ESMO recommendations on the standard methods to detect *NTRK* fusions in daily practice and clinical research

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ABSTRACT

NTRK1, NTRK2 and *NTRK3* fusions have been reported in a plethora of malignancies across different histologies and represent the most common mechanism of oncogenic activation of these receptor tyrosine kinases. These fusions result in chimaeric genes, where the 3' region of *NTRK* is joined with a 5' sequence of a fusion partner, resulting in a constitutively activated TRK with oncogenic properties. *NTRK* fusions have emerged as new targets for cancer therapy as they can be successfully inhibited by small molecule kinase inhibitors leading to durable responses across disease sites. Given the multitude of partners involved in *NTRK1/2/3* fusions, crucial to the administration of NTRK inhibitors is the development of optimal approaches for the detection of human cancers harbouring activating *NTRK1/2/3* fusion genes. The main techniques employed for *NTRK* fusion gene detection include immunohistochemistry, fluorescence *in situ* hybridization, RT-PCR, and both RNA-based and DNA-based next-generation sequencing. Each technique has advantages and limitations, and the choice of assays for screening and final diagnosis should also take into account the resources and clinical context. The European Society for Medical Oncology (ESMO) Translational Research and Precision Medicine Working Group (TR and PM WG) launched a collaborative project to review the available methods for the detection of *NTRK* gene fusions, their potential applications, and strategies for the implementation of a rational approach for the detection of *NTRK1/2/3* fusion genes in human malignancies. We present here recommendations for the routine clinical detection of targetable *NTRK1/2/3* fusions.

Keywords: NTRK1, NTRK2, NTRK3, immunohistochemistry, fluorescence *in situ* hybridization, next-generation sequencing.

Key message

Multiple techniques can detect *NTRK* fusions. In those tumours where *NTRK* fusions are highly recurrent FISH or RT-PCR are confirmatory techniques, whereas when a limited proportion of cases is expected to harbour *NTRK* fusions assays allowing fusion gene detection in an agnostic manner

are indicated: front-line sequencing or screening by immunohistochemistry followed by sequencing of positive cases.

INTRODUCTION

Neurotrophic tropomyosin-related kinases (NTRKs, or the commonly used alias TRKs) constitute a receptor tyrosine kinase family of neurotrophin receptors involved in neuronal development, including the growth and function of neuronal synapses and memory development [1]. After embryogenesis, TRK expression is limited primarily to the nervous system, where these kinases help regulate pain, proprioception, appetite and memory, and participate in the protection of neurons after ischemia or other types of injury [1, 2]. The three TRK family members described, namely NTRK1 (also known as TRKA), NTRK2 (also known as TRKB), NTRK3 (also known as TRKC) can be found in multiple tissue types (**Supplementary Table 1**) and are encoded by the *NTRK1*, *NTRK2*, and *NTRK3* genes, respectively. The TRK receptors are composed of an extracellular domain for ligand binding, a transmembrane portion and an intracellular domain with a kinase domain. Oligomerisation of the receptors and phosphorylation of specific tyrosine residues in the intracytoplasmic kinase domain are triggered when the ligand binds to the receptor, thus leading to the activation of signal transduction pathways, which results into proliferation, differentiation and survival in normal and neoplastic neuronal cells [3].

NTRK point mutations and indels have been described in various tumour types, including ovarian, colorectal, and lung cancers, as well as melanomas and myeloid leukaemias [4-8]. It should be noted, however, that the potential role of these mutations in promoting tumorigenesis and cancer progression is poorly understood. In fact, the vast majority of *NTRK* mutations are not known to be clinically actionable; however, some may constitute mechanisms of resistance to TRK inhibitors [9]. One in-frame deletion (ΔTRKA) and a splice variant (TRKAIII) in *NTRK1* have been reported as oncogenic in acute myeloid leukaemia and neuroblastoma, respectively [10, 11]. On the other hand, oncogenic fusions involving *NTRK1, NTRK2* and *NTRK3* have been reported in a plethora of malignancies across different histologies and represent the key mechanisms of oncogenic TRK activation [12]. These oncogenic fusions create chimaeric genes in which the 3' region of the *NTRK* gene is joined with a 5' sequence of a fusion partner gene, and may stem from intra-chromosomal or inter-chromosomal rearrangements [12, 13]. Despite the multitude of 5' fusion gene partners,

NTRK1/2/3 fusion genes share key characteristics, including the fact that the resulting genetic chimaera uniformly contains the *NTRK* kinase domain with the critical tyrosine docking sites (**Figure 1**), and results in an overexpressed and constitutively activated and overexpressed TRK kinase [12]. These fusion genes have been shown to possess oncogenic properties, including the induction of cancer cell proliferation and activation of critical cancer-related downstream signalling, such as the mitogen–activated protein kinase (MAPK) and PI3K/AKT pathways [12, 14-16]. Many 5' gene partners (at least 25) have been described; nevertheless, all rearrangements share an in-frame, intact TRK kinase domain [12, 17, 18].

Fusion genes affecting *NTRK1/2/3* are highly recurrent in certain rare malignancies. The best known form of *NTRK* fusion gene is the *ETV6-NTRK3*, which is present in >95% of secretory carcinomas of the breast [19] and of the salivary glands (i.e. mammary analogue secretory carcinoma of the salivary glands) [20], congenital fibrosarcoma [21] and cellular mesoblastic nephromas [22]. This fusion gene is the product of the t(12;15)(p13;q25) chromosomal translocation, which results in a chimeric transcript encompassing exon 4, 5 or 6 of *ETV6* and the kinase domain of *NTRK3* [19]. The *ETV6-NTRK3* fusion gene leads to constitutive activation of the TRKC kinase domain, with downstream activation of the PI3K/AKT and MAPK pathways [15, 23]. The *ETV6-NTRK3* fusion gene is also found in a small subset of acute myeloid leukaemias [24, 25], but the breakpoints are distinct from those found in solid malignancies. *NTRK1/2/3* fusion genes have also been detected in small subsets of common tumour types, and, in this context, the fusions typically occur in a mutually exclusive fashion with other strong mitogenic drivers, i.e. genetic alterations affecting the most common driver genes belonging to the MAPK signalling pathway (e.g. *KRAS*, *NRAS* and *BRAF*) [26-29]. They have also been reported as significantly more frequently encountered in MSI-high tumours in the context of colorectal carcinoma patients [28]. Interestingly, a recent study has shown that the association between *NTRK* fusions with MSI-high colorectal carcinomas seems to be strictly connected with MLH1 deficiency associated with *MLH1* promoter hypermethylation in the context of a non-Lynch syndrome scenario [29].

NTRK1/2/3 gene fusions have emerged as new targets for cancer therapy as they can be successfully inhibited by targeted kinase inhibitors [17, 18, 30, 31]. Of note, responses have been documented irrespective of the 5' gene partner and in a histology-agnostic fashion [9, 17, 30, 31]. Several compounds targeting TRKs are currently being explored in clinical trials and, notably, one of these compounds, larotrectinib (VITRAKVI), has received accelerated approval by the U.S. Food and Drug Administration (FDA) for adult and paediatric solid tumours with an *NTRK* fusion without known resistance mutations [32]. In addition, entrectinib has received breakthrough designation status by the U.S. FDA for the treatment of cancers harbouring *NTRK* fusions. Hence, there is an active interest in clinical oncology for *NTRK* fusions, which has prompted an urgent need to define the routine diagnostic testing to identify gene fusions as a companion diagnostic method to support clinical decision in this context. Therefore, the European Society for Medical Oncology (ESMO) Translational Research and Precision Medicine Working Group (TR and PM WG) launched a collaborative project to review the available methods that can be used to detect *NTRK1/2/3* gene fusions.

NTRK1/2/3 **FUSION GENE DETECTION**

A wide array of different techniques can be employed in the detection of *NTRK1/2/3* fusions and fusions (**Supplementary Figure 1**). Historically, gene fusions have been assayed by fluorescence *in situ* hybridization (FISH) and reverse transcriptase (RT)-PCR, and FISH assays for the detection of the *ETV6-NTRK3* fusion gene are commercially available. However, given the multitude of 5' partners involved in *NTRK1/2/3* fusion genes, assays that allow for the detection of multiple variants in a single test, including next-generation sequencing (NGS)-based RNA and DNA approaches, have been widely used in large academic centres in North America. The adoption of these NGS-based methods in other contexts has proven challenging, given the costs for the implementation and running of the assay, limited reimbursement by public or private payors, need for bioinformatics expertise and relatively longer turnaround time (1-3 weeks).

As a general approach, one could consider that in those malignancies where the *NTRK* fusions are described as pathognomonic or highly recurrent genetic alterations, such as the *ETV6-NTRK3* fusion gene in secretory carcinoma of the breast and of the salivary glands, congenital fibrosarcomas and cellular mesoblastic nephromas [19] (**Supplementary Table 1**), the detection of the fusion gene could be accomplished by FISH or RT-PCR. Conversely, in those neoplasms where a limited proportion of cases is expected to harbour an *NTRK1/2/3* fusion and the gene partner is unknown (**Supplementary Table 1**), assays allowing for the detection of fusion genes in an agnostic manner would be indicated, either in the form of front-line NGS testing or by using a two-step approach involving a screening by immunohistochemistry (IHC) followed by NGS of cases expressing TRKA/B/C.

From a technical standpoint, all the techniques have strengths and weaknesses (**Table 1**), as discussed in detail here below.

IN SITU ASSAYS

Immunohistochemistry (IHC)

Different antibodies are available to detect TRK expression in tissue samples. There are antibodies directed against specific NTRK proteins (TRKA or TRKB) [27, 33, 34], antibodies targeting an amino acid sequence common to TRKA, TRKB, and TRKC (pan-TRK antibodies) [35-38] or a pan-TRK antibody cocktail [39]. Positive controls for IHC include the cell lines KM12 (*TPM3-NTRK1*) [33], MO-91 (*ETV6-NTRK3*) and CUTO-3.29 (*MPRIP-NTRK1*) [40], and formalin-fixed, paraffin-embedded (FFPE) cell pellets can be used as external controls in immunohistochemical runs [41]. Peripheral nerves can serve as internal control, if present in the stroma (**Supplementary Figure 2**). Non-neoplastic tissues (skin, blood vessels, inflammatory cells) serve as negative internal controls [36].

The pattern of TRK expression detected by IHC can be variable in intensity and subcellular localisation (**Supplementary Figure 2**). There are data supporting the notion that the subcellular distribution of the chimaeric protein may vary with the 5' fusion gene partner, for instance, when

using a pan-TRK antibody five *LMNA-NTRK1* fusion-positive neoplasms displayed nuclear membrane accentuation [37], which was also observed in a *LMNA-NTRK1* fusion-positive uterine sarcoma assessed with an anti-TRKA antibody [35]. When using a panTRK antibody*, TM3/4* fusion positive tumours displayed cellular membrane accentuation [37], and an uterine sarcoma harbouring *TPR-NTRK1* fusion displayed strong and diffuse cytoplasmic expression with dot-like aggregates as well as rare nuclear expression and accentuation of the nuclear envelope [35]. Finally, half (3/6) of *ETV6-NTRK3* fusions displayed nuclear staining [37]. It should be emphasized, however, that further characterization of the subcellular localization according to the fusion gene partners is required before these patterns are adopted for diagnostic purposes.

Given the restricted expression of TRKA, TRKB and TRKC in adult tissues (i.e. smooth muscle, testes and neuronal components [42-44]), IHC has been proven highly sensitive (from 95% [37] to 100% [35]) and specific (from 93% [35] to 100% [37]) for the detection of *NTRK* fusions. It should be noted however that values for sensitivity and specificity reported so far derive from data on relatively small cohorts. Further validation in large cohorts will likely be more informative in the next future.

Hechtman et al. [37] analysed *NTRK* fusion-positive and *NTRK* fusion-negative cases, using DNAseq and RNAseq methods (see below). Pan-TRK IHC was positive in 20/21 cases with *NTRK* fusion transcripts. The discordant negative case was a mismatch repair-deficient colorectal carcinoma with an *ETV6-NTRK3* fusion. For all 20 RNAseq-negative cases concordant IHC results were observed. In addition, Chiang et al. [35] reported on 4 *NTRK* fusion-positive uterine sarcomas that showed features of fibrosarcoma and all displayed pan-TRK staining, with 3 of them also showing concurrent TRKA staining. In that study, 97 uterine spindle cell leiomyosarcomas were analysed: four fusion negative cases (as assessed by FISH and/or NGS) displayed weak and diffuse cytoplasmic TRKA expression, and 2 featured strong and diffuse cytoplasmic pan-TRK expression. Rudzinski et al. [36] observed a 97% sensitivity and a 98% specificity for the presence of an *NTRK* fusion when employing a pan-TRK antibody, whereas TRKA IHC (EP1058Y) was 100% sensitive and 63% specific. Consistent with Hechtman et al. [37], Rudzinski et al. [36] also identified a single

false negative *ETV6-NTRK3*-rearranged tumour when using pan-TRK antibodies; however, this tumour displayed diffuse, strong positivity for TRKA. Due to the lack of monoclonal anti-TRKC antibodies, it is uncertain as to whether these *ETV6-NTRK3* fusions that were independently detected in the two studies were indeed false negative IHC results rather than fusions that did not result in translation of the protein.

Taken together, these data suggest that pan-TRK IHC is a valuable tool to identify *NTRK*-rearranged neoplasms, and has several advantages as: i) it has relatively high sensitivity and specificity; ii) it represents a rapid method that can be easily employed in different laboratory environments with a quick turnaround time; iii) it is able theoretically to detect only transcribed and translated fusion proteins; iv) it is inexpensive and requires limited material.

However, in addition to the known limitations of immunohistochemical analyses in general, that include also possible issues related to the pre-analytical phase, IHC for TRKA, TRKB and TRKC has important caveats. First, a subset of cases, in particular those with smooth muscle and neuronal differentiation, expressing TRK proteins lack any identifiable fusions. Second, the interpretation of IHC results may prove challenging in tissues where TRKs are physiologically expressed. Third, a subset of fusion gene positive cases (mainly *NTRK3*) lack TRK protein expression as defined by IHC [37]. Fourth, there are no monoclonal, c-terminus TRKC antibodies, i.e. specific to *NTRK3* fusions, currently commercially available. Finally, the expression of the receptors is not diagnostic of an *NTRK1/2/3* fusion gene; it merely suggests that a fusion gene is likely present. Hence, some further considerations are required. In general, tumours harbouring *NTRK3* fusions had much weaker staining for pan-TRK than tumours with *NTRK1*/*NTRK2* fusions. This differential expression, as well as the presence of nuclear staining in *NTRK3*-rearranged tumours, may be useful for directing subsequent molecular testing strategies [36]. A possible algorithm in the use of pan-TRK IHC as a surrogate marker for *NTRK* fusions has been proposed: moderate to strong diffuse cytoplasmic pan-TRK IHC staining can be considered as a surrogate of the presence of *NTRK1*/*NTRK2* fusions (in the absence of muscle or neural differentiation of the lesion) and nuclear pan-TRK IHC can be considered a surrogate of *NTRK3* fusions. For tumours with only weak cytoplasmic expression of

pan-TRK, an *NTRK* fusion should be confirmed by other molecular/cytogenetic methods to ensure that a fusion is present in patients being considered for targeted therapeutic agents [36].

One of the approaches that has emerged consists of using IHC first, as a screening tool, followed by an RNA-based NGS approach to detect the specific fusion [38, 39]. The purpose of IHC screening is to distinguish in a rapid manner between patient specimens that are pan-negative for NRTKs and those that demonstrate a weak to strong level of tumour staining, which may harbour gene fusions. However, the overall cost-effectiveness of this method is debated, due to the rarity of *NTRK* fusions (<0.5% of cancers overall), in particular if additional targets are investigated. Outside of the rare tumour types with high-prevalence of *ETV6-NTRK3* fusions, up-front screening with NGS panels are ultimately the least expensive approach to uncover actionable driver alterations including the investigation of possible *NTRK* fusions. Nonetheless, this 2-step approach has allowed for the enrolment of patients in basket trials, such as the STARTRK trials [17]. In the context of STARTRK-2, a basket study of entrectinib for the treatment of patients with solid tumours harbouring *NTRK*1/2/3, *ROS1* or *ALK* fusion genes (ClinicalTrials.gov Identifier: NCT02568267), it is specified that for patients enrolled via local molecular testing, an archival or fresh tumour tissue is required to be submitted for independent central molecular testing at a central CLIA-certified laboratory. There are preliminary data reported by Potts et al. on the detection of *NTRK*, *ROS1* and *ALK* gene fusions in gastrointestinal tumour patients [38] with a pan-TRK antibody used to perform IHC locally and an AMP-based NGS assay (Archer FusionPlex™) used for confirmation of positives. The IHC positivity average rate was 8% across 15 tumour locations. Out of 157 gastrointestinal samples, no instances were detected where IHC yielded a negative result and fusion genes were detected by NGS (100% negative predictive value). In a study evaluating a cohort of samples of multiple histologies (n = 636) Murphy et al. [39] used an antibody cocktail consisting of monoclonal antibodies targeting Pan-TRK, ROS1 and ALK with confirmation using anchored multiplex PCR targeted RNAseq (Archer FusionPlex™ gene fusion assay). Approximately 30% showed positive staining and were tested by NGS. In a subgroup of 192 samples of colorectal, thyroid and lung cancers the pan-TRK IHC test had a 100% negative predictive value for gene fusion detection and the overall prevalence of the

fusion gene within the IHC positive population was 9%, demonstrating an enrichment of fusion positive samples within a population of clinical samples (*versus* the 4% frequency without the application of IHC screening).

As the prevalence reported here in these two studies is higher than that reported in the literature for these histologies (**Supplementary Table 1**), we cannot exclude that somehow the cohorts analysed were *a priori* "pre-enriched" for fusions.

Fluorescence *in situ* hybridization (FISH)

FISH is a commonly used method for detecting chromosomal rearrangements, and has been effectively used to detect *ALK*, *ROS1* and *RET* fusions in solid tumours. Either fusion or break-apart probes (**Supplementary Figure 3**) can be used screen for *NTRK1, NTRK2* or *NTRK3* fusions; nevertheless, split-apart fusion probes are invariably easier in FFPE samples. Similar to IHC, FISH cannot ascertain the 5' partner or whether the fusion results in a productive in-frame chimaeric transcript. Given that a multiplex FISH requires a great deal of experience in its interpretation, three separate FISH assays would have to be run in parallel, which become expensive and time consuming. FISH, however, can be very effective at identifying the presence of the *ETV6-NTRK3* fusion gene in the tumour types where it is highly prevalent.

The studies reported so far have used a wide array of probes detecting *NTRK* fusions, some constructed as home-brew assays [14, 26, 35, 45], others being commercially available specific break-apart probe kits mainly for *NTRK1* [34, 40] or *NTRK3*. When investigating *ETV6-NTRK3* fusions, there is also the possibility to use a mixed break-apart probe allowing detection of *ETV6*, *NTRK3* and other 13 genes breaks [46]. For instance, this approach was used in a study analysing *ETV6-NTRK3* fusion transcripts in 25 cases of secretory carcinoma of the salivary glands and the split-apart signals of the *NTRK3* gene were detected in 16 of 25 cases [46]. In 3 cases, the tissue was not analysable, and in 2 other cases analysis could not be performed because of lack of FFPE tissue. In the 4 remaining cases lacking *NTRK3* split-apart signals and *ETV6* split-positive, the fusion of *ETV6* gene to a non-*NTRK3* gene was suggested. To pursue possible fusion partners in these

cases, involvement of *NTRK1* and *NTRK2* genes was examined, however neither *NTRK1* nor *NTRK2* split-apart signals were identified.

Recommendations for scoring are those generally accepted for FISH for fusion gene detection. Sections should be 4um thick to avoid artefactual split-apart signals. Scoring should be performed by counting the number of fluorescent signals in at least 50 randomly selected non-overlapping tumour cell nuclei. Scoring by more than one observer is recommended. A cut-off value for gene break is set at 10% or 15% (i.e. cases can be considered as harbouring a gene fusion if >10% or >15% of nuclei display “split-apart signals”; red and green signals should be separated by a distance greater than the size of two hybridization probe signals) [20, 47].

Albeit being a robust technology for the detection of highly recurrent known fusion genes and confirmation of potential fusion genes, the utility of FISH for screening cancers with *NTRK1/2/3* fusions is limited, given the multitude of partners involved, the expertise required and its labour-intensive nature.

IN VITRO NUCLEIC ACID-BASED ASSAYS

In assays based on nucleic acids extracted from tumour tissues, an evaluation of tumour cellularity and specimen adequacy is of utmost importance. Additional pre-analytic parameters are equally important, including warm and cold ischemia, length of fixation and chemical properties of the fixatives employed. Therefore, robust approaches tailored for FFPE tissue samples are essential. RNA and DNA NGS assays can detect *de novo* fusion genes involving *NTRK1, NTRK2* and *NTRK3*, and define the 5’ partner. It should be noted, however, that for most of these assays, bioinformatics experience is required, and the turnaround times can be lengthier than those of more targeted molecular assays [37].

Reverse transcription (RT)-PCR

RT-PCR analyses of *NTRK* fusions have been reported in thyroid neoplasms [48, 49], glioblastomas [50], congenital fibrosarcomas [51], secretory carcinoma of the salivary glands [20, 46] and of the breast [52, 53]. Furthermore, RT-PCR has been used as an orthogonal validation method in studies exploring the genetic landscape of subgroups of neoplasms by high-throughput techniques [50].

Skalova et al. [46] analysed a series of 25 morphologically and immunophenotypically confirmed secretory carcinoma of the salivary glands with the absence of classical, exon 5-exon 15, *ETV6-NTRK3* fusion transcript as detected by standard RT-PCR. The classical fusion transcript was analysed by a more sensitive nested RT-PCR. In addition, atypical exon 4-exon 14 *ETV6-NTRK3* fusion transcripts, as well as possible combinations of exons involved in classical and atypical junction, were analysed by nested RT-PCR and/or RT-PCR. In 4 cases, the classical fusion transcript was found by nested RT-PCR. Five other cases harboured atypical, exon 4-exon 14 or exon 5- exon 14, *ETV6-NTRK3* fusion transcripts detected by both nested and/or standard RT-PCR. The rest of the cases remained negative on RT-PCR. FISH with *NTRK3* break-apart probes was also performed and a *NTRK3* gene split was detected in 16/25 cases. For 5 cases, the tissue was not analysable due to technical issues, but in the 4 remaining cases no evidence of *NTRK3* fusion was detected and *ETV6* split-apart signals were observed, thus suggesting the fusion of *ETV6* with a non-*NTRK3* gene. Regrettably, these cases were not subjected to NGS to elucidate the potential 3' partners of these potential fusion genes.

RNA next-generation sequencing assays

Given the chimaeric nature of the transcripts stemming from gene fusions, RNA sequencing constitutes an approach for the *de novo* detection of fusion genes that are transcribed. The primary concern with handling RNA is related to its labile nature, especially when dealing with archival FFPE samples. Highly damaged RNA is composed of fragments that are too short to be informative and/or will hamper library preparation and subsequent sequencing. RNA quality assessment is therefore a crucial step in this process in order to discriminate possible false negative results and to enable reproducibility of the test [39].

Although studies where whole transcriptome RNA sequencing to detect fusions affecting the *NTRK* genes are on record [1, 54], more common is the use of targeted assays. In recent years, anchored multiplex PCR (AMP), for which commercial ready-to-use kits as well customisable assays are available, has become a widely-adopted methodology for fusion gene detection. Owing to the initial adapter ligation step that facilitates priming without *a priori* knowledge of the gene fusion partner, the AMP method has been shown to have high technical sensitivity and specificity even in FFPE-derived RNA samples. In these assays, the sequencing library targets known fusion exons in multiple oncogenes including *NTRK1* and/or *NTRK3* [26, 35, 37, 45], or all of the three members of the *NTRK* family [34, 35, 37].

The impact of RNA quality was assessed in one study analysing 44 archival cases (infantile fibrosarcoma, congenital mesoblastic nephroma, secretory carcinoma of the salivary glands and secretory breast carcinoma) with sufficient tissue to be tested with NGS by an Archer DX AMP assay. Only 23/44 passed pre-sequencing quality control thresholds. Nevertheless, it should be mentioned that the likelihood that a case would fail quality control increased with sample aging, a feature expected to impact an assay using RNA extracted from FFPE tissue [26].

Anchored multiplex PCR has been employed also in larger studies aiming at screening for the presence of known fusion exons in multiple oncogenes: for instance, a panel including *ALK*, *ROS1*, *RET* and *NTRK1* fusion has been used in a phase I dose escalation study of entrectinib in adult patients with locally advanced or metastatic non-small cell lung carcinomas (NCT02097810). In this study, out of the 1,378 enrolled cases, two patients harboured *NTRK1* gene fusions (0.1%, 95% confidence interval 0.01%, 0.5%): a *TPM3-NTRK1* fusion previously described and a fusion transcript containing sequence from *SQSTM1* (sequestosome 1) and *NTRK1* [45].

In addition to the AMP technology, other NGS platforms can offer the possibility to test for *NTRK* fusions. These include the GeneTrails Solid Tumor Fusion Gene Panel (Knight Diagnostic

Laboratories), designed to detect fusions involving 20 target genes including *NTRK1*, *NTRK2*, *NTRK3* [55]; the Universal Fusion/Expression Profile (Neogenomics), an assay capable of detecting different classes of genomic abnormalities such as fusion transcripts and transcriptomic gene expression levels in 1,385 genes (*NTRK1*, *NTRK2*, *NTRK3* included); and the Oncomine assays (ThermoFisher Scientific), which cover fusion variants including *NTRK1*, *NTRK2* and *NTRK3* [56].

These panels require different amounts of RNA input. The choice of the technology must therefore take into account the amount of tissue available for testing.

Targeted next-generation DNA sequencing assays

Targeted NGS assays consist of panels of selected genes of interest where either all of the exons or hotspot regions only in selected exons for each gene are investigated. Several companies as well as academic centres have developed such assays, which can exploit distinct types of chemistry for sequencing. Some of the commercially available targeted sequencing panels offer the possibility to detect fusion genes and there are many examples where targeted DNA panels have been employed to detect *NTRK* fusions.

Some studies have been based on the Memorial Sloan Kettering Integrated Mutation Profiling of Actionable Cancer Targets (MSK-IMPACT™) assay, a deep-coverage hybridization capture-based assay encompassing the entire coding regions and selected intronic and regulatory regions of >400 key cancer genes [27, 37]. This tumour-profiling multiplex panel has been recently cleared by the U.S. Food and Drug Administration (FDA) as an *in vitro* diagnostic test that can identify somatic genetic alterations. MSK-IMPACT™ can detect missense mutations, indels, copy number alterations, and selected gene fusions. In this panel, probes for introns 3, and 7 to 12 of *NTRK1*, and intron 15 of *NTRK2* are included to detect fusions involving these 2 genes. In addition, probes for *ETV6* introns 4 and 5 are included to detect *ETV6-NTRK3* fusions. Of note, other introns affected by *NTRK* fusions could not be included because they are too large for a DNA-based capture approach (approximate upper limit: 25 kb). Zehir et al. [13] have reported on the use of MSK-IMPACT in a

prospective clinical sequencing of unselected metastatic patients and *NTRK1* and *NTRK3* fusions were detected in 18 patients (0.2%), a subgroup of whom were subsequently enrolled in targeted clinical trials.

A study focused on MSK-IMPACT applied to the analysis of 449 melanoma patients revealed four cases (0.9%) harbouring *NTRK* fusion genes; in three of these cases, the 3' partner was *NTRK1*, whereas in the remaining melanoma, *NTRK2* was rearranged [27].

There are other DNA-targeted sequencing assays that can be employed in the detecting of *NTRK* fusions. Some examples include the UW Oncoplex and the UCSF500 Cancer Gene Panel, both using probes for exons and selected introns of *NTRK1*, *NTRK2*, and *NTRK3*. Additional probes for *ETV6* exons and selected introns are included to detect *ETV6-NTRK3* fusions; the UW OncoPlex includes additional probes for selected exons and introns of *EML4* to detect *EML4-NTRK3* fusions [36]. In addition, the SmartGenomics Complete –(PathGroup) Expanded Solid Tumor, includes 160 genes profiled for mutations and 126 gene fusions. The Solid Tumor Focus Oncomine NGS Panel (Cancer Genetics) is designed to cover hotspot mutations of 35 unique genes in various different types of solid tumours including, but not limited to, lung cancer, colorectal cancer, skin cancer, breast cancer, bladder cancer, and thyroid cancer.

Finally, the FDA-approved FoundationOneCDx test (Foundation Medicine) allows for *NTRK* detection: it analyses 315 genes and selected fusions including those involving *NTRK1*, *NTRK2 and NTRK3*. *NTRK* fusions can also be detected by FoundationOne®Heme, a DNA- and RNA-based NGS assay that analyses 236 cancer-related genes and 19 genes commonly rearranged in cancer. Foundation Medicine has reported their experience on 2,031 tumour specimens from paediatric, adolescent and young adult patients affected by a variety of neoplastic conditions (leukaemias and solid tumours, including primary central nervous system (CNS) tumours) that were assayed with FoundationOne®Heme in the course of clinical care. From this data set, nine unique patients (0.44%) were identified to harbour *NTRK* fusions [57]. For four cases where a diagnosis of infantile fibrosarcoma was considered, FISH for *ETVT6-NTRK3* was performed and showed negative results in 3 of them.

Other commercially available DNA- and RNA-based panels that can detect *NTRK* fusions are listed in **Supplementary Table 2**.

Undoubtedly, DNA-based NGS has proven to be effective to detect gene rearrangements and predicted fusions; however, not all of the *NTRK* fusions can be practically detected using targeted assays, especially those fusions involving *NTRK2* and *NTRK3* where large intronic regions can render DNA-based detection challenging [9]. Further, many of the *NTRK* fusions detected by DNA-based sequencing are of unknown functional significance, requiring confirmation by another assay [37]. Hence, DNA-based targeted sequencing assays are often supplemented with RNA-sequencing methods. As mentioned above, FoundationOne®Heme incorporates both DNA- and RNA-based NGS approaches, and the Archer DX AMP assays is currently run in parallel with MSK-IMPACT™ for selected patients.

On the other hand, in a study comparing RNA-based and DNA-based NGS, 23 tumours of various histology where *NTRK* fusions had been identified on MSK-IMPACT were subjected to Archer RNA testing, which detected *NTRK* fusion transcripts in 21/23 cases. The two negative cases, a lung adenocarcinoma and a glioblastoma, both harboured a DNA level fusion that did not result in an RNA level fusion transcript (involving *NTRK1* exon 5/*P2RY8* exon2 and *NTRK3* exon 14/ZNF710 exon exon1, respectively) nor show TRK protein expression as assessed by IHC with a Pan-TRK antibody [37]). This highlights the need for NTRK IHC or RNA-based confirmation in cases of unusual or atypical *NTRK* genomic fusions detected by DNA-based testing, as some of these may represent non-functional bystander fusions.

NanoString technology

This digital barcode technology allows direct multiplexed measurement of analytes by the detection of hundreds of unique transcripts in a single reaction with the ability to analyse DNA, RNA, and protein even simultaneously [58]. No enzymes or library preparation are required to perform the assay. In terms of analysis of gene fusions, there is a commercially available ready-to-use nCounter

Lung Fusion Panel, which includes 63 probes, namely 35 for specific fusion detection (including *CD74-NTRK1* and *MPRIP-NTRK1*), 24 for positional gene expression imbalance detection, and four internal reference genes. As an alternative, it is possible to design custom panels. To the best of our knowledge, no studies specifically for the detection of *NTRK1/2/3* fusion genes have been conducted so far by using this technique.

CIRCULATING CELL-FREE DNA/RNA TESTING FOR *NTRK* FUSIONS

Peripheral blood might represent an alternative source of tumour-derived nucleic acids when a tumour specimen is not available. A number of DNA- or RNA-based NGS panels for the analysis of liquid biopsy are available. However, in most cases the coverage of *NTRK* fusions provided by these panels are limited. For example, the current versions of the DNA-based panels Guardant360 and AVENIO Extended ctDNA Analysis Kits cover only *NTRK1* fusions. The Oncomine Pan-Cancer Cell-Free Assay uses a single library from circulating DNA and RNA, and can detect selected fusions of the *NTRK1* and *NTRK3* genes. The limitations of these assays must be taken into account when screening for *NTRK* fusions.

CLINICAL PERSPECTIVES AND ONGOING TRIALS

Identification of *NTRK* fusions across different tumour types allowed for therapeutic interventions that are “age- and tumour-agnostic” and best exemplified by the design of “basket trials” for molecularly-defined subsets of patients [59]. Several tyrosine kinase inhibitors for TRKA, TRKB and/or TRKC are available and show a varying degree of activity towards actionable *NTRK* fusions (i.e. harbouring an in-frame and intact kinase domain). There are selective TRK inhibitors (larotrectinib) and multikinase inhibitors with anti-TRK activity [entrectinib, TPX-0005 (repotrectinib), crizotinib, cabozantinib, altiratinib, foretinib, ponatinib, nintedanib, merestinib, BAY2731954 (formerly known as LOXO-195), MGCD516, PLX7486, DS-6051b and TSR-011] (**Supplementary Table 3**) [30].

Larotrectinib and entrectinib represent the two compounds that are furthest in clinical development have been made so far. Larotrectinib (VITRAKVI®) has received accelerated approval by the U.S.

Food and Drug Administration (FDA) for adult and paediatric solid tumours with an *NTRK* fusion without known resistance mutations [32]. Entrectinib has received breakthrough designation status by the U.S. FDA for the treatment of cancers harbouring *NTRK* fusions.

Larotrectinib (LOXO-101) is a potent and highly selective small molecule inhibitor of all three TRK proteins and has been developed in parallel in adult and pediatric populations. It has been investigated in a phase I study (ClinicalTrials.gov Identifier: NCT02637687) [60], which provided the first proof of concept of safety and high response rates (tumour regressions in > 90%) in infants, children and adolescents with *NTRK* fusion cancers, thus establishing *NTRK* fusions as a tractable target in paediatric patients with solid or CNS tumours [60].

NTRK fusion gene testing was performed locally before enrolment, in a CLIA-certified laboratory, by FISH, RT-PCR or NGS. No central testing was performed in this protocol. Patients who did not have tumour samples available for such analyses were considered not to have *NTRK* fusions. Parallel trials focused on both adult and paediatric populations reported response rates of approximately 80% of cancers harbouring *NTRK* fusion genes (ClinicalTrials.gov numbers: NCT02122913, NCT02637687, and NCT02576431) [30] and *NTRK* fusion genes were detected by NGS following procedures and analytic pipelines established by each laboratory, or by FISH [30].

New data recently presented at ASCO further corroborate the tumour-agnostic efficacy and the favourable safety profile of larotrectinib in adult patients with *NTRK* fusion positive cancers [61], as well as its efficacy in CNS disease [62].

The potent oral inhibitor of the tyrosine kinases TRKA/B/C, ROS1 and ALK, entrectinib (RXDX-101), has been evaluated in two phase I studies (ALKA-372-001 and STARTRK-1) in patients with advanced or metastatic solid tumours, including patients with active CNS disease [17]. For patients enrolled via local molecular testing, an archival or fresh tumour tissue was required to be submitted for independent central molecular testing at Ignyta's CLIA laboratory post-enrolment. Entrectinib was shown to be well tolerated and active against those gene fusions in solid tumours, including in patients with primary or secondary CNS disease, given that entrectinib can cross the blood–brain

barrier. The analysis of the phase II–eligible population from ALKA-372-001 or STARTRK-1 (25 evaluable patients) showed objective response rates (using RECIST) ranging from 57%-100% [17]. More recently, pooled data from STARTRK-2, STARTRK-1 and ALKA-372-001 led to an integrated analysis of a global population (n=54 patients in total) that has confirmed the clinically meaningful, deep and durable systemic responses in patients with and without CNS metastases, showing a 57.4% objective response rate [31]. Moreover, very recent data reported at ASCO also provide further evidence of efficacy of entrectinib in CNS neoplastic lesions [63].

Ongoing, actively recruiting interventional phase I and phase II trials assessing the response rates of anti-TRKs are summarised in **Supplementary Table 3**.

Clinical trials assessing the efficacy of TRK inhibitors have also given the chance to investigate potential side effects that may arise from inhibition of the full-length TRK receptors in normal tissues. In theory, loss of normal regulation of TRKA, TRKB or TRKC receptor activity can result in numerous human diseases [12]. Nevertheless, the limited side-effect profile of larotrectinib reported so far and the tolerability demonstrated for entrectinib suggest that long-term administration of these agents is feasible. The most common treatment-related adverse events of Grade 3 in severity are fatigue/asthenia (reported for both larotrectinib and entrectinib) [17, 31], weight increase (reported for both larotrectinib and entrectinib) [17, 31] and anaemia (reported for entrectinib) [31]. Other side effects include paresthesias, dizziness, dysgeusia, diarrhoea, nausea, myalgias and arthralgias. Importantly, all related adverse events were reversible with dose modifications [17, 31].

Despite durable responses to TRK kinase–directed therapy in patients with *NTRK*-rearranged tumours, it is expected that acquired resistance to therapy will ultimately emerge in most patients [64]. Consistent with this expectation, previous reports have described the acquisition of secondary mutations in the TRK kinase domain after treatment with entrectinib in 2 patients: *NTRK1* G595R and G667C substitutions were identified in independent resistant clones from a patient with *LMNA*–*NTRK1* fusion–positive colorectal cancer, and a *NTRK3* G623R substitution (homologous to *TRKA* G595R) was identified in a patient with *ETV6–NTRK3* fusion–positive secretory carcinoma of the

salivary glands [65, 66]. In this scenario of resistance mediated by recurrent kinase domain mutations, BAY2731954 (LOXO-195) is a selective TRK TKI whose activity against these acquired mutations was confirmed in enzyme- and cell-based assays and *in vivo* tumour models [64]. In addition, as clinical proof of concept, the first 2 patients with NTRK fusion–positive cancers who developed acquired resistance mutations on larotrectinib were treated with BAY2731954 on a first-in-human basis, utilising rapid dose titration guided by pharmacokinetic assessments [64]. Of note, this approach led to rapid tumour responses and extended the overall duration of disease control achieved with NTRK inhibition in both patients. As BAY2731954 seems to be able to circumvent therapy resistance in *NTRK* fusion–positive cancers that acquired *NTRK* solvent front mutations, sequential treatment with distinct TRK inhibitors may constitute a viable therapeutic option (see also **Supplementary Table 3**).

Another emerging compound is TPX-0005 (repotrectinib), which was recently proven effective in overcoming resistance due to acquired solvent-front mutations involving *ROS1*, *NTRK1-3*, and *ALK* [67], thus representing a therapeutic option for patients who have progressed on earlier-generation TKIs.

In the scenario of lack of clinical response during first line anti-NTRK treatment, testing for *NTRK* mutations is recommended.

As an additional perspective, it is important to note that TRK inhibitors have been administered so far mainly to patients with metastatic disease; however, the efficacy of larotrectinib has been assessed also on selected patients with locally advanced disease. Two children with locally advanced infantile fibrosarcoma of the knee experienced substantial tumour shrinkage, allowing more conservative surgery with curative intent [30]. These data highlight the possible benefits of neoadjuvant therapy for patients with non-metastatic cancers bearing *NTRK* fusions [30].

CONCLUSIONS AND FUTURE PERSPECTIVES

NTRK fusions can be detected at high frequency in a handful of specific histologies, namely secretory carcinoma of the breast and of the salivary glands, congenital fibrosarcoma and cellular

mesoblastic nephroma, or identified at low frequency in a plethora of malignancies, and define a unique molecular subgroup of advanced solid tumours that can be targeted by specific agents. TRK inhibition has been proven highly effective leading to durable responses that have been observed without regard to the age of the patient, tumour tissue and fusion gene partner. To advance with these promising agents, it is critical to define optimal approaches for the identification of *NTRK1/2/3* fusion genes. The main techniques used in *NTRK* fusion detection are IHC, FISH, RNA-based and DNA-based NGS assays.

When using a diagnostic test to identify patient populations with low prevalence molecular alterations, efficiency and cost challenges should be considered. In this respect, pan-TRK immunohistochemistry has been demonstrated to represent a time-efficient and reliable screen for the detection of *NTRK* fusions and a two-step approach could be considered in clinical trials as well as in clinical practice. In cases displaying any degrees of protein expression, a multigene panel would be recommended to confirm or disprove the suspected genetic alteration. Nevertheless, gene panels offer the possibility to assess a relatively high number of actionable genes in a single assay, thus providing a higher degree of detail in the genomic landscape of the neoplastic lesions under investigation that may be key to plan the best therapeutic strategy for individual patients if used front-line. Among the different options of gene panels, with respect to detection of *NTRK* fusions targeted RNA-sequencing methods may represent the gold standard for screening, provided that the RNA quality is optimal. In addition, we should also consider that whenever tissue availability is limited, a DNA/RNA approach may be preferred if DNA and/or RNA has already been extracted and is available due to other molecular tests already performed on that tissue; IHC could be used to confirm the presence of the fusion.

Following the review of the literature on the available methods for the detection of *NTRK* gene fusions, the ESMO Translational Research and Precision Medicine Working Group recommendations for the implementation of a rational approach for the detection of *NTRK1/2/3* fusion genes in human malignancies are summarized in **Figure 2** and include:

1) In case *NTRK* fusions need to be assayed in a specific histological tumour type, where *NTRK* genes are known to be highly recurrently rearranged with specific partners (histology-based/confirmation-approach), any method is applicable as long as validated in a CLIA laboratory. In this scenario, FISH with specific probes or nested RT-PCR represent perhaps the most cost-effective assays. Another increasingly used method is targeted RNAseq.

2) In case *NTRK* fusions need to be screened in an unselected population (histology-agnostic screening approach):

a) scenario A – no availability of a targeted sequencing assay (multigene panel): perform IHC (if no smooth muscle or neuronal differentiation is present) and send to external sequencing any detected positivity (two-step approach);

b) scenario B – availability of a targeted sequencing assay (multigene panel): depending on the workload and cost-efficacy analyses performed at each Institution, perform either front-line sequencing assay or adopt a two-step approach (IHC followed by targeted sequencing). If front-line sequencing is feasible, RNA sequencing methods represent the gold standard for screening, provided that the RNA quality is optimal. Nevertheless, we should acknowledge that the most exhaustive approach would be to: i) use targeted DNA sequencing assay (multigene panel) upfront in all patients; ii) for mitogenic driver-negative patients, perform targeted RNAseq (multigene panel); iii) use IHC to confirm protein expression of *NTRK* fusions, as we should keep in mind that the protein kinase is the pharmacological target.

A final consideration should be dedicated to the definition of the population that should be tested. At present, systematic analyses of large cohorts of metastatic cancer patients for the presence of *NTRK1/2/3* fusion genes across cancer types have yet to be carried out. Therefore, phenotypic features of cancers harbouring *NTRK1/2/3* fusion genes have yet to be fully characterized. Some guidelines, and in particular those by the National Comprehensive Cancer Network on non-small cell lung cancer, have already included a recommendation for *NTRK* gene fusion testing in patients with metastatic disease [68].

Based on these premises we believe that, outside those tumour types where *NTRK* fusions are expected at high frequency, a more conservative approach for the time being should be applied not to miss patients harbouring these targetable genetic alterations. Therefore, we would argue that the population to be tested should be represented by “any malignancy at an advanced stage, in particular if it has been proven wild type for other known genetic alterations tested in routine practice, and especially if diagnosed in young patients”.

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FIGURE LEGENDS

Figure 1: Schematic representation of the different *NTRK* fusion genes, illustrating the *NTRK* domains maintained across fusion genes regardless of the 5' partner. Tumour types in which the different fusions have been described are reported. Data are extrapolated from Vaishnavi et al. [12], Prabhakaran et al. [69], Qaddoumi et al. [70], Milione et al. [34] and Zehir et al. [13]. Fusion proteins are not drawn to scale.

Legend: GB: glioblastoma; CNS: central nervous system; CRC: colorectal carcinoma; CUP: cancer of unknown primary; KD: kinase domain; MASC: mammary analogue of secretory carcinoma (i.e. secretory carcinoma of the salivary glands); PTC: papillary thyroid carcinoma; TM: transmembrane. *Colorectal carcinoma, papillary thyroid carcinoma, paediatric glioma, sarcoma, lung adenocarcinoma; **: astrocytoma, including pylocitic astrocytoma, paediatric high-grade glioma and adult glioblastoma; ***: secretory breast carcinoma, secretory carcinoma of the salivary glands, acute myeloid leukaemia, papillary thyroid carcinoma, paediatric gliomas, congenital fibrosarcoma, cellular mesoblastic nephroma, and colorectal carcinomas.

Figure 2: Summary of the ESMO Translational Research and Precision Medicine Working Group recommendations. Following the review of the literature on the available methods for the detection of *NTRK* gene fusions, the Working Group has agreed on a strategy for the implementation of a rational approach for the detection of *NTRK1/2/3* fusion genes in human malignancies, as depicted here. In the scenario where the presence of an *NTRK* fusion needs to be confirmed (this happens for patients affected by tumours in which *NTRK* fusion are known to be highly prevalent if not pathognomonic of the lesion) any technique could work in principle, nevertheless the best options as confirmatory techniques are FISH, RT-PCR or RNA-based targeted panels.

In the scenario where the challenge is the identification of *NTRK* fusions in an unselected population, the possibility to use an NGS targeted panel (DNA- or RNA-based) that reliably detects *NTRK* fusions would be ideal. In particular, if the RNA quality is optimal targeted RNA sequencing methods may represent the gold standard for screening. If an *NTRK* fusion is identified, then the most

exhaustive approach would include the use of IHC to confirm protein expression of the detected *NTRK* fusions, as the protein kinase is the pharmacological target.

Alternatively, a “two-step approach” could be considered, which includes IHC first and confirmation of any positivity detected with IHC by NGS (a service that could be externalized).

*: this population would be likely represented by “any malignancy at an advanced stage, in particular if it has been proven wild type for other known genetic alterations tested in routine practice, and especially if diagnosed in young patients”.

Figure 1

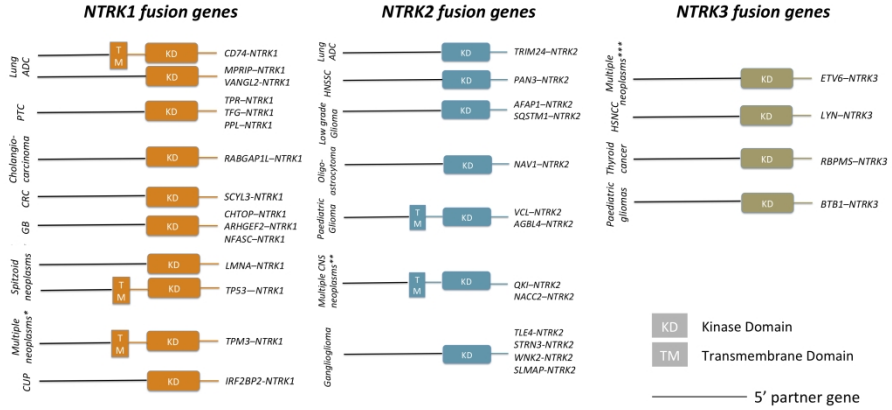

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Figure 2

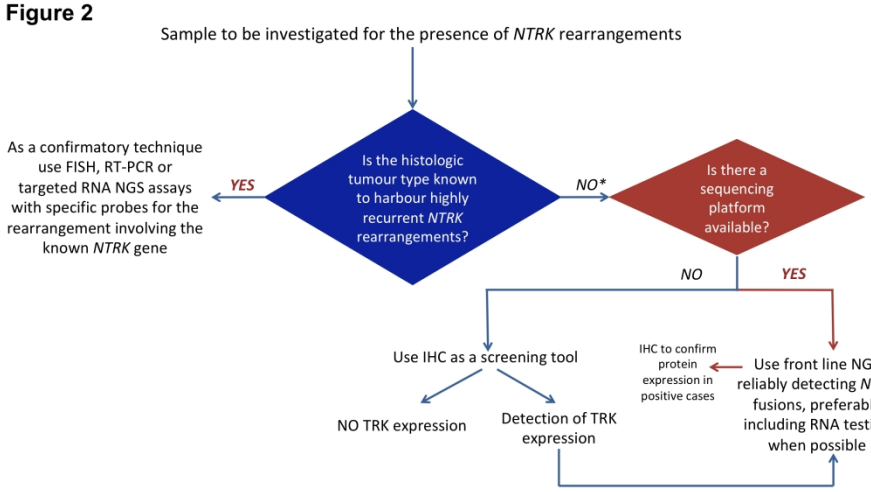

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Table 1: Summary of main features, strengths and weaknesses of all available techniques to detect *NTRK* rearrangements.

Method	Sensitivity	Specificity	Detection of all fusion genes	Detecton of partner	Detection of expression	Screening
IHC	High[1]	High[2]	Yes	No	Yes	Yes
FISH[3]	High	High	One per probe	No	No	No
RNA seq NGS	High	High	Yes	Yes	Yes	Yes
DNA seq[3]	Moderate	High	Yes	Yes	No	Yes

[1]false negatives reported mainly in *NTRK3* fusions;
[2]in the absence of smooth muscle/ neuronal differentiation;
[3]detected rearrangements by DNA-based assays may not result in fusions, correlation with surgical pathology and predicted transcript (for sequencing) is needed.