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The Use of Pan-Tropomyosin Receptor Kinase Immunohistochemistry as a Screening Tool for the Detection of Neurotrophic Tropomyosin-Related Kinase Fusions: Real-World Data from a National Multicentric Retrospective Study

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Keywords

Pan-tropomyosin receptor kinase · Neurotrophic tropomyosin-related kinase · Immunohistochemistry · Gene fusion · Next-generation sequencing

Abstract

Introduction: The neurotrophic tropomyosin-related kinase (*NTRK*) genes encode the tropomyosin receptor kinases (TRKs). Patients with solid tumors harboring an oncogenic *NTRK* fusion are eligible for treatment with TRK inhibitors. *NTRK* fusion is often associated with TRK overexpression. Pan-TRK immunohistochemistry (IHC) is used to screen for *NTRK* fusions, but immunoreactivity patterns are poorly defined. ***Methods:*** Data on pan-TRK immunoreactivity patterns in 2,669 solid tumors (comprising carcinomas, sarcomas, and melanocytic lesions) were retrospectively collected by nine laboratories and comprised tumor type, percentage of pan-TRK-positive tumor cells, staining intensity, cytoplasmic, membrane and/or nuclear staining pattern, and the presence or absence of *NTRK* fusion. ***Results:*** Overall, 2,457 tumors (92%) were pan-TRK negative and 212 neoplasms (8%) were pan-TRK positive. Twenty-two pan-TRK-positive tumors (0.8%) harbored an *NTRK* fusion, representing 10% of all pan-TRK-positive tumors. Cytoplasmic immunoreactivity was most often observed, followed by membrane immunoreactivity. Nuclear pan-TRK positivity was least frequent, but was most often (33%) associated with *NTRK* fusion. ***Conclusion:*** Pan-TRK IHC can be used to screen for *NTRK* fusions, especially in commonly diagnosed solid tumors with low

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NTRK fusion prevalence. In case of pan-TRK immunoreactivity, regardless of its intensity and tumor cell percentage, subsequent molecular tests should be performed to formally confirm the presence or absence of *NTRK* fusions.

Introduction

The neurotrophic tropomyosin-related kinase (*NTRK*) gene family (*NTRK1*, *NTRK2*, and *NTRK3*) encodes the tropomyosin receptor kinases (TRKs) A, B, and C (TRKA, TRKB, and TRKC, respectively) which are predominantly expressed in human neuronal tissue [1]. These neurotrophin receptors are involved in neuronal development during embryogenesis [2]. In adults, they play a role in the homeostasis of the central nervous system (CNS) and the peripheral nervous system [3]. Each TRK protein is constituted by an extracellular domain for ligand binding, a transmembrane domain, and an intracellular tyrosine kinase domain [2, 3]. Despite their homology, each receptor has a high affinity for a particular ligand: neurotrophin nerve growth factor for TRKA, neurotrophin-4 or brain-derived neurotrophic factor for TRKB, and neurotrophin-3 for TRKC [4]. Receptor activation by ligand binding results in receptor homodimerization, phosphorylation, and activation of several downstream signaling pathways, including the RAS/MAPK/ERK and the PLCγ/PI3K pathway by TRKA and TRKB, respectively, and the PI3K/AKT pathway by TRKC [1, 3]. These signaling pathways are involved in the prevention of apoptosis and cellular proliferation [1].

Although somatic *NTRK* mutations and splice variants were described in several cancers [4], the main cause of constitutional TRK activation in oncogenesis is based on the fusion of the 3′ region of an *NTRK* gene with the 5′ region of an unrelated partner gene, by intra- and interchromosomal rearrangements [5, 6]. The resulting chimeric fusion protein contains the tyrosine kinase domain of the TRK protein, which is joined in-frame with the fusion partner. This novel protein is often aberrantly expressed and/or constitutively active, and can, therefore, act as an oncogenic driver [6]. Patients with locally advanced unresectable or metastatic tumors harboring such a fusion protein are eligible for treatment with targeted therapies, comprising the highly selective TRK inhibitor, larotrectinib, and the small-molecule entrectinib, which inhibits TRK, ROS proto-oncogene 1, and anaplastic lymphoma kinase protein activity [6–10]. Next-generation inhibitors, such as repotrectinib and selitrectinib, were designed to abut resistance to these FDA-approved first-generation TRK inhibitors, which are mainly attributed to acquired mutations in the kinase domain [11].

Various studies reported a low prevalence of *NTRK* fusions in more commonly diagnosed tumors, such as melanomas and adenocarcinomas of the gastrointestinal tract, lungs, and breast [12–18]. Contrariwise, *NTRK* fusions are highly prevalent (or even pathognomonic) in several rare adult and pediatric tumor types such as the secretory carcinomas of the breast and salivary gland, infantile fibrosarcoma, cellular mesoblastic nephroma, and uterine and vaginal sarcomas resembling fibrosarcoma [19–23]. *NTRK* fusions can be identified by DNA-based or RNA-based next-generation sequencing, real-time polymerase chain reaction, or fluorescence in situ hybridization (FISH) using break-apart or fusion probes [3]. Although highly accurate, these molecular tests are often not widely available, and they are rather time-consuming and expensive [20]. Since many *NTRK* fusions result in TRK overexpression, pan-TRK immunohistochemistry (IHC) can, therefore, be considered as a cheap and fast alternative screening tool [24]. However, not all tumors with TRK immunoreactivity harbor an *NTRK* fusion, and some *NTRK* fusions – *NTRK3* rearrangements in particular – do not cause diffuse TRK overexpression [25, 26]. Because of this variable – often tumor type dependent – sensitivity and specificity, it is generally recommended to perform molecular testing in tumor types with a high frequency of *NTRK* fusions and to confirm a positive immunohistochemical result by a molecular test in tumor types with a low frequency of *NTRK* fusions [3, 24, 25, 27].

Given the rarity of *NTRK* fusions and its associated TRK overexpression in common carcinoma types, little is known about the immunoreactivity patterns in these neoplasms. A recent study reported heterogeneous staining in several tumor types [28]. To date, immunoreactivity patterns are better characterized in rare neoplasms which frequently show *NTRK* fusions. For instance, pediatric mesenchymal neoplasms with *NTRK1* or *NTRK2* rearrangement show predominantly cytoplasmic positivity, whereas *NTRK3*-rearranged neoplasms predominantly show nuclear immunoreactivity with or without cytoplasmic staining [20]. In the present multicentric retrospective study, we investigated pan-TRK immunoreactivity in a large series of solid neoplasms. Pan-TRK expression was correlated with the available molecular test results. As such, we aimed to identify a particular threshold for subsequent "reflex" molecular testing in neoplasms with a low incidence of *NTRK* fusions. Such a "pan-TRK staining atlas" could be useful for pathologists in daily routine practice.

Materials and Methods

Tumor Samples

Data on pan-TRK IHC were retrospectively collected via an online password-protected platform provided by Modis Belgium (Temse, Belgium). Eligible samples comprised formalin-fixed, paraffin-embedded (FFPE) tissue samples originating from solid tumors with available pan-TRK IHC, with or without available information on subsequent molecular *NTRK* testing. Cases were diagnosed between January 1, 2019, and November 30, 2020. Data were anonymously provided by the Departments of Pathology of nine different Belgian institutions: the Antwerp University Hospital (Antwerp), AZ Delta (Roeselare), the Centre Hospitalier Universitaire (CHU) de Liège (Liège), the Cliniques universitaires Saint-Luc (Brussels), the Ghent University Hospital (Ghent), the Institut de Pathologie et de Génétique (IPG, Gosselies), the Jules Bordet Institute (Brussels), the Onze Lieve Vrouwziekenhuis Aalst (Aalst), and the University Hospitals Leuven (Leuven). The contributing laboratories reported the tumor type (if known), the site of primary origin (if known) in the case of metastatic tumors, the pan-TRK status (including the staining pattern, the percentage of positive tumor cells, and the staining intensity), and the presence or absence of an *NTRK* fusion (if any molecular test had been performed). Information on heterogeneous immunoreactivity and the sample type (biopsy vs. resection specimen vs. cytology) was not available. Given its retrospective descriptive, noninterventional, and anonymous nature, this study was exempt from informed consent and/or approval by the local Ethics Committees, in accordance with Article 3 of the Belgian law of May 7, 2004, concerning experiments on human beings [29].

Pan-TRK Immunohistochemistry

Pan-TRK IHC was either performed for diagnostic purposes at the discretion of the pathologist or requested by the treating oncologist. In each participating laboratory, pan-TRK IHC was performed on 3–5-µm-thick FFPE tissue sections mounted on positively charged glass slides, by using the VENTANA pan-TRK assay (rabbit monoclonal antibody, clone EPR17341) on an automated BenchMark instrument (Ultra or XT; Ventana Medical Systems, Tucson, AZ, USA). The recommended staining protocol can be found in online supplementary Table 1 (for all online suppl. material, see www.karger.com/doi/10.1159/000522426). Appendix or cerebral cortex were used as an external positive on-slide control. Nerves and ganglion cells were required to show at least weak to moderate cytoplasmic immunoreactivity; lymphocytes, epithelial cells, smooth muscle cells, and adipocytes had to be negative. The percentage of tumor cells showing immunoreactivity for pan-TRK was noted for each solid tumor. Tumors were considered positive if ≥1% of tumor cells showed immunoreactivity, regardless of the intensity. Staining intensity was registered as negative (0), weak (1+), moderate (2+), or strong (3+), as previously described [30]. The staining pattern, comprising cytoplasmic, membranous, and nuclear immunoreactivity, was noted as well. Due to the anonymized data collection, a post hoc central review of the slides was not performed.

Molecular Confirmation of NTRK Gene Fusion

The pan-TRK-positive samples of three laboratories (CHU Liège, OLV, UZA) were subjected to targeted RNAseq with the Oncomine Focus Assay (Thermo Fisher Scientific, San Francisco,

Table 1. Carcinomas, mesotheliomas, and neuroendocrine neoplasms without pan-TRK immunoreactivity

Tumor type	*N*
Carcinoma (NOS)	439
Breast	1
Colon and rectum	6
Head and neck region (NOS)	9
Kidney (NOS)	1
Liver (NOS)	1
Lung	177
Esophagus	7
Ovary	2
Pancreas	2
Salivary glands	2
Skin	3
Small bowel	2
Thyroid	26
Unknown origin (not specified)	198
Uterus	2
Adenoid cystic carcinomas of various origins	7
Adenocarcinoma	869
Appendix	1
Bladder	1
Breast	104
Cervix	3
Cholangiocarcinoma	49
Colon and rectum	108
Gallbladder	4
Head and neck region (NOS)	2
Kidney (NOS)	5
Liver (NOS)	5
Lung	356
Esophagus	17
Ovary	12
Pancreas	96
Prostate	9
Salivary glands	5
Small bowel	4
Stomach	25
Unknown origin (not specified)	47
Uterus	14
Vulva	2
Adenosquamous carcinoma of the lung	5
Nonsmall-cell lung cancer (NOS)	591
Squamous cell carcinoma	61
Head and neck	11
Lung	38
Esophagus	2
Penis	1
Cervix	1
Origin unknown	8
Urothelial cell carcinoma	7
Mesothelioma of the pleura	2
Neuroendocrine neoplasms	33
Neuroendocrine carcinoma (NOS)	8
Neuroendocrine tumor	11
Small-cell neuroendocrine carcinoma	6
Large-cell neuroendocrine carcinoma	8

NOS, not otherwise specified; pan-TRK, pan-tyrosine receptor kinase.

Table 2. Melanocytic lesions, mesenchymal neoplasms, and tumors of the CNS without pan-TRK immunoreactivity

Tumor type	*N*
Melanocytic lesions	45
Melanoma	42
Spitz naevus	3
Tumors of soft tissues and bone	97
Sarcoma (NOS)	27
Adamantinoma	1
Alveolar soft part sarcoma	1
Angiosarcoma	4
Chondrosarcoma	2
Clear cell sarcoma	2
Congenital mesoblastic nephroma	1
Dedifferentiated liposarcoma	6
Dermatofibrosarcoma protuberans	1
Desmoid fibromatosis	2
Desmoplastic small round cell tumor	2
Epithelioid hemangioendothelioma	1
Ewing sarcoma	2
Fibrillary sarcoma	1
Fibromyxoid sarcoma	1
GIST of the stomach	3
Histiocytic sarcoma	1
Inflammatory myofibroblastic tumor	1
Intimal sarcoma of the heart	2
Kaposi sarcoma	1
Leiomyosarcoma	9
Liposarcoma	2
Low-grade endometrial stromal sarcoma	2
Malignant peripheral nerve sheath tumor	2
Myofibroblastic sarcoma	1
Myxofibrosarcoma	4
Myxoinflammatory fibroblastic sarcoma	2
Nodular fasciitis/myofibroma of the skin	1
Osteosarcoma	4
Plexiform fibrohistiocytic tumor	1
Rhabdomyosarcoma	3
Sclerosing epithelioid fibrosarcoma	1
SMARCA4-deficient sarcoma	1
Solid fibrous tumor	1
Synoviosarcoma	1
Tenosynovial giant cell tumor	1
Tumors of the CNS	97
Glioblastoma	41
(Anaplastic) astrocytoma	6
(Anaplastic) oligodendroglioma	3
Chordoma	3
Ependymoma of the spine	3
Glioma	21
Medulloblastoma	1
Meningioma	12
Neuroblastoma	1
Oligoastrocytoma	1
Pilocytic astrocytoma	1
Pleomorphic xanthoastrocytoma	3
Schwannoma	1
Hodgkin's lymphoma	1
Pheochromocytoma of the adrenal gland	1
Thymoma	4
Tumor type and localization unknown	198

NOS, not otherwise specified; pan-TRK, pan-tropomyosin receptor kinase.

CA, USA) on an S5 instrument, according to the manufacturer's instructions [30]. One laboratory (UZG) used the Archer FusionPlex Expanded Sarcoma Assay (ArcherDx, Boulder, CO, USA) for targeted RNAseq on the Illumina MiSeq platform, with subsequent data analysis using the Archer Analysis Software. Three laboratories (Institut Jules Bordet, CUSL, UZL) used the Archer FusionPlex comprehensive thyroid and lung panel (ArcherDX) for targeted RNAseq to investigate the presence of *NTRK* fusions, as was previously described [31]. One laboratory (AZ Delta) used an in-house developed gene panel for the identification of somatic mutations in 56 target genes, in combination with amplicon-based RNAseq (Illumina Focus panel). Two laboratories (IPG, UZL) performed FISH analysis on 3–4-μm-thick FFPE tissue slides of pan-TRK-positive tumor samples. Details on these procedures can be found in the online supplementary Materials and Methods.

Results

Immunoreactivity Patterns of Pan-TRK IHC

Nine participating laboratories provided information on pan-TRK IHC in 2,669 solid tumors. Overall, 2,457 tumors (92%) did not show any pan-TRK immunoreactivity and were designated as pan-TRK negative. These histological subtypes, if known, are listed in Tables 1 and 2.

Pan-TRK immunoreactivity was observed in 212 tumors (8%). The histological subtypes, if known, are shown in Tables 3–5. Cytoplasmic staining was most frequently observed, regardless of the intensity; 161 out of the 212 pan-TRK-positive tumors (76%) showed at least weak cytoplasmic staining. The percentage of pan-TRK-positive tumor cells varied from 1% to 100%. Thirty-one out of the 161 cases (19%) showed strong cytoplasmic immunoreactivity; 55 cases (34%) and 74 cases (46%) showed intermediate and weak expression, respectively. Cytoplasmic staining intensity was not reported for 1 case (1%).

Nuclear staining was least common with only 12 out of the 212 solid tumors (6%) presenting with nuclear immunoreactivity in 1%–100% of the tumor cells. In 5 out of these 12 cases (42%), nuclear immunoreactivity was isolated, and in 7 cases (58%), cytoplasmic immunoreactivity was observed as well. Strong nuclear staining was observed in 3 cases (25%). Four (33%) and 5 cases (42%) presented with intermediate and weak staining intensity, respectively.

Membrane staining was observed in 94 out of the 212 cases (44%). The percentage of positive tumor cells varied from 1% to 100%. Combined membrane and nuclear staining without cytoplasmic immunoreactivity were not observed. Fifty out of the 94 cases (53%) showed combined membrane and cytoplasmic immunoreactivity.

Table 3. Overview of the types of carcinoma and immunoreactivity patterns of locally advanced or metastatic carcinomas designated as positive for pan-TRK IHC

Origin	*N*	Immunoreactivity for pan-TRK		
		cytoplasmic staining, *n*	nuclear staining, *n*	membrane staining, *n*
Lung				
NSCLC (NOS)	63	32	2	46
SCLC	4	4	0	2
Carcinoid (NET)	1	1	0	0
Invasive breast cancer	9	6	0	3
Adenoid cystic carcinoma (site NOS)	5	5	0	0
Esophageal cancer (NOS)	6	5	0	4
Gastric carcinoma	1	0	0	1
Pancreatic carcinoma	1	1	0	0
Colorectal carcinoma	2	2	0	1
Cholangiocarcinoma	4	3	0	1
Hepatocellular carcinoma	1	1	0	1
Urothelial carcinoma – bladder	1	1	0	0
Prostate cancer	1	1	0	1
Parotid gland tumors				
Acinic cell carcinoma	1	0	0	1
Pleomorphic adenomas	2	2	0	0
Adenocarcinoma (NOS)	1	1	0	0
Secretory carcinoma	2	1	1	0
Thyroid carcinoma	7	7	0	4
Squamous cell carcinoma[a]	5	3	0	5
Gynecological tract				
Low-grade serous carcinoma	2	2	0	1
High-grade serous carcinoma	4	3	2	2
Carcinosarcoma	2	1	0	1
Endometrioid carcinoma	4	3	1	1
Small-cell NEC, site unknown	2	2	0	2
Adenocarcinoma, CUP	1	1	0	0

CUP, carcinoma of unknown primary origin; NEC, neuroendocrine carcinoma; NET, neuroendocrine tumor; NOS, not otherwise specified; NSCLC, nonsmall-cell lung cancer; SCLC, small-cell lung cancer. [a] Nonpulmonary, site not otherwise specified.

The remaining 44 cases (47%) showed isolated membrane staining. Ten out of the 94 cases (11%) showed strong membrane immunoreactivity. Intermediate and weak staining was observed in 32 (34%) and 52 (55%) cases, respectively.

Identification of NTRK Gene Fusions

Twenty-two of the pan-TRK-negative solid tumors were subjected to targeted RNAseq to exclude other gene fusions. The RNAseq failed for three tumors because of poor RNA quality. The 19 remaining samples did not harbor *NTRK* fusions. Information about any molecular analysis (either by FISH or by RNAseq) was not available for 22 of the 212 pan-TRK-positive tumors (10%; Fig. 1). *NTRK* fusions were detected in 22 pan-TRK-positive tumors (10%; Table 5), whereas 168 pan-TRK-positive tumors (80%) did not harbor *NTRK* fusions (Fig. 2). Table 5 displays the details of the different immunoreactivity patterns in the fusion-positive solid tumors. The staining pattern was unknown for one papillary thyroid carcinoma. Four *NTRK*-rearranged tumors (18%) presented with nuclear immunoreactivity for pan-TRK, comprising one glioblastoma, one spindle cell tumor (not otherwise specified, NOS), and two infantile fibrosarcomas (Fig. 3). Twenty tumors showed cytoplasmic staining, which was strong in 16 cases, and which was diffuse (≥80% of neoplastic cells) in 13 cases. The percentage of neoplastic cells with cytoplasmic immunoreactivity was not specified in 2 cases. Two colorectal carcinomas and one spindle cell tumor (NOS) showed diffuse and strong membrane staining, which was combined with diffuse and strong cytoplasmic staining. The distribution of the percentage of

Table 4. Overview of the types of melanocytic lesions, soft tissue tumors, and CNS tumors, designated as positive for pan-TRK IHC, and their immunoreactivity patterns

Origin	*N*	Immunoreactivity for pan-TRK		
		cytoplasmic staining, *n*	nuclear staining, *n*	membrane staining, *n*
Melanocytic lesions	3			
Spitz naevus	1	0	0	1
Melanoma	2	2	0	0
CNS tumors	27			
Glioblastomas	15	13	0	5
Anaplastic astrocytoma	3	3	0	1
Astrocytoma (NOS)	2	2	0	0
Diffuse leptomeningeal glioneuronal tumor	1	1	0	0
Medulloblastoma	1	1	0	1
Meningothelial meningioma	1	1	0	0
Oligodendroglioma	1	1	0	0
Neuroblastoma	2	2	0	0
Pilocytic astrocytoma	1	1	0	0
Soft tissue tumors	28			
BCOR fusion-positive sarcoma	1	1	0	0
Dermatofibrosarcoma	1	1	0	0
Desmoplastic small round cell tumor	4	4	1	0
Ewing (-like) sarcoma	4	3	0	2
Extra-skeletal myxoid chondrosarcoma	1	1	0	0
GIST	2	2	0	1
Leiomyosarcoma	3	2	1	0
Liposarcoma	1	1	0	0
MPNST	1	1	0	0
Myofibroblastic tumor	1	*	*	*
Myxoid chondrosarcoma	1	1	0	0
PEComa	1	1	0	1
Post-irradiation sarcoma	1	1	0	0
Rhabdomyosarcoma	1	1	0	0
Sarcoma (NOS)	2	2	0	1
Schwannoma	1	1	0	0
SFT	1	1	0	0
Synovial sarcoma	1	1	0	0

Molecular work-up was either not performed, or did not identify an *NTRK* gene fusion. *BCOR*, BCL-6 corepressor; GIST, gastrointestinal stromal tumor; NOS, not otherwise specified; PEComa, perivascular epithelioid cell tumor; SFT, solitary fibrous tumor. * Not mentioned in the database.

tumor cells is shown in Figure 4 per staining pattern. Although information on heterogeneous immunoreactivity was not available, the percentage of positive tumor cells might reflect heterogeneous staining in some cases.

Overall, nuclear pan-TRK staining was most often associated with the presence of an *NTRK* fusion where four out of twelve neoplasms (33%) with nuclear immunoreactivity had a fusion. Membrane and cytoplasmic immunoreactivity were associated with *NTRK* fusions in 3 out of 94 cases (3%) and 20 out of 161 cases (12%), respectively.

Discussion

We report the experience of nine institutions with the assessment of pan-TRK IHC in routine practice. In this retrospective multicentric study, comprising 2,669 solid tumors, the global pan-TRK positivity rate was 8%. In this "real-world" cohort, pan-TRK IHC was either performed for diagnostic purposes at the discretion of the pathologist or requested by the treating oncologist. There was no ad hoc definition of eligible tumor types. This cohort was, therefore, not enriched in cancers with uncommon histology, low mutational burden, or a depletion of concur-

Table 5. Overview of 22 tumors with variable immunoreactivity for pan-TRK and the presence of an *NTRK* gene fusion as determined by molecular testing

Tumor type	Immunoreactivity for pan-TRK IHC						Molecular work-up		
	cytoplasm		nucleus		membrane		molecular test type	*NTRK* gene (exon)	partner gene (exon)
	intensity	percentage	intensity	percentage	intensity	percentage			
Carcinomas									
Colorectal carcinoma	Strong	100	–	0	Strong	100	FISH & RNAseq	*NTRK1* (*10*)	*PLEKHA6* (*21*)
Colorectal carcinoma	Strong	100	–	0	Strong	100	RNAseq	NTRK1 (*10*)	*TPM3* (*7*)
Colorectal carcinoma	Strong	*	–	0	–	0	RNAseq	NTRK3 (*14*)	*EML4* (*2*)
NSCLC (NOS)	Strong	100	–	0	–	0	RNAseq	*NTRK3*	*EML4*
NSCLC (NOS)	Strong	100	–	0	–	0	RNAseq	*NTRK3* (*14*)	*EML4* (*2*)
Papillary thyroid carcinoma	*	*	*	*	*	*	RNAseq	*NTRK3* (*14*)	*EML4* (*2*)
Secretory carcinoma (SG)	Strong	100	–	0	–	0	RNAseq	*NTRK1* (*10*)	*IRF2BP2* (*1*)
Secretory carcinoma (SG)	Strong	100	–	0	–	0	RNAseq	*NTRK3*	*ETV6*
Secretory carcinoma (SG)	Strong	50	–	0	–	0	RNAseq	*NTRK1*	*IRF2BP2*
Secretory carcinoma (SG)	Weak	25	–	0	–	0	RNAseq	*NTRK3* (*14*)	*ETV6* (*2*)
Soft tissue tumors									
Infantile fibrosarcoma	Strong	100	–	0	–	0	FISH	Unknown	*ETV6*
Infantile fibrosarcoma	Strong	80	Moderate	20	–	0	RNAseq	*NTRK3*	*ETV6*
Infantile fibrosarcoma	Strong	90	Moderate	20	–	0	RNAseq	*NTRK3*	*ETV6*
Spindle cell tumor (NOS)	Strong	100	Strong	100	–	0	RNAseq	*NTRK1*	*TMP3*
Spindle cell tumor (NOS)	Strong	95	–	0	Strong	95	RNAseq	*NTRK3* (*14*)	*TFG* (*4*)
LLNT	Strong	*	–	0	–	0	RNAseq	*NTRK3* (*14*)	*SPECC1L* (*11*)
Melanocytic lesions									
Reed naevus	Weak	25	–	0	–	0	FISH	*NTRK3*	Unknown
Atypical Spitz naevus	Strong	100	–	0	–	0	RNAseq	*NTRK1*	*LMNA*
Atypical Spitz naevus	Intermediate	50	–	0	–	0	RNAseq	*NTRK1*	*KLC1*
CNS tumors									
Glioblastoma	–	0	Strong	100	–	0	RNAseq	NTRK2 (*16*)	PATZ1 (*3*)
Glioma	Intermediate	3	–	0	–	0	RNAseq	NTRK3 (*15*)	ETV6 (*5*)
PXA	Strong	100	–	0	–	0	RNAseq	NTRK2 (*15*)	TPM4 (*7*)

FISH, fluorescence in situ hybridization; LLNT, lipofibromatosis-like neural tumor; NOS, not otherwise specified; NSCLC, nonsmall-cell lung cancer; *NTRK*, neurotrophic tyrosine kinase; pan-TRK, pan-tropomyosin receptor kinase; PXA, pleomorphic xanthoastrocytoma; RNAseq, ribonucleic acid sequencing; SG, salivary gland. * Not mentioned in the database.

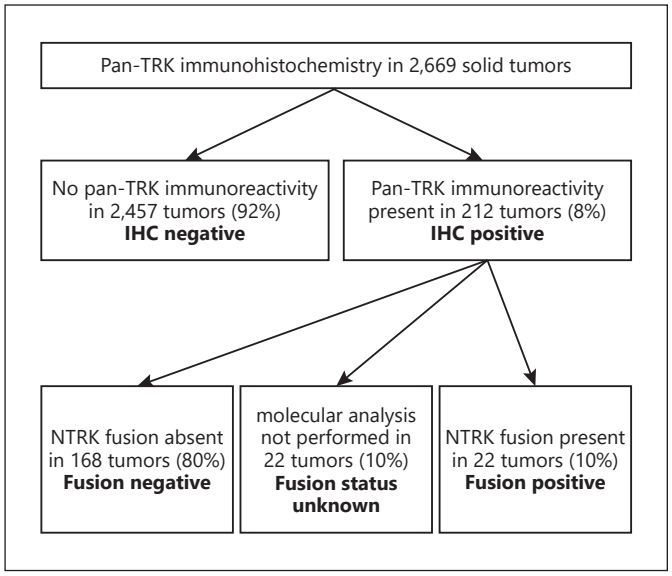
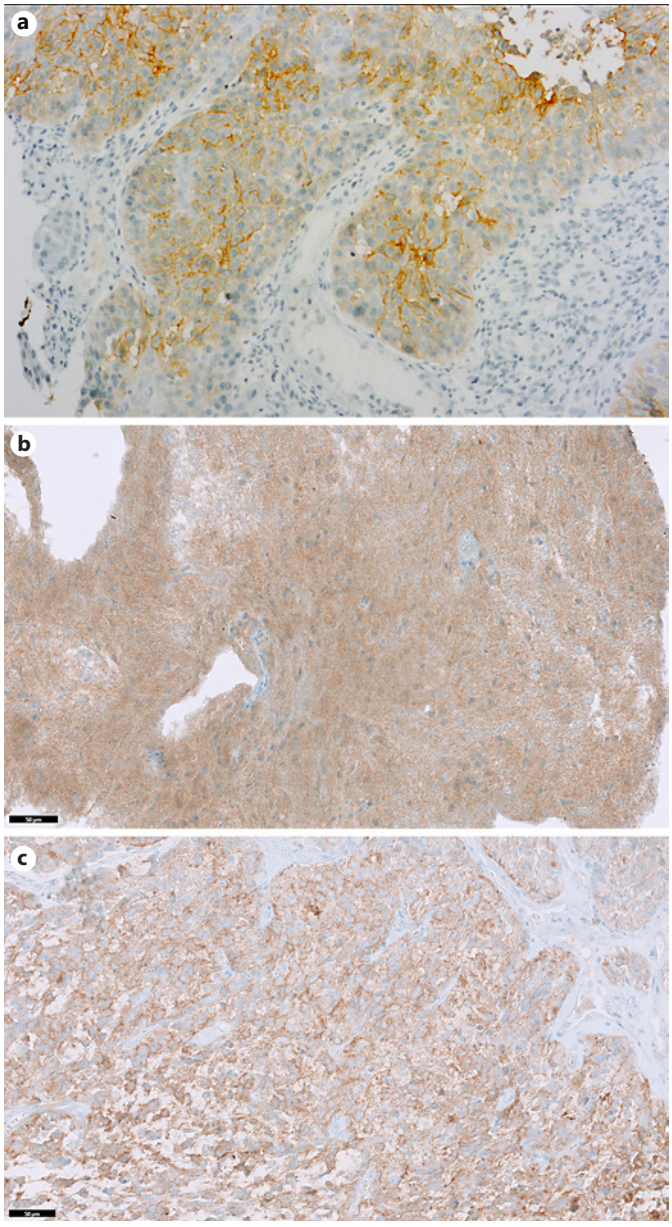


Fig. 1. Flowchart illustrating the screening by pan-TRK IHC in 2,669 solid tumors, followed by molecular analysis for the detection of *NTRK* gene fusions. IHC, immunohistochemistry.



Color version available online

Fig. 2. Photomicrographs of pan-TRK IHC in four solid tumors without identified *NTRK* fusion: weak cytoplasmic and moderate cytoplasmic immunoreactivity in a pharyngeal squamous cell carcinoma (**a**), diffuse cytoplasmic staining in a low-grade glioma (**b**), and diffuse cytoplasmic immunoreactivity in a grade IV glioblastoma (**c**).

rent oncogenic drivers [32]. Since *NTRK* testing was often performed together with other molecular tests during the work-up of advanced tumors, the observed frequency of *NTRK* fusions is similar to the one previously reported in large cohorts [26]. The percentage of positive tumor cells varied from 1% to 100%, and the staining intensity varied from weak to intense. Although most solid tumors harboring *NTRK* fusions presented with diffuse and strong immunoreactivity, similar immunoreactivity patterns were also noted in pan-TRK-positive *NTRK* fusion-negative solid tumors. This observation not only confirms that pan-TRK IHC can be used as an initial screening tool for the detection of *NTRK* gene rearrangements but it also emphasizes the need for subsequent confirmatory molecular tests, such as targeted RNAseq or FISH, as has been proposed by others [3, 24, 25, 27]. The retrospective data collection in this study precluded central review of the pan-TRK IHC. Therefore, inter-laboratory variations due to inter-observer variability cannot be excluded, nor were we able to study heterogeneity in the immunoreactivity patterns. However, the variable percentage of positive tumor cells suggests heterogeneous immunoreactivity in some cases. Another major weakness of this retrospective study is the lack of systematic molecular testing for each solid tumor sample, which precludes calculations of the precise specificity and sensitivity of pan-TRK IHC in this large "real-world" cohort. As "reflex molecular testing" was only performed on pan-TRK-positive solid tumors, we cannot exclude that we missed an *NTRK*-rearranged tumor due to false-negative pan-TRK IHC. Nevertheless, our study provides some interesting observations concerning pan-TRK immunoreactivity patterns

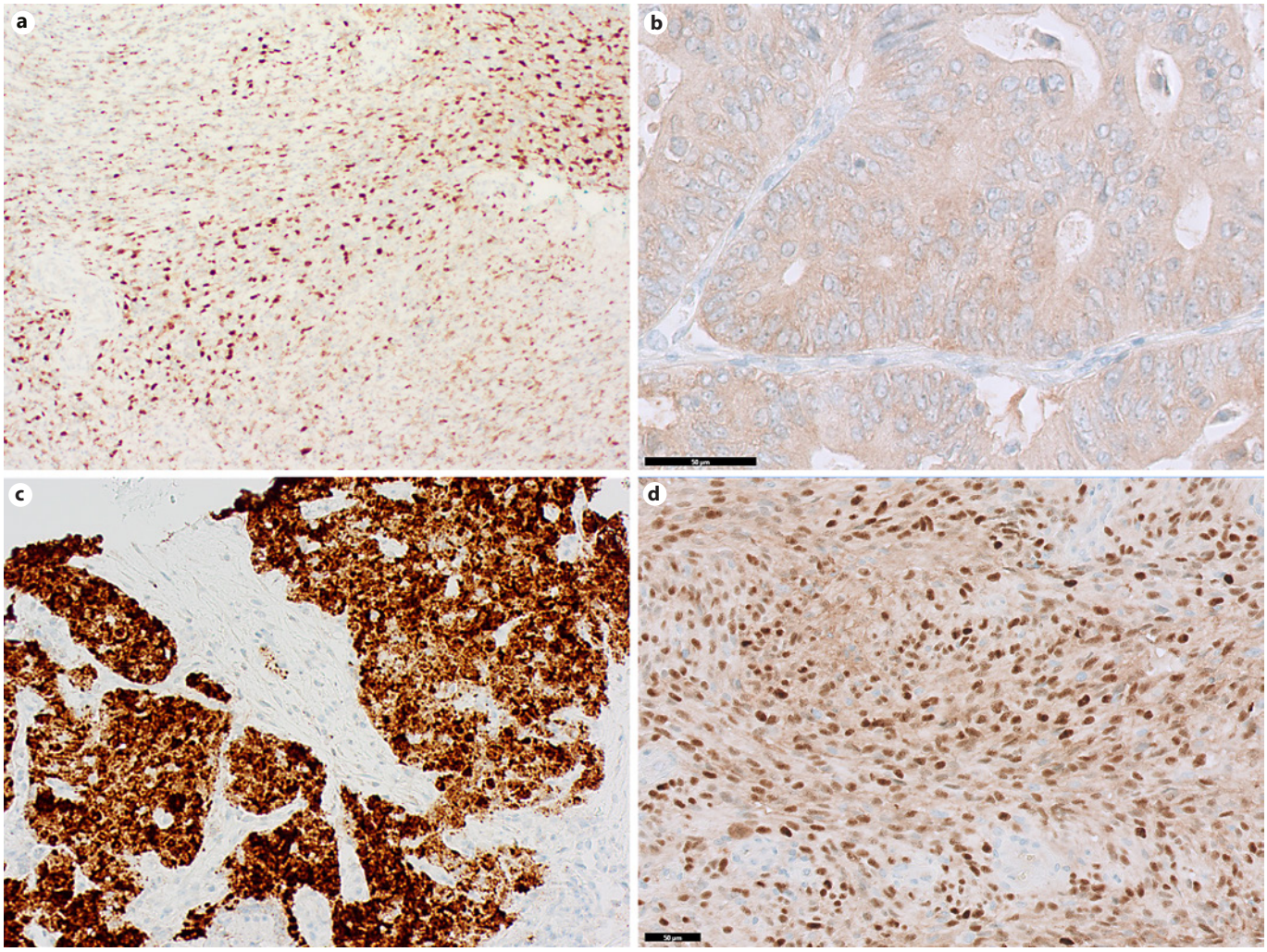


Fig. 3. Photomicrographs of pan-TRK IHC in four solid tumors with an identified *NTRK* fusion. **a** *ETV6-NTRK3* rearranged fibrosarcoma with strong cytoplasmic immunoreactivity in 80% of tumor cells and moderate nuclear immunoreactivity in 20% of tumor cells (original magnification ×100). **b** *EML4-NTRK3* rearranged colorectal adenocarcinoma with diffuse cytoplasmic pan-TRK immunoreactivity. **c** Strong diffuse cytoplasmic immunoreactivity in an *IRF2BP2-NTRK1*-rearranged secretory carcinoma of the parotid salivary gland (original magnification ×200). **d** *PATZ1-NTRK2*-rearranged glioblastoma with diffuse strong nuclear immunoreactivity. *ETV6,* ETS transcription factor variant 6*; IRF2BP2,* interferon regulatory factor 2-binding protein 2.

in fusion-positive and fusion-negative solid tumors. The sensitivity and specificity of pan-TRK IHC were variable in large cohorts, and we refer to these studies for further details [20, 26, 28, 33].

As expected, pan-TRK immunoreactivity was enriched in some rare solid tumor types, wherein *NTRK* fusions are considered to be highly prevalent or even pathognomonic. Three infantile fibrosarcomas harbored the pathognomonic ETS transcription factor variant 6-*NTRK3* fusion, which is consistent with previous reports [22, 34–37]. This gene fusion is also pathognomonic for congenital mesoblastic nephroma and secretory carcinoma of the breast, wherein predominantly nuclear pan-TRK expression was reported [38, 39]. Our series does not contain these tumor types, but we did identify four salivary gland secretory carcinomas. Two of these presented with the commonly detected ETS transcription factor variant 6-*NTRK3* fusion [40–44], which was accompanied by weak to strong cytoplasmic pan-TRK immunoreactivity. The remaining two salivary gland secretory carcinomas harbored an *NTRK1* rearrangement with the interferon regulatory factor 2-binding protein 2 gene

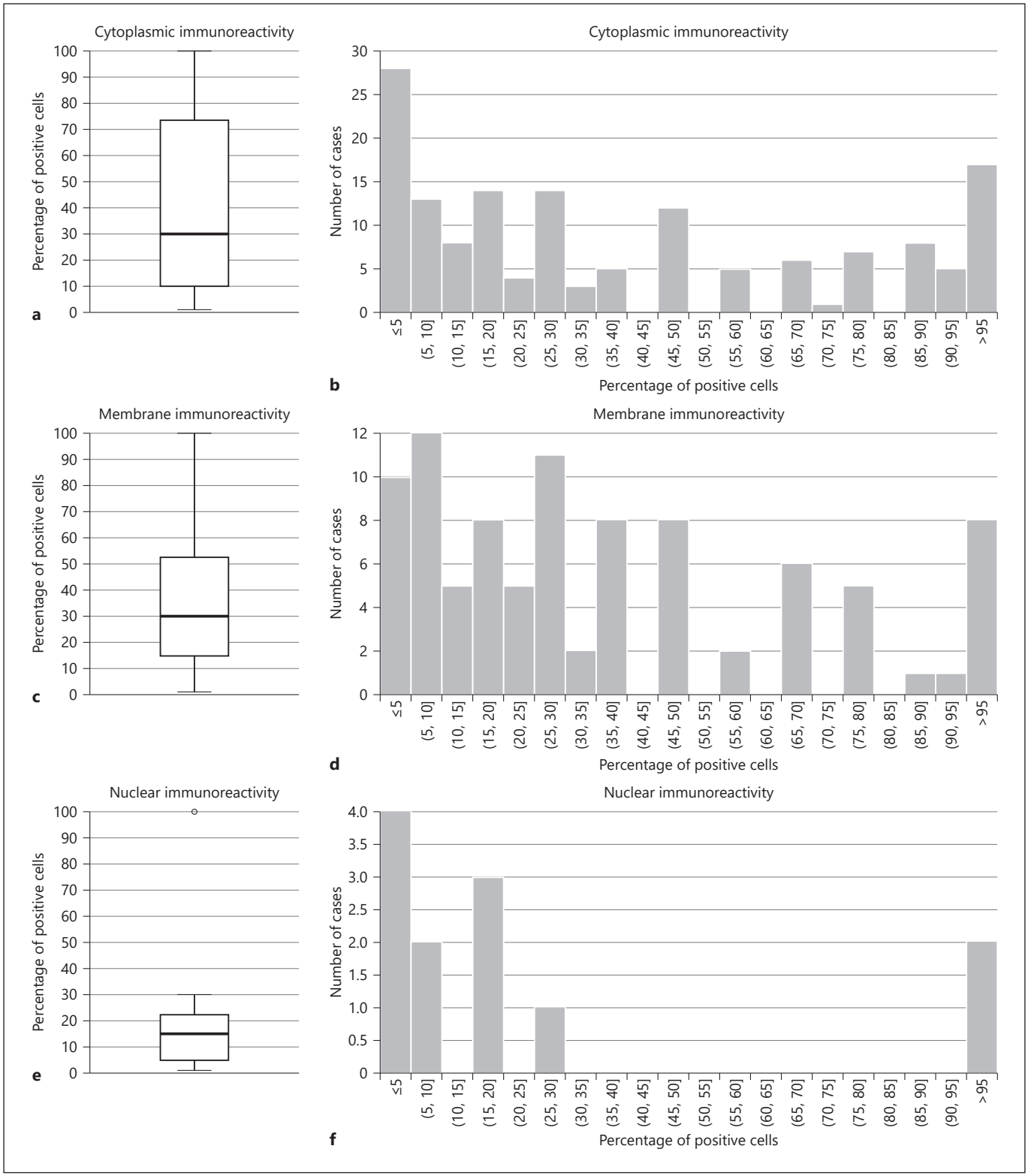


Fig. 4. Box-and-whisker plots (**a**, **c**, **e**) and histograms (**b**, **d**, **f**) illustrating the distribution of the percentage of positive cells for each pan-TRK immunoreactivity pattern. The median percentage of positive cells was 30% for cytoplasmic staining (**a**), 30% for membrane staining (**c**), and 15% for nuclear staining (**e**). **e** Circles represent outliers.

as a fusion partner. Additionally, two secretory carcinomas of the salivary gland presented with pan-TRK immunoreactivity, without detectable *NTRK* fusion in 1 case and without available molecular test results in the other case. Although previous studies reported predominantly nuclear immunoreactivity in salivary gland secretory carcinomas [43, 44], the four fusion-positive cases in our series presented with cytoplasmic staining only. The interferon regulatory factor 2-binding protein 2-*NTRK1* rearrangement has previously been described by Hechtman et al. [33] in a pulmonary adenocarcinoma with cytoplasmic pan-TRK staining, by Brcic et al. [45] in a skin fibrohistiocytic tumor with diffuse strong cytoplasmic pan-TRK staining and by Zhao et al. [46] in a 9-year-old patient with a diffuse sclerosing variant of a papillary thyroid carcinoma.

We identified three melanocytic pan-TRK-positive, fusion-positive lesions, comprising two *NTRK1*-rearranged atypical Spitz naevi and an *NTRK3*-rearranged Reed naevus with an unknown fusion partner. Previously, Yeh et al. [47] reported the occurrence of *NTRK3* fusions in Spitz naevi. However, VandenBoom et al. [48] observed that *NTRK3* fusions are more common in Reed naevi (57%) than in Spitzoid tumors (3%). Spitz tumors with *NTRK1* fusions were reported to present with distinctive histopathological characteristics, including elongated, thin and branched filigree-like rete ridges, rosette-like configurations of dermal melanocytes, and marked reduction in melanocyte size descending into the dermis [49].

Thirty out of 127 (24%) CNS tumors in our series were reported to present with at least focal pan-TRK immunoreactivity. The thirty pan-TRK-positive CNS tumors that were subjected to molecular testing revealed an *NTRK* fusion in 3 cases (10%), comprising a glioma, a glioblastoma, and a pleomorphic xanthoastrocytoma. Given the physiologic expression of TRKs in the CNS and the peripheral nervous system in children and adults [3], this high pan-TRK positivity rate is not surprising. In fact, we cannot exclude the presence of false-positive cases due to extensive background pan-TRK staining in the surrounding normal neuronal tissue. Vice versa, some diffusely infiltrating pan-TRK-positive tumors might have been designated as false-negative, since the observed immunoreactivity could have been attributed to background staining in pre-existent normal neuronal tissue [26]. The threshold for reflex molecular testing to exclude *NTRK* fusions should, therefore, be low in CNS tumors, since interpretation of pan-TRK IHC is very challenging.

As for *NTRK* fusions in commonly diagnosed adenocarcinomas, we detected two *NTRK3*-rearranged nonsmall-cell lung carcinomas (NSCLCs) and three *NTRK*-rearranged colorectal adenocarcinomas. Given the overall pan-TRK positivity rate of 5.2% and an *NTRK* fusion rate of only 0.2% in 1,246 NSCLCs in our series, our observation confirms the previously reported extreme rarity of *NTRK* fusions in pulmonary adenocarcinomas and squamous cell carcinomas [14, 17, 50, 51]. Similarly, *NTRK* fusions are rare in colorectal adenocarcinomas, as we detected a pan-TRK positivity rate of 4.2% and an *NTRK* fusion rate of 2.5% in 119 colorectal adenocarcinomas. There is a higher incidence of *NTRK* fusions in mismatch repair-deficient and *RAS/BRAF* wild-type colorectal carcinomas, which are mainly associated with sporadic MutL Homolog 1 (*MLH1*) promoter hypermethylation rather than Lynch syndrome [52]. Chou et al. [53] confirmed that the group of *MLH1/PMS2/BRAF*V600E triple-negative colorectal adenocarcinomas was enriched in pan-TRK-positive cases (5.3%), whereas the pan-TRK positivity rate amounted to only 0.02% in colorectal adenocarcinomas without this phenotype. Due to the retrospective nature of our study, we cannot verify how many pan-TRK-positive and -negative colorectal adenocarcinomas in our series presented with this triple-negative phenotype, but additional reports suggest that *NTRK* fusion screening by pan-TRK IHC could be limited to this particular subgroup [15, 54].

The question, therefore, remains whether it is necessary to systematically perform upfront pan-TRK IHC on all locally advanced and metastatic solid tumors samples, given the rarity of *NTRK* fusions in commonly diagnosed carcinomas in adults. For instance, the extreme rareness of *NTRK* rearrangements in NSCLCs alludes toward a negative answer. However, pulmonary adenocarcinomas are often subjected to targeted RNAseq to explore for the potential presence of other gene rearrangements, such as anaplastic lymphoma kinase (ALK), rearranged during transfection (RET) proto-oncogen, and ROS proto-oncogene 1, and the use of multigene panels comprising the *NTRK* genes will, therefore, reveal the occasional "zebra" among the proverbial herd of horses. The observations on *NTRK* fusion enrichment in a particular subgroup of colorectal adenocarcinomas also plea for a selective application of pan-TRK immunohistochemical screening. However, additional large-scale studies with integrated detailed cost-benefit analysis are required to provide a robust and definitive answer, potentially taking into account the number of "quality-adjusted life years" gained by patients treated with TRK inhibitors.

Conclusion

The results of this nation-wide multicentric study confirm the previously proposed algorithms for *NTRK* fusion screening in solid tumors [3, 24, 27], i.e., pan-TRK IHC can be applied as a screening tool for *NTRK* rearrangements, especially in commonly diagnosed solid tumors with low *NTRK* fusion prevalence. However, in the case of pan-TRK immunoreactivity, regardless of its intensity and tumor cell percentage, subsequent molecular tests are recommended to establish a conclusive diagnosis of *NTRK* rearrangement. We, as well as others, have shown that many different solid tumor types can present with some extent of pan-TRK positivity in the absence of *NTRK* fusions. Although the retrospective nature of our national study precluded the calculation of the "real-world" sensitivity and specificity, we demonstrated here that pan-TRK immunoreactivity patterns strongly overlap between fusion-positive and fusion-negative cases. It, therefore, seems as a safe approach to keep the current threshold for pan-TRK positivity at ≥1% of positive tumor cells, regardless the staining pattern, to warrant additional "reflex" molecular testing.

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Statement of Ethics

This descriptive retrospective study was conducted ethically in accordance with the World Medical Association Declaration of Helsinki. Ethics approval and informed consent were not required, in accordance with the Belgian law as detailed in the manuscript, since this retrospective study did not involve human studies or experiments involving animals.

Conflict of Interest Statement

M.R. Van Bockstal received a renumeration from Roche Diagnostics Belgium for her contribution to the present study. The other authors have no conflicts of interest to declare.

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Author Contributions

M.R.V.B: conceptualization, supervision, analysis of the data, arrangement of the figures, and writing – original draft of the manuscript. All authors: acquisition of the data, interpretation of the data, resources, writing – review and editing, and final approval.

Data Availability Statement

All data reported in this article are available from the corresponding author (M.R.V.B.) upon reasonable request.

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