TP53*,[16] *LMNA*[16]		*ETV6*,[17] *MYH9*,[17] *MYO5A*[17]
Paediatric high-grade gliomas	*TPM3*[36]	*AGBL4*,[36] *VCL*[36]	*ETV6*,[36] *BTB1*[36]
Low frequency (<5%)			
Ganglioglioma		*TLE*[38]	
Astrocytoma		*NACC2*,[37] *QK1*[37]	

myosin phosphatase Rho-interacting protein (*MPRIP*) gene fused to the 3' portion of *NTRK1* or rearrangement between *CD74* and *NTRK1*.[7 9]

Epidemiology of TRK fusion cancer

NTRK gene fusions may occur in as many as 1% of all solid tumours.[7 10] They are found in numerous tumour types in both adult and paediatric patients[2 7 10] (table 1). Two main categories of tumours are identified: rare cancers with a high frequency (>80%) of *NTRK* gene fusions and more common cancers with a lower frequency of *NTRK* gene fusions (either 5%–25% or <5%; table 1). A high frequency of *NTRK* gene fusions have been identified in mammary analogue secretory carcinomas (90%–100%)[11] and secretory breast carcinomas (>90%)[12] in adult patients, and in infantile fibrosarcomas (91%–100%),[34] other mesenchymal tumours (100%)[41] and congenital mesoblastic nephromas (83%)[42] in paediatric patients. *NTRK* gene fusions are found at a lower frequency in radiation-associated papillary thyroid cancer (14.5%)[43] in adult patients and papillary thyroid cancer (26%)[35] and Spitzoid tumours (16%)[16] in paediatric or adolescent patients. The reported frequency of *NTRK* gene fusions in common cancer types is generally <5%, including head and neck cancer (0.2%),[9] lung cancer (0.2%–3.3%),[7 9] colorectal cancer (0.7%–1.5%),[9 44] skin cutaneous melanoma (0.3%),[9] and sarcoma (1%).[9]

Treatments targeting *NTRK* gene fusions

A number of TRK inhibitors are emerging which can be subdivided into those that are selective inhibitors for TRK and those that are multi-kinase inhibitors active against a range of targets including TRK.[45] Larotrectinib is currently the only selective TRK inhibitor and was approved by the Food and Drug Administration (FDA) in November 2018.[46] Data on 55 larotrectinib-treated paediatric and adult patients with TRK fusion-positive advanced solid tumours, representing 17 unique cancer types, have been evaluated.[10] Objective tumour responses,

based on independent radiologic review, were seen in 75% of patients.[10] At 1 year, 71% of the responses were ongoing and 55% of patients remained progression-free.[10] The median duration of response had not been reached after a median follow-up of 8.3 months.[10] The same was true for median progression-free survival after a median follow-up of 9.9 months.[10] Larotrectinib was well tolerated. Adverse events were predominantly of grade 1 and no patient discontinued larotrectinib due to drug-related adverse events.[10] Furthermore, no adverse event of grade 3 or 4 that was considered by the investigators to be related to larotrectinib occurred in more than 5% of patients.[10] Among infants, children and adolescents (n=24), larotrectinib was well tolerated and showed a high response rate in those with advanced, TRK fusion-positive solid tumours (n=17).[47] Five of these children (median age, 2 years; range, 0.4–12 years) with locally advanced soft tissue tumours achieved a partial response to larotrectinib (RECIST v1.1) and underwent surgical resection after a median of six cycles (range, 4–9 cycles) of treatment.[48] Similar findings were reported by Drilon et al[10] for two children with locally advanced infantile fibrosarcoma. Larotrectinib treatment resulted in sufficient tumour shrinkage to allow for limb-sparing surgery with pathologic assessment confirming negative margins (R0 surgery). Both patients were progression-free without larotrectinib treatment after 4.8 months and 6.0 months of follow-up.

Favourable preliminary results were seen with entrectinib in two Phase I clinical trials of paediatric and adult patients with *NTRK, ROS1* or *ALK* fusions[21] leading to further investigations in patients with *NTRK* gene fusions. TRK inhibitors developed to overcome acquired resistance to first-generation TRK inhibitors are already in development.[45] LOXO-195 (BAY 2731954) has demonstrated efficacy against treatment-resistant alleles of *NTRK* gene fusions in patients with TRK fusion-positive cancers.[49] Repotrectinib, a TRK, ROS1 and ALK inhibitor, has demonstrated confirmed responses in patients with *ROS1* or *NTRK3* fusion-positive cancers who had relapsed on earlier-generation inhibitors.[50]

TESTING METHODS FOR TRK FUSION CANCERS

For optimal clinical efficacy of TRK inhibitors, an effective diagnostic strategy to detect *NTRK* gene fusions in tumour samples is essential to guide treatment selection. Approaches that may be used to directly or indirectly detect the presence of a gene fusion in clinical tissue samples[51] include immunohistochemistry (IHC), fluorescence in situ hybridisation (FISH), reverse transcriptase polymerase chain reaction (RT-PCR) and next-generation sequencing (NGS) using DNA or RNA (table 2).[10 18 32 34 52–58]

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IHC enables detection of TRK overexpression as a surrogate for the presence of an *NTRK* gene fusion and provides a time-efficient and tissue-efficient technique that may be used for routine screening[18] (figure 2A). Studies employing pan-TRK monoclonal antibody cocktails have demonstrated positive TRK expression in tumour samples.[18 59–61] However, some studies indicate that interpretation of IHC data may be more challenging than initially ascertained.[62] In an analysis of 11,502 formalin-fixed paraffin-embedded (FFPE) tumour samples of various cancer types for the presence of gene fusions, 31 cases (0.27%) with *NTRK* gene fusions were identified[62] by NGS. Of the 28 cases that were assessed by pan-TRK IHC, 21 scored positive (≥1% of tumour cells staining at any intensity above background), giving a sensitivity of only 75%, and 45% of tumours with *NTRK3* fusions scored negative by IHC. False negative cases could be related to sample preparation, for example, fixation. Therefore, it is important to check if internal controls such as endothelial cells are positive, or to use external controls such as positive cell lines. Similarly, positive IHC results must be followed with confirmatory testing using a molecular method to verify the presence of a fusion, as overexpression of wildtype TRK proteins may also be detected.

FLUORESCENCE IN SITU HYBRIDISATION

Break-apart FISH is a well-established method for detecting clinically relevant gene fusion events[52] and is of value in tumours

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

Assay	Advantages	Disadvantages
IHC	Low cost[52 53] Readily available[34] Detects TRKA, B and C[18] Turnaround time 1–2 days[53]	May not be specific for *NTRK* gene fusion as it detects both wild-type and fusion proteins[18] Possible false positives[34] Possible false negatives for fusions involving TRKC[60] There is no standardisation of scoring algorithms[52]
FISH	The location of the target within the cell is visible[54 55] Several targets can be detected in one sample using several fluorophores[54] Requires knowledge of only one of the two fusion partners when using break-apart probes *NTRK* gene fusions with unknown partners can be detected using break-apart FISH FISH is readily available in most laboratories and institutes	The target sequence must be known for conventional FISH otherwise three separate tests are required for *NTRK1*, *NTRK2* and *NTRK3*[56] Complex chromosomal translocations can result in false positive signals[56] False negative results may be above 30%[63]
RT-PCR	High sensitivity and specificity[34] Low cost per assay[52]	Target sequences must be known (i.e., cannot readily detect novel fusion partners)[32 52] A comprehensive multiplex RT-PCR assay might be challenging because of the potentially large number of possible 5′ fusion partners[52 57]
NGS	May detect novel fusion partners (depending on the assay used)[32] Can be used to evaluate multiple actionable targets simultaneously while preserving limited tissue[32] Currently used for *NTRK* testing[10] RNA-NGS is preferred over DNA-NGS as sequencing for RNA-based testing is focused on coding sequences not introns[56]	Commercially available DNA-based NGS platforms may not be capable of identifying all *NTRK* gene fusions, especially those involving *NTRK2* and *NTRK3,* which have large intronic regions[58] DNA-NGS is limited by intron size[56] RNA-NGS is limited by RNA quality[56]

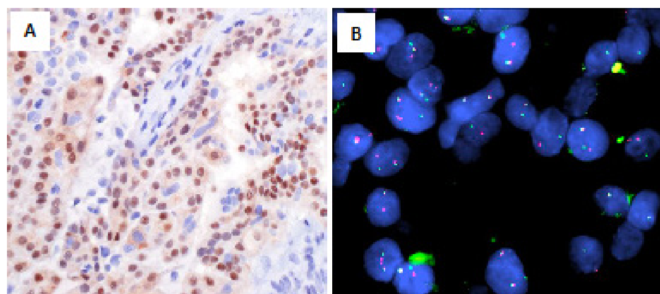

Figure 2 Secretory carcinoma of the breast aka juvenile carcinoma: low-grade basal tumour. (A) Immunohistochemistry. Nuclear staining of TRK detected by pan-TRK IHC. (B) FISH. t(12:15) *ETV6-NTRK3* fusion using an *ETV6 b*reak-apart probe. Due to the prevalence of *ETV6-NTRK3* gene fusions, an *ETV6* break-apart probe is typically used. FISH image provided by courtesy of Dr Hanina Hibshoosh, Columbia University. FISH, fluorescence in situ hybridisation; IHC, immunohistochemistry; TRK, tropomyosin receptor kinase.

with a high prevalence of *NTRK* gene fusions involving recurrent fusions[24] (figure 2B). The *ETV6-NTRK3* gene fusion was one of the first *NTRK* gene fusions reported and has been identified in numerous cancer types:[7] it is amenable for detection using break-apart FISH (figure 2B). As FISH is largely limited to the detection of a single gene fusion, a separate break-apart FISH probe is required for each of the three *NTRK* genes.[56] Furthermore, the 5' gene fusion partner will not be identified.[56] False negatives may result if the deletion is small enough to leave enough of the complementary regions for hybridisation of both FISH probes or if there is a complex FISH pattern with numerous nuclei showing atypical doublet fusion signals and only a few nuclei with split signals.[56] Indeed, in one study *ETV6* FISH was associated with a 36% false negative rate.[63]

REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION

RT-PCR provides an alternative or complementary approach to FISH, detecting *NTRK* gene fusions using primers in the coding sequence of the 5' fusion partner and the *NTRK* kinase domain.[56 57] A disadvantage of RT-PCR is that the large number of possible 5' fusion partners may make a comprehensive multiplex RT-PCR assay challenging.[57] An alternative approach could be to assess the ratio of 5' and 3' amplicons of each of the *NTRK* genes by multiplex RT-PCR reactions, with an imbalance in the ratio for a specific gene suggesting a possible fusion event.[57]

NEXT-GENERATION SEQUENCING

NGS provides a precise method to detect *NTRK* gene fusions, with high sensitivity and specificity compared with other testing methods.[57] An advantage of NGS is that multiple oncogenic events in addition to *NTRK* gene fusions can be identified from a single tumour sample.[57] A wide variety of NGS-based approaches are available for fusion testing with the primary distinguishing factor being whether they are RNA- or DNA-based.[56] Access to NGS in a clinical setting may be limited as availability of this technique varies between regions and countries.

DNA-based next-generation sequencing

Although DNA-based NGS panels may detect multiple oncogenic genomic events from one sample, not all DNA-based NGS platforms can identify all *NTRK* gene fusions, especially those involving *NTRK2* and *NTRK3* where detection of gene fusions is complicated by the presence of large introns that are typically inadequately sequenced and difficult to analyse[56 58] (figure 1B).

RNA-based next-generation sequencing

The advantage of RNA-based NGS over DNA-based NGS is that sequencing is focused on the mature mRNA hence is not affected by intron size.[56] A disadvantage is the high reliance on RNA quality, which can be poor if obtained from FFPE samples.[56] Many NGS assays now include RNA fusions in their gene panels, and it is likely that NGS diagnostics that depend on RNA for fusion detection will increasingly be used in clinical practice to test for *NTRK* gene fusions.

NTRK gene fusion testing algorithm

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

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

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

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

Conclusions and future directions

NTRK gene fusions have been identified across a range of tumour types and occur at a high frequency in certain rare cancers.[2 7 9 20 34 36 42] More common cancers have a low but significant frequency of *NTRK* gene fusions[2 7 9 20 34 36 42] and thus represent a sizeable at-risk patient population. With the recent FDA approval of the selective TRK inhibitor, larotrectinib (Vitrakvi), along with the continued development of multi-kinase inhibitors with activity in TRK fusion cancer, testing for *NTRK* gene fusions should become part of the standard diagnostic process. Marked differences in the prevalence of *NTRK* gene fusions across tumour types mean that clinical diagnostic strategies will vary accordingly but will rely on IHC, FISH and NGS assays. The

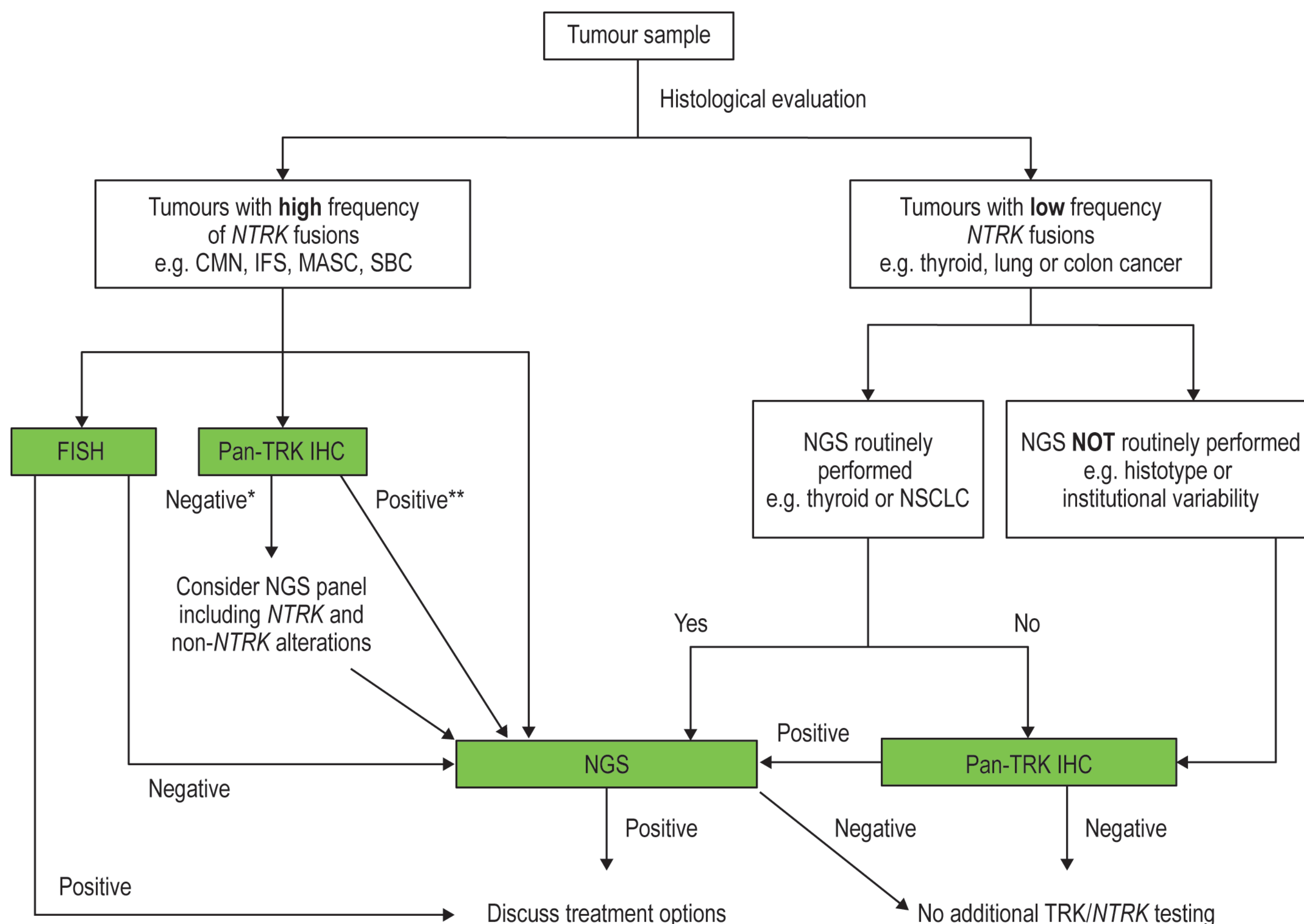

Figure 3 Testing algorithm for TRK fusion cancer. CMN, congenital mesoblastic nephroma; FISH, fluorescence in situ hybridisation; IFS, infantile fibrosarcoma; IHC, immunohistochemistry; MASC, mammary analogue secretory carcinoma; NGS, next-generation sequencing; NSCLC, non-small cell lung cancer; *NTRK*,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

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

Take home messages

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

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