

Detection of NTRK gene fusions in sarcomas: a comparative study of Pan-TRK immunohistochemistry, FISH, and RNA-Based NGS

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Summary

Background. Fusion involving *NTRK1*, *NTRK2* and *NTRK3* are oncogenic driver occurring in several adult and pediatric tumor types. In sarcomas they are mostly found in infantile fibrosarcoma, inflammatory (IFS), inflammatory myofibroblastic tumor (IMT) and in the so-called “*NTRK*-rearranged spindle cell neoplasm” entity described in the current WHO (2020) classification, including lipofibromatosis-like neural tumor, fibrosarcoma-like and malignant peripheral nerve sheath tumor-like spindle cell neoplasms.

Methods. We retrospectively reviewed 92 soft tissue and bone sarcomas diagnosed at the Rizzoli Institute between 2019 and 2023, in which pan-TRK IHC was performed. 17 tumours showed positive staining and were further assessed using FISH for *NTRK1*, *NTRK2*, and *NTRK3* rearrangements. A subset of 12 cases underwent RNA-based NGS for fusion detection.

Results. In total, we collected data from 17 patients who tested positive for pan-TRK antibody and compared pan-TRK IHC and molecular testing for the detection of *NTRK* rearrangement in sarcomas. FISH analysis detected *NTRK* rearrangements in 4/17 cases (23.5%), while NGS confirmed *NTRK* fusions in 3/12 cases (25%). All *NTRK* fusion-positive cases confirmed by both FISH and NGS showed diffuse pan-TRK staining. Two additional cases exhibited pan-TRK diffuse positivity but were *NTRK* wild-type by NGS and harboured *BCOR::MAML1* and *EWSR1::NACC2* fusions, respectively. 1 case with focal positivity by immunohistochemistry and *NTRK* rearrangement by FISH was not confirmed by NGS.

Conclusions. Pan-TRK IHC can be considered an initial screening tool to identify sarcomas potentially harbouring *NTRK* fusions. However, the presence of diffuse pan-TRK immunoreactivity in *NTRK*-wild-type tumours highlights the need for cautious interpretation of IHC results. FISH may represent a useful intermediate diagnostic tool when NGS is unavailable but requires cautious interpretation particularly in cases with atypical or isolated signals. NGS-based molecular confirmation remains essential for the definitive identification of *NTRK* fusions.

Keywords: *NTRK* fusion, pan-TRK immunohistochemistry (IHC), fluorescent in situ hybridisation (FISH), next-generation sequencing (NGS), sarcoma

Introduction

The tropomyosin receptor kinase (TRK) family comprises transmembrane proteins – TRKA, TRKB, and TRKC – that are physiologically expressed in neural tissues and play a crucial role in neuronal development and neurophysiological functioning. These receptors are encoded

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by the *NTRK1*, *NTRK2*, and *NTRK3* genes, located on chromosomes 1q23.1, 9q21.33, and 15q25.3, respectively. Each TRK receptor consists of three main domains: an extracellular ligand-binding domain, a single transmembrane region, and an intracellular domain containing the conserved tyrosine kinase (TK) region, which is essential for signal transduction^{1,2}.

Wild-type TRK receptors are activated through homo-dimerization and transphosphorylation, processes that are mediated by the binding of neurotrophin ligands such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin-3 (NT-3). The dimerization of TRK leads to the activation of multiple downstream signal transduction pathways, including the MAPK pathway, the PI3K-AKT pathway, and the phospholipase C-gamma (PLCγ) pathway. These pathways regulate key cellular functions such as proliferation, differentiation, and survival in both normal and neoplastic neuronal cells³.

NTRK genes are implicated in oncogenic fusions that result in ligand-independent constitutive activation of TRK signaling. Such fusions have been identified in a wide spectrum of pediatric and adult tumors. Among mesenchymal neoplasms, they are mostly found in infantile fibrosarcoma (IFS), inflammatory myofibroblastic tumors (IMTs), and in a provisional tumour category introduced in the 2020 WHO Classification of Soft Tissue and Bone Tumors: “spindle cell neoplasm with *NTRK* fusion.” This latter category encompasses lipofibromatosis-like neural tumors (LPFNTs), lipofibromatosis-like-neural tumors (LPFNTs) fibrosarcoma-like and malignant peripheral nerve sheath-like tumors⁴⁻⁷.

The prevalence of *NTRK* fusions in both pediatric and adult sarcomas is reported to be less than 1%^{8,9}. To date, various types of *NTRK* fusions have been described in sarcomas, including *LMNA::NTRK1* and *EML4::NTRK3*. The *LMNA::NTRK1* fusion has been identified in low-grade sarcomas with myopericytoma-like features and in uterine spindle cell sarcomas, whereas the *EML4::NTRK3* fusion has been reported in infantile fibrosarcoma^{5,10}.

The *ETV6::NTRK3* fusion, resulting from the t(12;15) (p13;q25) translocation, is one of the earliest identified and most well-characterized *NTRK* rearrangements. It represents the canonical fusion for infantile fibrosarcoma, occurring in approximately 90% of cases⁷.

These fusions result from either intrachromosomal or interchromosomal rearrangements involving *NTRK* genes and lead to the in-frame juxtaposition of the 3' region of an *NTRK* gene (encoding the C-terminal tyrosine kinase domain) with the 5' sequence of a fusion partner gene (typically encoding an N-terminal dimerization/ oligomerization domain). This fusion

configuration enables constitutive activation or overexpression of the kinase domain, thereby contributing to oncogenic potential.

The partner gene generally contributes the 5' promoter region, driving aberrant expression of the chimeric transcript^{1,11,12}. *NTRK* gene rearrangements have recently emerged as actionable targets for molecular therapy with novel compounds that selectively inhibit the constitutively active fusion proteins generated by these genetic alterations¹¹.

Entrectinib and larotrectinib are the first tumour-agnostic tyrosine kinase (TK) inhibitors to receive FDA and EMA approval for the treatment of unresectable, locally advanced, or metastatic solid tumors harboring an *NTRK* gene fusion. Larotrectinib is the most specific of the two, selectively targeting TRKA, TRKB, and TRKC, whereas entrectinib additionally inhibits ROS1 and ALK kinases. These agents have demonstrated antitumour efficacy irrespective of the *NTRK* fusion type, tumour histology, or anatomical site of origin, in both adult and pediatric populations.

Given this broad therapeutic applicability, it is crucial to implement diagnostic strategies that can be routinely used to screen tumors for the presence of *NTRK* fusions^{13,14}.

The detection of *NTRK* gene fusions for patient selection in targeted therapy can be performed on clinical specimens using various methodologies. Current international guidelines from ASCO and ESMO recommend immunohistochemistry (IHC) as an initial screening tool, followed by confirmation with RNA-based next-generation sequencing (NGS), which is considered the most sensitive and specific approach^{15,16}. Another available method for the detection of *NTRK* rearrangement is fluorescence in situ hybridization (FISH)^{16,17}.

In the present study, we reviewed a series of soft tissue and bone sarcomas that were evaluated for pan-TRK expression by immunohistochemistry (IHC) and for *NTRK1*, *NTRK2*, and *NTRK3* rearrangements by FISH. The objective was to assess and compare the diagnostic utility of IHC and FISH in identifying *NTRK* gene rearrangements within this group of tumors. Furthermore, we correlated IHC and FISH positivity with the presence of *NTRK* fusions confirmed by next-generation sequencing (NGS).

Materials and methods

PATIENTS

Following approval by the institutional ethics committee (protocol code: CE AVEC: 59/2024 Oss/IOR;

approval date: 14 February 2024), we retrospectively analyzed a series of 92 soft tissue and bone sarcomas diagnosed at the Rizzoli Institute over a 5-year period (2019-2023), in which pan-TRK immunohistochemistry had been performed for diagnostic or predictive purposes. The study cohort included tumors with spindle cell morphology not clearly suggestive of a specific diagnostic entity and clinically aggressive mesenchymal tumors, such as dedifferentiated chondrosarcomas in which pan-TRK testing was requested for predictive purposes.

PAN-TRK IMMUNOHISTOCHEMISTRY (IHC)

All tumor specimens were fixed in 10% buffered formalin and routinely processed for histological evaluation. Pan-TRK immunohistochemical staining for TRKA, TRKB, and TRKC expression was performed using a commercially available assay (rabbit monoclonal antibody, clone EPR17341, RTU 28 µg/ml, Roche, Ventana, Tucson, AZ, USA). This antibody targets a homologous region near the C-terminus that is preserved in both fusion and wild-type TRK proteins.

Formalin-fixed paraffin-embedded (FFPE) tissue blocks were sectioned at 3 µm thickness, mounted on slides, and incubated at 58 °C for 2 hours. All staining procedures were carried out on the BenchMark Ultra automated staining platform according to the manufacturer's instructions (Ventana Medical Systems, Tucson, AZ, USA), using the OptiView DAB Detection Kit, as previously described by Benini et al.¹⁸.

Non-neoplastic brain tissue was included as a positive control in each run. The antibody was incubated for 16 minutes at 36 °C, following antigen retrieval with Cell Conditioning 1 (CC1, pH 8) for 88 minutes at 100 °C. All stained slides were independently evaluated and scored by two pathologists (MG and AR). Immunoreactivity was assessed using a semi-quantitative scale as detailed in Table I¹⁹.

Cases were considered positive if more than 1% of tumor cells showed staining at any intensity above background, in any subcellular localization pattern, including membranous, cytoplasmic, nuclear or combinations thereof¹⁷.

Table I. Semiquantitative scoring system used for interpreting pan-TRK immunohistochemical staining in tumor cells.

Percentage of stained tumor cells (%)	Interpretation
1-10	Rare Positive
11-49	Focal Positive
> 50	Diffuse Positive

FLUORESCENCE IN SITU HYBRIDIZATION (FISH)

Fluorescence in situ hybridization (FISH) for the detection of *NTRK1*, *NTRK2*, and *NTRK3* gene rearrangements was performed on interphase nuclei using three commercially available break-apart probe kits: ZytoLight SPEC *NTRK1* Dual Colour Break-Apart Probe, ZytoLight SPEC *NTRK2* Dual Colour Break-Apart Probe, and ZytoLight SPEC *NTRK3* Dual Colour Break-Apart Probe (ZytoVision, Bremerhaven, Germany). Each probe is designed to label the 3' region with ZyOrange and the 5' region with ZyGreen, enabling detection of gene rearrangements involving *NTRK1*, *NTRK2*, and *NTRK3*, respectively.

For each case, representative 3-µm-thick FFPE tissue sections were prepared. Slides were incubated overnight at 60 °C, deparaffinized in xylene, and dehydrated in a graded ethanol series. Pretreatment was performed using the Histology FISH Accessory Kit (Dako, Glostrup, Denmark), according to the manufacturer's protocol, as previously described²¹.

A serial hematoxylin-eosin-stained section was prepared from each tumour, and areas of viable, non-necrotic neoplastic tissue were marked by a pathologist to guide the hybridization procedure.

To interpret FISH results, at least 100 non-overlapping tumor nuclei exhibiting strong, well-delineated signals were visually examined under an Olympus BX41 fluorescence microscope (Tokyo, Japan) at 100× magnification using dual-spectrum filters (Spectrum Green and Spectrum Orange). A case was considered positive for *NTRK* rearrangement based on the identification of one of the following two signal patterns: (1) A classic break-apart pattern, defined by one intact fusion signal along with two split signals (3' orange and 5' green) separated by a distance of at least three signal diameters. (2) An atypical pattern, consisting of one fusion signal and a single isolated 3' orange signal in the absence of a corresponding green signal. Isolated orange signals are indicative of either 5' *NTRK* region deletion or unbalanced translocations involving this chromosomal segment. (3) Genomic aberration due to small deletion, duplications or inversion might result in inconspicuous patterns. These signal patterns should be further investigated.

To minimize subjectivity, each case was independently evaluated in a double-blinded manner by at least two experienced analysts. Any discrepancies in signal interpretation were subsequently resolved through consensus review.

Tumors were defined as FISH-positive if at least 10% of tumor cells demonstrated either of these rearrangement patterns.

Copy number alterations for *NTRK1*, *NTRK2*, and *NTRK3* genes were also assessed. Copy number gain

was defined as a mean of 3-5 signals in $\geq 10\%$ of analysed nuclei. Images were acquired using a Colour View III CCD camera (Olympus) and processed with CytoVision imaging software, version 7.5 (Leica Biosystems, Richmond, USA).

ANCHORED MULTIPLEX PCR (AMP)-BASED TARGET NGS

Twelve samples were analyzed using an RNA-based anchored multiplex sequencing assay, as previously described¹⁸. Briefly, total RNA was extracted from FFPE specimens using the RNeasy FFPE Tissue Kit (Qiagen GmbH, Hilden, Germany), following the manufacturer's protocol. Sections measuring 6-8 μm in thickness were cut from representative paraffin blocks for RNA extraction.

RNA quantity was determined using a Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). Libraries were prepared using the Archer FusionPlex Sarcoma v2 panel, which includes 659 unidirectional gene-specific primers (GSPs) targeting 63 genes known to be involved in oncogenic fusions in sarcomas.

For library construction, 200 ng of total RNA was used as input, in accordance with the manufacturer's instructions. Initial RNA quality was assessed using the Archer PreSeq RNA Quality Control (QC) assay. cDNA libraries were generated only if the sample met the threshold for acceptable quality (PreSeq qPCR crossing point ≤ 30). In this assay, the quality of first-strand cDNA synthesis is used as a direct measure of RNA integrity. Briefly, prior to library preparation, all samples undergo real-time PCR-based quality control using VCP primers (Archer® PreSeq™ RNA QC Assay), as required by the Archer protocol for assessing RNA quality. Only samples that meet the VCP threshold proceed to library construction. Specifically, a Ct value > 30 (PreSeq qPCR crossing point ≤ 30) indicates low RNA quality, and such samples are deemed insufficient for testing. After sequencing, the Archer Analysis software performs additional quality control assessments within the analysis pipeline. Key quality metrics, including Fusion QC, are provided based on a pool of Gene-Specific Primers (GSPs) targeting four control gene transcripts: CHMP2A, GPI, RAB7A, and VCP. Known to be moderately expressed across most tissue type. The Fusion QC score represents the average number of unique RNA start sites calculated per control GSP2. A Fusion QC score greater than 10 is required to ensure adequate RNA quality for reliable assay results. Notably, the Fusion QC score increases as the Ct value decreases, reflecting higher RNA integrity. All purification steps during library preparation were performed using Agencourt AMPure XP beads

(Beckman Coulter, Brea, CA, USA). Purified libraries were quantified using the Ion Library TaqMan Quantitation Kit (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) and subsequently pooled to achieve equimolar concentrations of 25 pM.

The prepared libraries (anchored multiplex PCR amplicons) were loaded onto an Ion 510 chip using the Ion Chef System (ThermoFisher Scientific, Waltham, MA, USA) and sequenced on the Ion Torrent S5 next-generation sequencing platform.

Sequencing reads were aligned to the human reference genome (GRCh37/hg19), and data files (BAM format) were analysed using the Archer Analysis software (Web Application v7.1.0.38; Pipeline Version 7.1.0-14; ArcherDx, Invitae, San Francisco, CA) under default settings.

Tumors were considered *NTRK1-3* fusion-positive when an in-frame fusion involving the kinase domain of *NTRK* was identified.

STATISTICAL ANALYSIS

Data were analyzed descriptively. Results are reported as absolute numbers and percentages. Concordance between pan-TRK immunohistochemistry, FISH, and NGS was assessed descriptively (observed agreement among the cases tested). Due to the retrospective design and stepwise application of molecular testing only to Pan-TRK-positive cases, formal calculations of sensitivity, specificity, or predictive values were not possible.

Results

PATHOLOGICAL DIAGNOSIS

The majority of cases were classified as malignant spindle cell mesenchymal neoplasms, arranged in fascicles to herringbone pattern, and lacking specific morphological and immunohistochemical differentiating features. Exceptions included cases 10, 12, and 13, which showed pleomorphic morphology with immunohistochemical expression of myogenic markers; case 5, characterized by pleomorphic features with pleomorphic lipoblasts; four malignant peripheral nerve sheath tumor (MPNST) showing loss of H3K27me3 expression; and case 11, showed MDM2 amplification. These pleomorphic cases belonged to the subgroup of tumors selected for predictive purposes and were molecularly investigated based on clinical indications related to tumor aggressiveness rather than diagnostic uncertainty.

PAN-TRK IMMUNOHISTOCHEMISTRY

We retrieved 92 patients with a diagnosis of soft tissue and bone sarcoma in which Pan-TRK expression was tested by immunohistochemistry (IHC). Of these, 17 patients exhibited positive pan-TRK IHC results. Staining patterns was diffuse (5/17), focal (11/17) and rare positive cells (1/17). Regarding subcellular localisation, 16 samples showed cytoplasmic TRK protein expression; among these, five also demonstrated additional membranous expression.

One sample (case 9) showed both cytoplasmic and nuclear membrane expression, while another sample (case 7) showed only membranous expression (Fig. 1). Notably, isolated cytoplasmic staining was the most common pattern occurring in 10 out of 17 cases. Immunohistochemical patterns and the distribution of subcellular localization for TRK protein are summarized in Table II. It is also worth noting that two cases (case 3 and case 4) had been previously reported^{20,21}.

FISH ANALYSIS

Interphase FISH analysis for *NTRK1*, *NTRK2*, *NTRK3* was performed in all 17 cases; however, one case (case 13) was not evaluable due to poor signal quality and was therefore classified as not adequate (NA).

NTRK1 rearrangements were identified in three cases (cases 8, 9, and 16) and an *NTRK3* rearrangement in one case (case 4); all positive cases met the criterion of > 10% tumor cells exhibiting break-apart signals.

In case 8 and 9, *NTRK1* FISH analysis revealed the presence of one single orange signal mapping to the 3' region of *NTRK1*, combined with a loss of the green signal corresponding to the 5' region (Fig. 1). One case (case 16) showed *NTRK1* split-apart signals approximately in 15% of analyzed tumor cells along with copy number gain. Regarding the *NTRK3*-positive tumor (case 4), a classic break apart signal was observed in approximately the 50% of tumor cells, with a separation distance of at least three signal diameters together with an increase of the 3' region of *NTRK3*. Copy number gain involving *NTRK1*, *NTRK2*, and *NTRK3* was

Table II. Summary of histopathological, immunohistochemical and molecular data.

Case ID	Pathologic diagnosis	Pan TRK staining pattern	Pan-TRK subcellular pattern	NTRK FISH	FISH Pattern	Fusion identified by NGS sequencing
1	MPNST	Focal positive	C	Neg	-	No fusion detected
2	Spindle cell sarcoma	Rare positive	C	Neg	-	NT
3	MPNST	Diffuse positive	C+M	Neg	-	BCOR::MAML1
4	<i>NTRK</i> -rearranged spindle cell neoplasm	Diffuse positive	C+M	Pos <i>NTRK3</i>	Break-apart + extra 3'	EML4(e2):: <i>NTRK3</i> (e14)
5	Pleomorphic liposarcoma	Focal positive	C	Neg	-	No fusion detected
6	Spindle cell sarcoma	Diffuse positive	C+M	Neg	-	EWSR1(e7)::NACC2(e3)
7	Spindle cell sarcoma	Focal positive	M	Neg	-	No fusion detected
8	<i>NTRK</i> -rearranged spindle cell neoplasm	Diffuse positive	C+M	Pos <i>NTRK1</i>	Isolated 3'	LMNA(e2):: <i>NTRK1</i> (e11)
9	<i>NTRK</i> -rearranged spindle cell neoplasm	Diffuse positive	C+NM	Pos <i>NTRK1</i>	Isolated 3'	LMNA(e2):: <i>NTRK1</i> (e11)
10	Leiomyosarcoma	Focal positive	C	Neg	-	No fusion detected
11	Dedifferentiated liposarcoma	Focal positive	C	Neg	CNG <i>NTRK1</i> / <i>NTRK2</i> / <i>NTRK3</i>	NT
12	Leiomyosarcoma	Focal positive	C+M	Neg	-	NT
13	Leiomyosarcoma	Focal positive	C	NA	-	NT
14	MPNST	Focal positive	C	Neg	-	No fusion detected
15	Lipofibromatosis	Focal positive	C	Neg <i>NTRK1</i>	<i>NTRK2</i> / <i>NTRK3</i> NT	No fusion detected
16	Spindle cell sarcoma	Focal positive	C	Pos <i>NTRK1</i>	CNG <i>NTRK1</i>	No fusion detected
17	MPNST	Focal positive	C	Neg	-	NT

MPNST, Malignant Peripheral Nerve Sheath Tumor; C, cytoplasm; M, cell membrane; NM, nuclear membrane; Pos, positive; Neg, negative; FISH, Fluorescence *in situ* hybridization; NA, not adequate; NT, not tested; CNG, copy number gain; NGS, Next Generation Sequencing; e, exon.

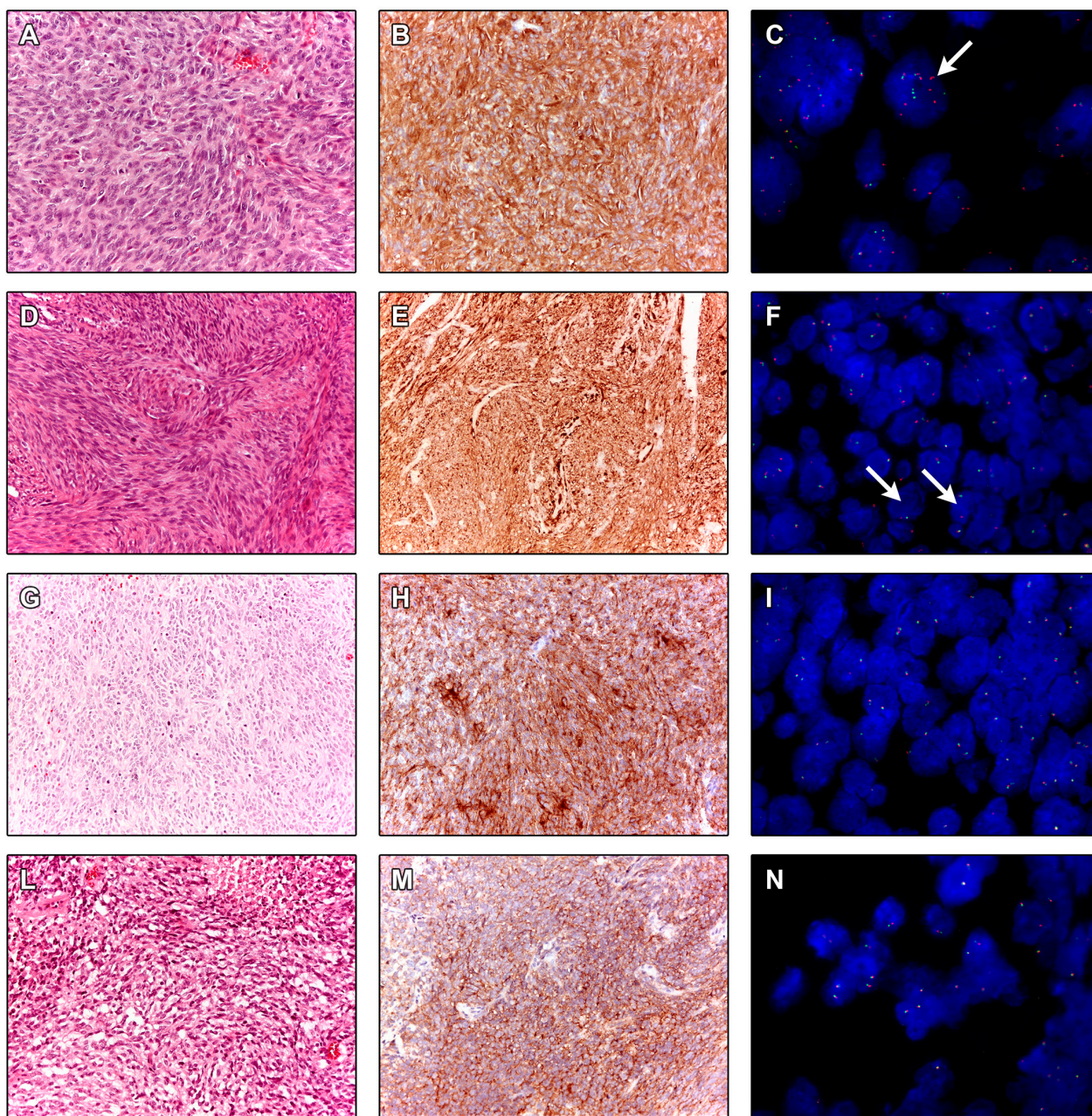


Figure 1. (A, D, G, L): Hematoxylin and eosin (H&E) staining of sarcomas harbouring *EML4::NTRK3* (case 4) (A), *LMNA::NTRK1* (case 8) (D), *BCOR::MAML1* (case 3) (G), and a fusion-negative sarcoma (case 7) (L). Original magnification: 20×. (B, E, H, M): Immunohistochemical staining with pan-TRK antibody showing diffuse cytoplasmic and membranous positivity in fusion-positive cases (cases 4, 8, 3) and focal membranous staining in the fusion-negative case (case 7). Original magnification: 20×. (C, F, I, N): Representative FISH images for *NTRK1* and *NTRK3*. (C): *NTRK3* FISH showing an atypical rearrangement pattern (white arrow) with one or more fusion signals and additional 5' (green) or 3' (red) signals in the *EML4::NTRK3* fusion-positive tumour (case 4). (F): *NTRK1* FISH demonstrating an atypical pattern (white arrow) with one fusion signal and a single 3' (red) signal in the absence of the 5' (green) signal, suggesting an interstitial deletion on chromosome 1q22 in the *LMNA::NTRK1* fusion (case 8). (I, N): Typical wild-type FISH pattern with two fusion signals observed in the *BCOR::MAML1* fusion-positive sarcoma (case 3) (I) and in the fusion-negative sarcoma (case 7) (N). Original magnification: 100×.

detected in case 11. Sample 15 had insufficient tumor tissue for a complete FISH analysis. No *NTRK2* rearrangements were identified in this series.

Overall, among the pan-TRK IHC-positive cases, concordant FISH results were observed in 4 of 17 cases (23.5%).

NGS FUSION DESCRIPTION

NGS RNA analysis was successfully performed in 12 patients. The remaining five cases were not analyzed by NGS due to lack of available biological material. (Tab. I). An *EML4::NTRK3* fusion was detected in the case (case 4) that exhibited an *NTRK3* rearrangement by FISH, whereas an *LMNA::NTRK1* fusion was identified in two patients (cases 8 and 9) with *NTRK1* rear-

rangements confirmed by FISH (Fig. 2). The *EML4::NTRK3* fusion detected in this analysis involved exon 2 of *EML4* (NM_019063.3) and exon 14 of *NTRK3* (NM_002530.3).

The genomic coordinates of the RNA fusion junction localised to Chr2:42472827 and Chr15:88576276, respectively. Notably, both the coiled-coil domain of the *EML4* gene and the tyrosine kinase domain at the C-terminus of *NTRK3* were preserved. In the intrachromosomal *LMNA::NTRK1* fusion, exon 2 of *LMNA* (NM_005572.3) was joined to exon 11 of *NTRK1* (NM_002529.3) in both patients (cases 8 and 9). In these two *LMNA::NTRK1* fusion-positive cases, the genomic coordinates of the RNA fusion junction were localized to Chr1:156100564 for *LMNA* and

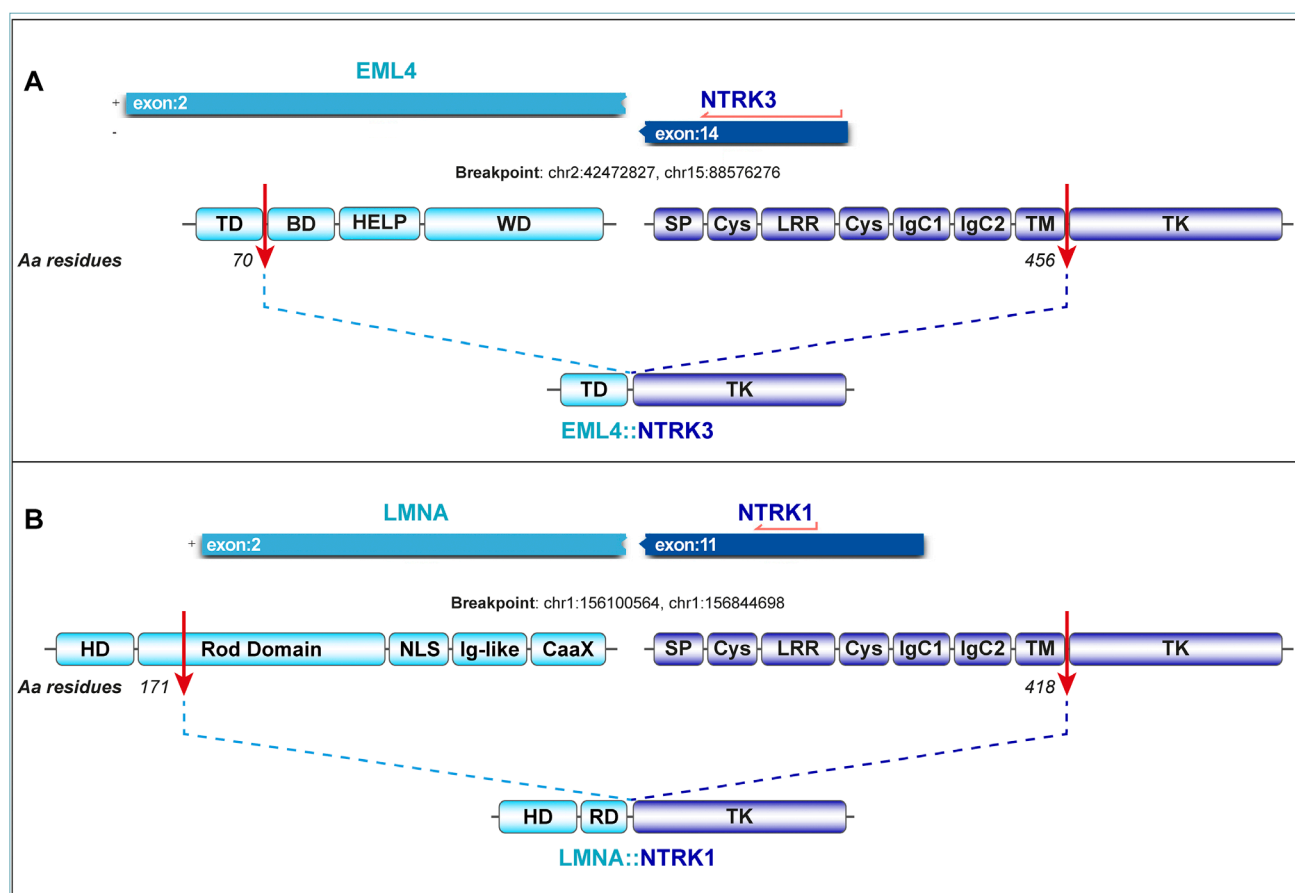


Figure 2. Visualization of *NTRK* fusion transcripts detected by RNA-based next-generation sequencing using the Archer Fusion-Plex Sarcoma Panel. The diagram illustrates the genes and exons involved, genomic breakpoint locations, and predicted functional domains. Horizontal red arrows in panels (A) and (B) indicate the nucleotide locations of the gene-specific primers (GSPs) targeting *NTRK3* and *NTRK1*, respectively, as used in the Archer FusionPlex assay. (A) Anchored multiplex PCR results showing the *EML4::NTRK3* fusion transcript involving exon 2 of *EML4* and exon 14 of *NTRK3*. The schematic representation shows the retention of the trimerisation domain (TD/coiled-coil domain) of *EML4* and the complete tyrosine kinase domain (TK) of *NTRK3*. (B) Anchored multiplex PCR results showing the *LMNA::NTRK1* fusion transcript involving exon 2 of *LMNA* and exon 11 of *NTRK1*. The resulting fusion retains part of the alpha-helical rod domain of *LMNA* and the complete TK domain of *NTRK1*.

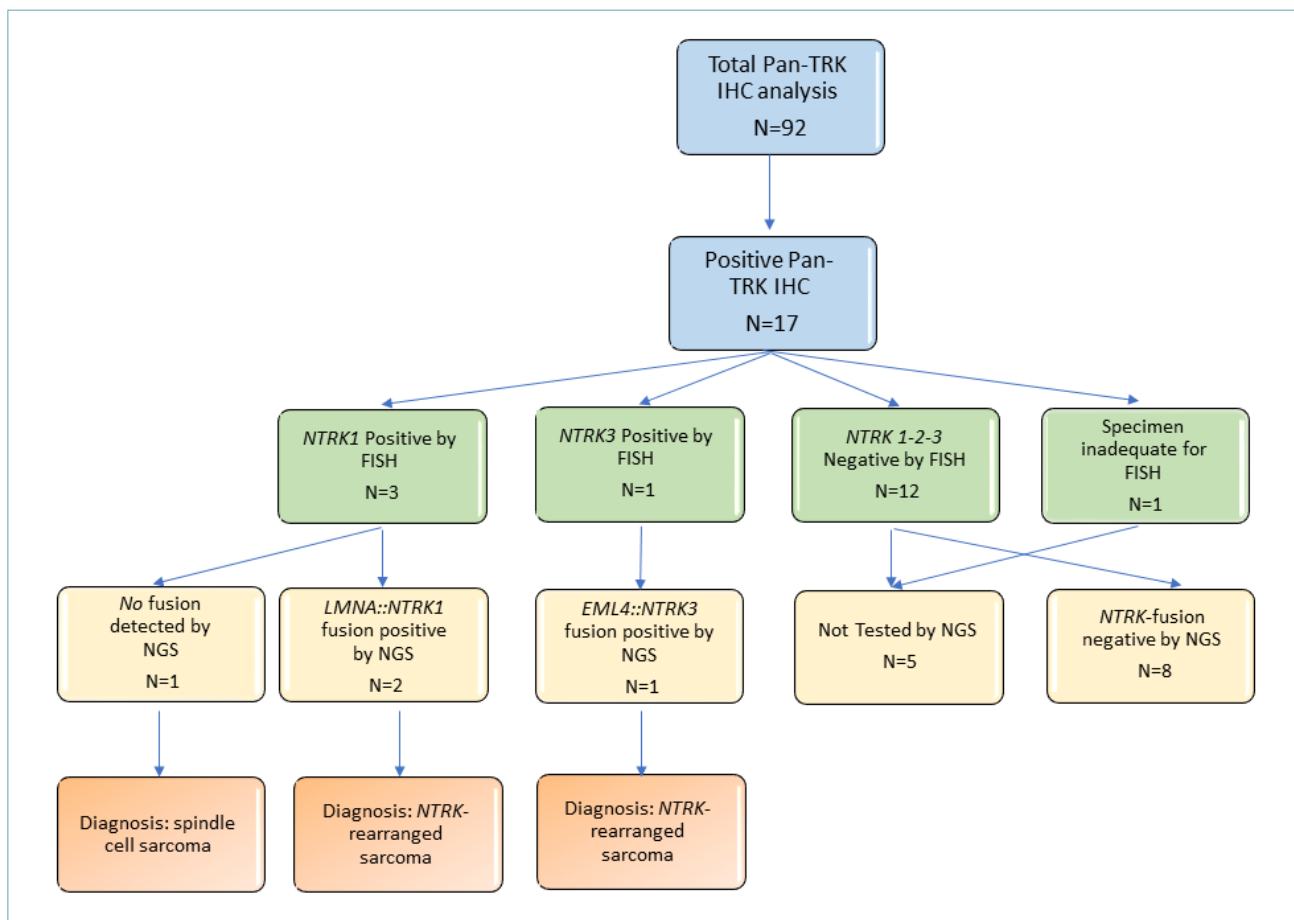


Figure 3. Overview of the diagnostic workflow applied in the study.

Chr1:156844698 for *NTRK*. The resulting fusion transcript retained a portion of the alpha-helical rod domain of *LMNA* and the kinase domain of *NTRK1*.

Each *NTRK* fusion event was predicted to be in-frame according to Archer software. The frame status was further confirmed by manual inspection, comparing the sequences to the consensus coding sequence (CCDS) available in the NCBI database (<https://www.ncbi.nlm.nih.gov/>).

Patient 3 harbored a *BCOR::MAML1* fusion with breakpoints located in exon 15 of the *BCOR* gene and exon 1 of *MAML1*. This rearrangement has previously been described by Cocchi et al.²¹. In patient 6, Archer software identified a novel in-frame *EWSR1::NACC2* fusion, involving exon 7 of *EWSR1* and exon 3 of *NACC2*. No gene fusions were identified in seven cases, while the remaining five cases were not tested. With regard to immunohistochemical correlation, all tumors harboring *NTRK* fusions showed diffuse pan-TRK immunoreactivity; however, diffuse staining was also observed in two cases with non-*NTRK* gene fu-

sions (*BCOR::MAML1* and *EWSR1::NACC2*).

In addition, *LMNA::NTRK1* fusion-positive tumors showed distinct subcellular localization patterns, with predominant cell membrane expression in case 8 and nuclear membrane expression in case 9. The single case harbouring an *EML4::NTRK3* fusion (case 4) showed membranous staining in addition to cytoplasmic staining. Tumors harboring *BCOR::MAML1* and *EWSR1::NACC2* fusions exhibited combined cytoplasmic and membranous pan-TRK expression. Overall, *NTRK* fusions were confirmed by NGS in 3 of the 12 cases analyzed (25%), all of which showed diffuse pan-TRK immunoreactivity providing a descriptive measure of IHC-NGS agreement within the tested subset. However, diffuse pan-TRK staining was also observed in two additional cases that were *NTRK* wild-type by NGS and harbored non-*NTRK* gene fusions. No *NTRK* rearrangements were identified among cases displaying focal pan-TRK staining or rare positive cells.

Figure 3 provides a concise overview of the diagnostic

workflow, integrating results from multiple methodologies and outlining the final diagnosis.

Discussion

In the most recent edition of the WHO Classification of Soft Tissue and Bone Tumors, *NTRK*-rearranged spindle cell neoplasm (excluding infantile fibrosarcoma, IFS) has been recognized as an emerging entity. More recently, it has been demonstrated that *NTRK* gene fusions represent actionable therapeutic targets in cancer, with promising results observed using highly selective tyrosine kinase inhibitors (TKIs) such as larotrectinib and entrectinib^{13,14}. The choice of methodology for detecting *NTRK* fusions must consider both the histological features of the tumor and the resources available within the diagnostic laboratory.

In the context of sarcomas, some morphological and immunohistochemical criteria are suggestive for *NTRK*-rearranged spindle cell neoplasms, as outlined in the WHO Classification of Soft Tissue and Bone Tumors (5th edition), in the chapter on *NTRK*-rearranged spindle cell neoplasm²². Following international guidelines (ESMO, ASCO) we employed pan-TRK immunohistochemistry (IHC) as an initial screening tool to identify sarcomas potentially harbouring *NTRK* fusions¹⁵. Pan-TRK IHC is considered an appropriate first-line assay to triage cases for subsequent molecular confirmation.

The FDA- and CE-IVD-approved clone EPR17341 is currently the most widely used antibody for pan-TRK detection. It targets a conserved C-terminal domain shared by TRKA, TRKB, and TRKC proteins, which is retained in both wild-type and fusion variants^{1,14,16}. Previous large immunohistochemical studies have reported highly variable sensitivity and specificity of pan-TRK expression across a wide range of solid tumors¹⁷.

With respect to sarcomas, low specificity has been reported, particularly in tumors exhibiting neural or myogenic differentiation, as wild-type TRK proteins are physiologically expressed in neural and smooth muscle tissues¹⁶. Additional false-positive staining can occur in non-*NTRK*-fused tumors, including leiomyosarcomas and *BCOR*-rearranged sarcomas^{23,24}. False pan-TRK IHC positivity has been reported to be associated with increased expression of *NTRK* genes, particularly *NTRK3*, which may be upregulated in sarcomas independently of gene rearrangements²⁴. In contrast, false-negative pan-TRK immunohistochemical results have been reported to be relatively uncommon, as shown by Hechtman et al.¹⁷. The diagnostic performance of the pan-TRK antibody clone EPR17341 has been evaluated in large series, showing high sensitivity

for *NTRK* fusion-positive tumors but variable specificity, particularly in mesenchymal neoplasms²⁵.

In our cohort, five pan-TRK-positive tumors were not assessed by NGS due to the unavailability of biological material, a limitation related to the retrospective study design, therefore, reliable estimation of the overall sensitivity of the pan-TRK assay cannot be determined.

As described in recently published studies, different subcellular staining patterns may be observed with pan-TRK immunohistochemistry, all of which are considered positive¹⁷. Notably, subcellular staining patterns have been shown to correlate with the fusion partner, as the latter can influence protein localization, leading to atypical subcellular distribution in contrast to the membrane-associated expression of wild-type TRK proteins¹⁷.

In our series, cytoplasmic staining represented the predominant pattern. Cell membrane and nuclear membrane staining were also observed in a subset of cases.

For patients to be eligible for targeted therapies, confirmation of the fusion event through molecular techniques is required. FISH is a rapid and accessible method for the detection of *NTRK* rearrangements and may be employed for further selection of cases for RNA-based NGS analysis, particularly when upfront NGS testing is unavailable.

However, several interpretative pitfalls may arise with FISH, especially in the presence of subtly split signals or isolated 3' signals which are more common in intrachromosomal rearrangements. These patterns can be challenging to interpret due to their borderline nature, especially when they involve subtle separation of the green and orange signals. Notably, isolated 3' signals do not necessarily correlate with an active fusion event and may instead reflect proximal deletions near the *NTRK1* breakpoint region or unbalanced rearrangements.

Such cases require careful interpretation and, whenever possible, additional molecular validation such as NGS, to confirm true structural rearrangements and their biological relevance. Additional challenges including signal copy number gains, may further complicate interpretation. Given the potential for such ambiguous results, intra-observer reproducibility is essential. In this study, each case was independently reviewed by two experienced analysts to ensure consistency and accuracy in FISH interpretation, thereby minimizing the risk of misinterpretation associated with atypical signal patterns. In our series, all tumors underwent FISH analysis.

Notably, in our series, the two cases showing isolated 3' *NTRK1* signals were those in which an *NTRK1* fu-

sion was subsequently confirmed by molecular analysis, underscoring the diagnostic relevance of these borderline FISH patterns.

Overall, *NTRK* rearrangements were identified in only four cases (23.5%), with *NTRK1* rearrangements observed in three cases and an *NTRK3* rearrangement detected in one case.

These findings support the notion that *NTRK* rearrangements occur in only a small subset of mesenchymal tumors exhibiting pan-TRK positivity.

Beyond the detection of rearrangements, our analysis also revealed increased copy number of *NTRK* genes in two cases. However, as centromeric copy number was not assessed, the presence of polysomy cannot be excluded. Further studies are needed to clarify the biological and clinical significance of this observation. Regarding NGS analysis, two cases with *NTRK1* rearrangement identified by FISH tested positive for an *LMNA::NTRK1* fusion by NGS. In both cases, the *LMNA::NTRK1* fusion was in-frame, involving exon 2 of *LMNA* and exon 11 of *NTRK1*. This represents one of the shortest *LMNA::NTRK1* fusion transcripts reported in the literature. The fusion results from an intrachromosomal rearrangement involving loci on chromosome 1q22 (*LMNA*) and chromosome 1q23.1 (*NTRK1*). Exons 1-2 of *LMNA* encode the N-terminal globular domain and part of the alpha-helical rod domain, suggesting a potential role in the functional activation of the resulting chimeric protein.

In sarcomas, *LMNA::NTRK1* represents the most frequently reported genetic alteration in lipofibromatosis-like neural tumors.

In our experience, one tumor harbouring an *LMNA::NTRK1* fusion exhibited nuclear membrane immunostaining. This pattern appears inconsistent with the nature of the fusion, which involves the loss of the genomic region encoding the nuclear localization signal (NLS). Notably, this staining pattern was not observed in the second case with *LMNA::NTRK1* fusion, which instead demonstrated cell membrane immunoreactivity. Of particular interest, one case displaying focal pan-TRK positivity and an *NTRK1* rearrangement by FISH was negative by NGS.

This finding may reflect structural rearrangements detected by FISH that do not generate a stable or expressed fusion transcript. This phenomenon can be explained by several biological mechanisms, including short-range intrachromosomal rearrangements or microdeletions. Alternatively, low transcript abundance or breakpoints not covered by the targeted RNA panel may have impaired NGS detection. RNA degradation in FFPE tissue is a well-known limitation of fusion assays; however, as detailed in the Methods section, the likelihood that degradation contributed to this NGS-negative

result is minimized in our Archer FusionPlex workflow through specific RNA quality-control steps.

This case highlights that such discordances, although uncommon, can occur and support the use of complementary diagnostic approaches.

This raises the possibility that not all aberrant *NTRK* FISH patterns correspond to true fusion events, highlighting the need for molecular confirmation in selected cases.

In sarcomas, in addition to *LMNA*, *NTRK1* forms fusions with various gene partners, including *TPM3* and *TPR*. All of these fusions result from intrachromosomal rearrangements involving chromosome 1q23.1 (*NTRK1* locus) and either chromosome 1q21.3 (*TPM3*), 1q22 (*LMNA*), or 1q31.1 (*TPR*). These rearrangements can occur via interstitial deletion, as in *LMNA::NTRK1*, or through chromosomal inversion, as in *TPM3::NTRK1* or *TPR::NTRK1*⁴.

The *NTRK3* FISH-positive case in our cohort corresponded to an *EML4::NTRK3* fusion, which was previously reported by Palmerini et al.²⁰. This fusion involves exon 2 of *EML4* joined to exon 14 of *NTRK3*, encompassing the coiled-coil domain of *EML4* and the tyrosine kinase (TK) domain of *NTRK3*. This fusion has been reported in congenital fibrosarcoma (CFS), cervical sarcoma, and spindle cell sarcoma^{26,27}.

In our study, pan-TRK IHC for the *EML4::NTRK3* case demonstrated both cytoplasmic and membranous staining of tumor cells. This staining pattern differs from that observed in other *EML4::NTRK3*-positive tumors, such as CFS, in which both cytoplasmic and nuclear localization have been described.

The presence of a complete *NTRK* tyrosine kinase (TK) domain without frameshift variants is essential for maintaining the oncogenic potential of *NTRK* gene fusions. All *NTRK* fusions identified in this study were predicted to be in-frame, with preservation of the TK domain. Notably, each *NTRK* fusion-positive cases demonstrated diffuse pan-TRK immunoreactivity.

However, we also report two cases displaying diffuse pan-TRK positivity that were *NTRK* wild-type by NGS. These tumors harboured *BCOR::MAML1* and *EWSR1::NACC2* fusions, respectively. In the context of *BCOR*-rearranged sarcomas, pan-TRK positivity has been described by Kao et al., and was attributed to *NTRK3* upregulation²⁴.

Consistently, RNA expression analysis of both *BCOR::MAML1* and *EWSR1::NACC2* fusion-positive cases revealed increased relative expression of *NTRK3*, indicating that pan-TRK immunoreactivity in these tumors is driven by *NTRK3* overexpression rather than by *NTRK* gene fusions (Supplementary Figure 1).

Limitations section

This study was conducted on a retrospective cohort, and only a subset of IHC-positive cases underwent NGS analysis due to clinical factors unrelated to assay performance. Although RNA quality from FFPE tissue is an inherent challenge in targeted RNA-based approaches, the workflow used in this study includes internal controls and quality metrics to ensure technical reliability. Nonetheless, rare fusion variants, very low transcript expression, or breakpoints not represented in the targeted design may theoretically escape detection. While these considerations did not account for the absence of NGS testing in our cohort, they remain relevant in routine diagnostic practice and should be considered when interpreting discrepancies between IHC, FISH, and NGS results.

Conclusion

In this study, we compared pan-TRK immunohistochemistry and molecular testing for the detection of *NTRK* rearrangements in sarcomas. FISH analysis performed on the 17 IHC-positive cases yielded positive results in four cases, while NGS confirmed *NTRK* fusions in three of these. Notably, some cases with diffuse pan-TRK staining were *NTRK* wild-type, highlighting the potential for false-positive IHC results, particularly in sarcomas harboring *BCOR* or other non-*NTRK* fusions. These findings suggest that, although FISH is less specific than NGS, it remains a valuable intermediate diagnostic tool in cases with pan-TRK positivity, particularly when access to NGS is limited or unavailable. Overall, pan-TRK IHC should be considered a screening assay in sarcomas and interpreted with caution, with molecular confirmation recommended to establish the presence of *NTRK* rearrangements. These results also underscore the need for careful interpretation of staining patterns and awareness of known IHC pitfall in mesenchymal tumors.

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CONFLICTS OF INTEREST STATEMENT

The authors declare that they have no known com-

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AUTHOR CONTRIBUTIONS

Conceptualization, S.C., S.B., and M.G.; methodology, G.G., E.S.; software, A.R., and G.M.; validation, G.G.; resources, A.R., G.M., and E.S.; data curation, S.C., G.G., M.M., A.P., S.B.; writing-original draft preparation, S.C., S.B., and M.G.; writing-review and editing, S.C., S.B., and M.G. All authors have read and agreed to the published version of the manuscript.

ETHICAL CONSIDERATION

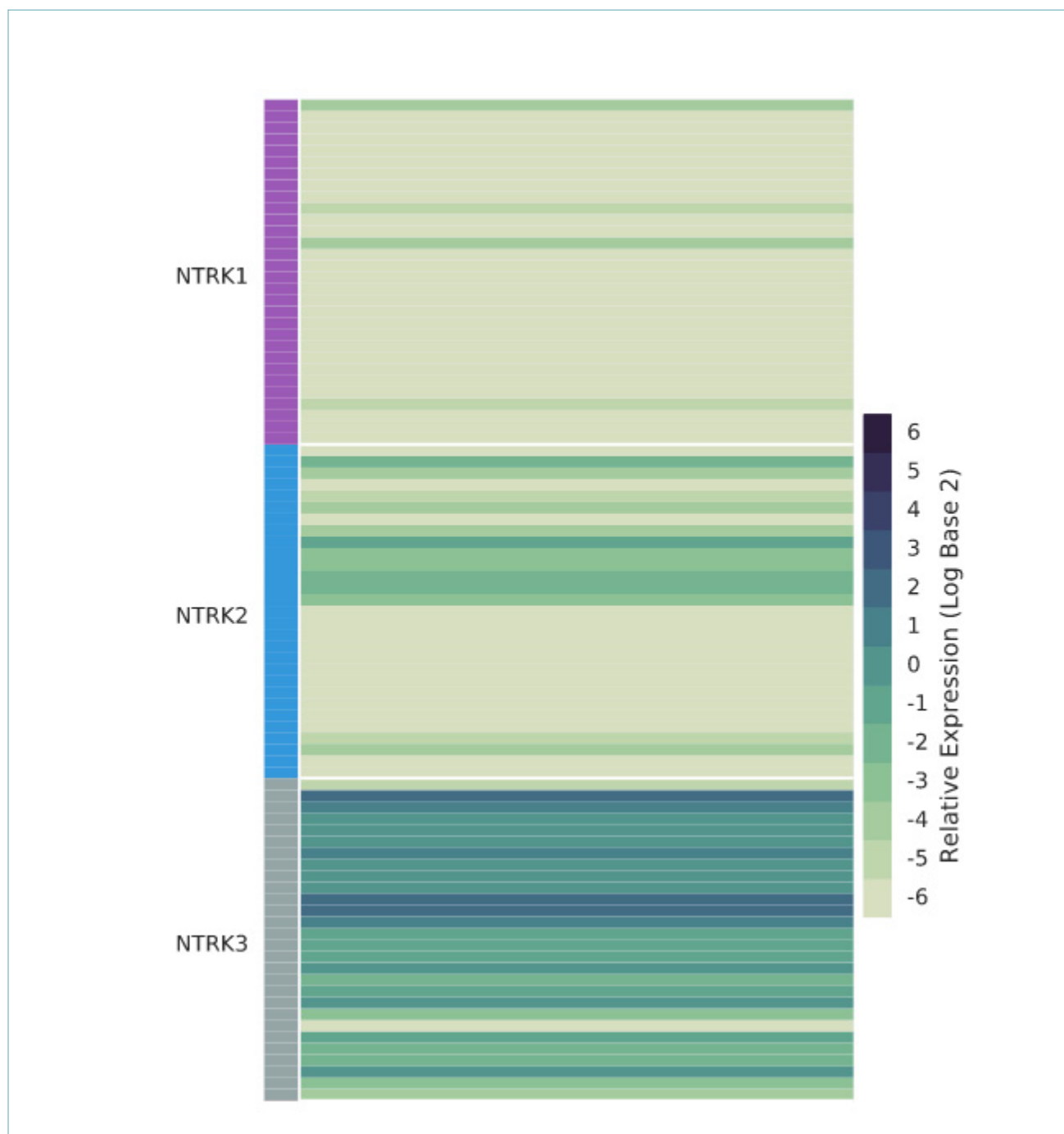
This study was approved by Institutional Ethics Committee of Area Vasta Emilia Centro della Regione Emilia-Romagna (code: CE AVEC:59/2024 Oss/IOR). The research was conducted ethically, with all study procedures being performed in accordance with the requirements of the World Medical Association's Declaration of Helsinki.

Written informed consent was obtained from each participant/patient for study participation and data publication.

References

- Gatalica Z, Xiu J, Swensen J, et al. Molecular characterization of cancers with NTRK gene fusions. *Mod Pathol* 2019;32:147-153. <https://doi.org/10.1038/s41379-018-0118-3>
- Amatu A, Sartore-Bianchi A, Bencardino K, et al. Tropomyosin receptor kinase (TRK) biology and the role of NTRK gene fusions in cancer. *Ann Oncol* 201;30:viii5-viii15. <https://doi.org/10.1093/annonc/mdz383>
- Khotskaya YB, Holla VR, Farago AF, et al. Targeting TRK family proteins in cancer. *Pharmacol Ther* 2017;173:58-66. <https://doi.org/10.1016/j.pharmthera.2017.02.006>
- Agaram NP, Zhang L, Sung YS, et al. Recurrent NTRK1 Gene Fusions Define a Novel Subset of Locally Aggressive Lipofibromatosis-like Neural Tumors. *Am J Surg Pathol* 2016;40:1407-1416. <https://doi.org/10.1097/PAS.0000000000000675>
- Sbaraglia M, Bellan E, Dei Tos AP. The 2020 WHO Classification of Soft Tissue: news and perspectives. *Pathologica* 202;11370-84. <https://doi.org/10.32074/1591-951X-213>
- Kallen ME, Hornick JL. The 2020 WHO Classification: What's New in Soft Tissue Tumor Pathology? *Am J Surg Pathol* 2021;45:e1-e23. <https://doi.org/10.1097/PAS.0000000000001552>
- Davis JL, Al-Ibraheemi A, Rudzinski ER, et al. Mesenchymal neoplasms with NTRK and other kinase gene alterations. *Histopathology* 2022;80:4-18. <https://doi.org/10.1111/his.14443>
- Marchetti A, Ferro B, Pasciuto MP, et al. NTRK gene fusions in solid tumors: agnostic relevance, prevalence and diagnostic strategies. *Pathologica* 2022;114:199-216. <https://doi.org/10.32074/1591-951X-787>

- ⁹ Siozopoulou V, Marcq E, De Winne K, et al. NTRK fusions in a sarcomas series: Pathology, molecular and clinical aspects. *Pathol Oncol Res* 2022;28:1610423. <https://doi.org/10.3389/pore.2022.1610423>
- ¹⁰ Church AJ, Calicchio ML, Nardi V, et al. Recurrent EML4-NTRK3 fusions in infantile fibrosarcoma and congenital mesoblastic nephroma suggest a revised testing strategy. *Mod Pathol* 2018;31:463-473. <https://doi.org/10.1038/modpathol.2017.127>
- ¹¹ Amatu A, Sartore-Bianchi A, Siena S. *NTRK* gene fusions as novel targets of cancer therapy across multiple tumour types. *ESMO Open* 2016;1:e000023. <https://doi.org/10.1136/esmoopen-2015-000023>
- ¹² Cocco E, Scaltriti M, Drilon A. NTRK fusion-positive cancers and TRK inhibitor therapy. *Nat Rev Clin Oncol* 2018;15:731-747. <https://doi.org/10.1038/s41571-018-0113-0>
- ¹³ Drilon A, Siena S, Ou SI, et al. Safety and Antitumor Activity of the Multitargeted Pan-TRK, ROS1, and ALK Inhibitor Entrectinib: Combined Results from Two Phase I Trials (ALKA-372-001 and STARTRK-1). *Cancer Discov* 2017;7:400-409. <https://doi.org/10.1158/2159-8290.CD-16-1237>
- ¹⁴ Drilon A, Laetsch TW, Kummar S, et al. Efficacy of Larotrectinib in TRK Fusion-Positive Cancers in Adults and Children. *N Engl J Med* 2018;378:731-739. <https://doi.org/10.1056/NEJMoa1714448>
- ¹⁵ Marchiò C, Scaltriti M, Ladanyi M, et al. ESMO recommendations on the standard methods to detect NTRK fusions in daily practice and clinical research. *Ann Oncol* 2019;30:1417-1427. <https://doi.org/10.1093/annonc/mdz204>
- ¹⁶ Solomon JP, Benayed R, Hechtman JF, et al. Identifying patients with NTRK fusion cancer. *Ann Oncol* 2019;30:viii16-viii22. <https://doi.org/10.1093/annonc/mdz384>
- ¹⁷ Hechtman JF, Benayed R, Hyman DM, et al. Pan-Trk Immunohistochemistry Is an Efficient and Reliable Screen for the Detection of NTRK Fusions. *Am J Surg Pathol* 2017;41:1547-1551. <https://doi.org/10.1097/PAS.0000000000000911>
- ¹⁸ Benini S, Gamberi G, Cocchi S, et al. The Efficacy of Molecular Analysis in the Diagnosis of Bone and Soft Tissue: A 15-Year Mono-Institutional Study. *Int J Mol Sci* 2022;24:632. <https://doi.org/10.3390/ijms24010632>
- ¹⁹ Piazzini M, Bavelloni A, Greco S, et al. Expression of the double-stranded RNA-dependent kinase PKR influences osteosarcoma attachment independent growth, migration, and invasion. *J Cell Physiol* 2020;235:1103-1119. <https://doi.org/10.1002/jcp.29024>
- ²⁰ Palmerini E, Frega G, Gambarotti M, et al. NTRK rearranged sarcoma of the bone. Role for larotrectinib in the neoadjuvant setting of an ultra-rare tumor: a case report. *Front Oncol*. 2023 Oct 9; 13:1252359. <https://doi.org/10.3389/fonc.2023.1252359>
- ²¹ Cocchi S, Gambarotti M, Gamberi G, et al. The utility of FISH analysis in the diagnosis of BCOR-rearranged sarcomas. *Pathol Res Pract*. 2024 Mar;255:155209. <https://doi.org/10.1016/j.prp.2024.155209>
- ²² Suameijer AJH, Antonescu CR. NTRK-rearranged spindle cell neoplasm. In: WHO Classification of Tumours Editorial Board. WHO Classification of Soft Tissue and Bone Tumours. 5th edition. Lyon: International Agency for Research on Cancer (IARC);2020:287-289.
- ²³ Chiang S, Cotzia P, Hyman DM et al. NTRK fusions define a novel uterine sarcoma subtype with features of fibrosarcoma. *Am J Surg Pathol* 2018 Jun;42:791-798. <https://doi.org/10.1097/PAS.0000000000001055>
- ²⁴ Kao YC, Sung YS, Argani P, et al. NTRK3 overexpression in undifferentiated sarcomas with YWHAE and BCOR genetic alterations. *Mod Pathol* 2020;33:1341-1349. <https://doi.org/10.1038/s41379-020-0495-2>
- ²⁵ Haberecker M, Kuerten P, Vetter VK, et al. A practical approach to better identify NTRK 1-3 fusion-positive mesenchymal neoplasms by pan-Trk immunohistochemistry. *Virchows Arch* 2025;487:671-680. <https://doi.org/10.1007/s00428-025-04102-9>. Epub 2025 Apr 15.
- ²⁶ Dang X, Xiang T, Zhao C, et al. EML4-NTRK3 Fusion Cervical Sarcoma: A Case Report and Literature Review. *Front Med (Lausanne)* 2022;9:832376. <https://doi.org/10.3389/fmed.2022.832376>
- ²⁷ Tannenbaum-Dvir S, Glade Bender JL, Church AJ, et al. Characterization of a novel fusion gene EML4-NTRK3 in a case of recurrent congenital fibrosarcoma. *Cold Spring Harb Mol Case Stud* 2015;1:a000471. <https://doi.org/10.1101/mcs.a000471>



Supplementary Figure 1. Heatmap showing the relative expression levels (log2 scale) of *NTRK1*, *NTRK2*, and *NTRK3* genes across the sarcoma samples analyzed. The scale bar on the right represents log2-transformed expression values ranging from -6 (low expression) to +6 (high expression). Each row corresponds to a gene and each column to a sample. Increased *NTRK3* expression is observed in BCOR::MAML1 fusion-positive case. The heatmap was generated using Archer Analysis software Pipeline Version: 7.1.0-14 Web Application Version: 7.1.0-38.