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

Mieke R. Van Bockstal\textsuperscript{a,b} Gabriela Beniuga\textsuperscript{c} Ligia Craciun\textsuperscript{d} David Creytens\textsuperscript{e,f} Franceska Dedeurwaerdere\textsuperscript{g} Philippe Delvenne\textsuperscript{h} Pieter Demetter\textsuperscript{d} Bart De Wiest\textsuperscript{i} Koen Dewinne\textsuperscript{j} Lionel Habran\textsuperscript{h} Patrick Pauwels\textsuperscript{j} Ivan Theate\textsuperscript{c} Sara Vander Borght\textsuperscript{k} Kris Van Der Steen\textsuperscript{i} Birgit Weynand\textsuperscript{k}

\textsuperscript{a}Department of Pathology, Cliniques Universitaires Saint-Luc (CUSL), Woluwé-Saint-Lambert, Brussels, Belgium; \textsuperscript{b}Institute of Clinical and Experimental Research (IREC), Université Catholique de Louvain, Brussels, Belgium; \textsuperscript{c}Institut de Pathologie et de Génétique (IPG), Charleroi, Belgium; \textsuperscript{d}Department of Pathology, Institut Jules Bordet, Brussels, Belgium; \textsuperscript{e}Department of Pathology, Ghent University Hospital (UZG), Ghent University, Ghent, Belgium; \textsuperscript{f}Cancer Research Institute Ghent, CRIG, Ghent University Hospital, Ghent, Belgium; \textsuperscript{g}Department of Pathology, AZ Delta, Roeselare, Belgium; \textsuperscript{h}Anatomopathology Department, University Hospital of Liège (CHU Liège), Liège, Belgium; \textsuperscript{i}Department of Pathology, Onze-Lieve-Vrouwziekenhuis (OLV) Aalst, Aalst, Belgium; \textsuperscript{j}Department of Pathology, Antwerp University Hospital (UZA), Edegem, Belgium; \textsuperscript{k}Department of Pathology, University Hospitals Leuven (UZL), Leuven, Belgium

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

Correspondence to: Mieke R. Van Bockstal, mieke.vanbockstal@saintluc.uclouvain.be
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.

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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 neurotrophic 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/P13K pathway by TRKA and TRKB, respectively, and the P13K/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 inter-chromosomal 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 selotrectinib, 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 Vrouweziekenhuis 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, CA, USA). The results were analyzed and interpreted according to the recommendations of the clinical laboratories involved. The gene fusion status was determined as positive or negative based on the RNAseq results.

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.
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. 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 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 |
| 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 fascitsis/myofibroma of the skin | 1 |
| Osteosarcoma | 4 |
| Pleomorphic 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 |
| Hodgin’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.
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), 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

| Origin                          | N   | Immunoreactivity for pan-TRK |
|---------------------------------|-----|-----------------------------|
|                                 |     | cytoplasmic staining, n     |
| Lung                            |     | nuclear staining, n         |
| 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 ¹       | 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   |

¹ Nonpulmonary, site not otherwise specified.

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.
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 | NTRK gene (exon) | partner gene (exon) |
|-----------------------------|----------------------------------|-------------------|------------------|---------------------|
|                             | cytoplasm intensity | percentage | nucleus intensity | percentage | membrane intensity | percentage | molecular test type | NTRKgene (exon) | partner gene (exon) |
| Carcinomas                  |                                |                  |                  |                  |                   |          |                   |                 |                    |
| Colorectal carcinoma        | Strong 100                    | 0                | Strong 100       | 0                | FISH & RNAseq     | NTRK1 (10) | PLEKH6 (21)         |
| Colorectal carcinoma        | Strong 100                    | 0                | Strong 100       | 0                | RNAseq            | NTRK1 (10) | TPM3 (7)            |
| Colorectal carcinoma        | Strong *                      | 0                | –                 | –                | RNAseq            | NTRK3 (14) | EML4 (2)            |
| NSCLC (NOS)                 | Strong 100                    | 0                | –                 | 0                | RNAseq            | NTRK3 (14) | EML4 (2)            |
| NSCLC (NOS)                 | Strong 100                    | 0                | –                 | 0                | RNAseq            | NTRK3 (14) | EML4 (2)            |
| Papillary thyroid carcinoma | Strong 100                    | 0                | Strong 100       | –                | RNAseq            | NTRK3 (14) | ETV6 (2)            |
| Secretory carcinoma (SG)   | Strong 100                    | 0                | –                 | 0                | RNAseq            | NTRK1 (10) | IRF2BP2 (1)         |
| Secretory carcinoma (SG)   | Strong 100                    | 0                | –                 | 0                | RNAseq            | NTRK3      | ETV6 (2)            |
| Secretory carcinoma (SG)   | Strong 100                    | 0                | –                 | 0                | RNAseq            | NTRK3      | ETV6 (2)            |
| Secretory carcinoma (SG)   | Strong 100                    | 0                | –                 | 0                | RNAseq            | NTRK3      | 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                    | 0                | Strong 100       | –                | RNAseq            | NTRK1      | TMP3               |
| Spindle cell tumor (NOS)    | Strong 95                     | 0                | Strong 95        | 0                | RNAseq            | NTRK3 (14) | TGF (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            | NTRK3      | LMNA               |
| Atypical Spitz naevus       | Intermediate 50               | 0                | –                 | 0                | RNAseq            | NTRK3      | KLC1               |
| CNS tumors                  |                                |                  |                  |                  |                   |          |                   |                 |                    |
| Glioblastoma                | –                               | 0                | Strong 100       | –                | 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.
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.
Pan-TRK Immunohistochemical Screening for NTRK Fusions

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. 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

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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). 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/PM2S/BRAFV600E 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 RNAsseq 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.

Acknowledgment

The authors thank Ms. Carolien Boeckx, Immuno-onco-biomarker Liaison from Roche Diagnostics Belgium (Diegem, Belgium), for critically reading the manuscript.

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.

Funding Sources

M.R. Van Bockstal received a postdoctoral clinical mandate (2019-089) from the not-for-profit organization “Foundation Against Cancer” (Brussels, Belgium), and a renumeration from Roche Diagnostics Belgium for her contribution to the present study (data analysis, writing and editing of the manuscript, article processing costs). The other authors did not receive funding for their contribution.

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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