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Original Research

The sensitivity of pan-TRK immunohistochemistry in solid tumours: A meta-analysis

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Abstract ***Introduction:*** Since the approval of neurotrophic tropomyosin receptor kinase (*NTRK*) tyrosine kinase inhibitors for fist-line advanced stage pan-cancer therapy, pathologists and molecular biologists have been facing a complex question: how should the large volume of specimens be screened for *NTRK* fusions? Immunohistochemistry is fast and cheap, but the sensitivity compared to RNA NGS is unclear.

Methods: We performed RNA-based next-generation sequencing on 1,329 cases and stained 24 *NTRK*-rearranged cases immunohistochemically with pan-TRK (ERP17341). Additionally, we performed a meta-analysis of the literature. After screening 580 studies, 200 additional *NTRK*-rearranged cases from 13 studies, analysed with sensitive molecular diagnostics as well as pan-TRK IHC, were included.

Results: In the included 224 NTRK-rearranged solid tumours, the sensitivity for pan-TRK IHC was 82% and the false-negative rate was 18%. *NTRK3* fusions had more false negatives (27%) compared to *NTRK1* (6%) and *NTRK2* (14%) ($p = 0.0006$). Membranous, nuclear and peri-nuclear staining patterns strongly correlated with different fusion products, with membranous staining being more prevalent in *NTRK1* and *NTRK2*, nuclear in *NTRK3*, and perinuclear in *NTRK1*.

Conclusion: Despite a reduction in the number of molecular analysis, using pan-TRK immunohistochemistry as a prescreening method to detect NTRK fusions in solid tumours will miss 18% of all *NTRK*-fused cases (especially involving *NTRK3*). Therefore, the most comprehensive

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and optimal option to detect NTRK fusions is to perform molecular testing on all eligible cases. However, in case of financial or logistical limitations, an immunohistochemistry-first approach is defensible in tumours with a low prevalence of *NTRK* fusions.

1. Introduction

Neurotrophic tropomyosin receptor kinase (*NTRK*) fusions are powerful oncogenic drivers, which are common in rare tumour types, e.g., infantile fibrosarcoma and secretory breast cancer, but rare (with prevalence estimates below 1%) in some common tumours, e.g., lung adenocarcinoma and colorectal carcinoma [1,2].

In recent clinical trials, a remarkable survival benefit of *NTRK* tyrosine kinase inhibitors (TKIs) was observed, with high response rates and durable, long-term progression-free survival in patients with *NTRK*-rearranged cancers [3–6]. Therefore, *NTRK* TKIs have been approved for first-line treatment in all *NTRK*-rearranged advanced stage cancers. This approval is independent of cancer type, making *NTRK* one of the first tumour-agnostic targets [7,8].

In addition, several tumour types, such as Spitz tumours and secretory carcinoma, are (in part) characterised by the presence of *NTRK* fusions. Without the ability to sensitively detect *NTRK* fusions in diagnostics, patients with these tumour types could end up with the wrong diagnosis and – in selected cases – even suboptimal treatment.

The gold standard for fusion detection is targeted RNA-based next-generation sequencing (RNA NGS) or whole genome sequencing (WGS), but these molecular techniques are expensive and time-consuming and have limited worldwide accessibility [9]. As an alternative, pan-TRK immunohistochemistry (IHC) with the ERP17341 antibody (Abcam) has been investigated as a potential screening tool, as it is much faster, has lower costs, and is more widely available than molecular diagnostics [10].

Several recent studies report that pan-TRK IHC screening is a reliable alternative for molecular analysis [11–13]. However, other studies report a problematic false-negative rate over 15% [14–18], potentially leading to underdetection. However, due to the overall low prevalence of *NTRK* fusions in solid tumours, most studies included only a limited number of *NTRK*-rearranged cases, which makes that robust recommendations for using pan-TRK IHC as a screening method for the detection of *NTRK* fusions are lacking [11–24].

In this study, we describe a cohort of 1329 solid tumours that were analysed for *NTRK* fusions with anchored multiplex PCR (AMP)-based targeted RNA NGS in routine diagnostics in our institution. In addition, we performed a meta-analysis for studies comparing pan-TRK IHC with molecular analysis for the detection of *NTRK* fusions. The aim of our study was to robustly describe the sensitivity and false-negative rate of pan-TRK IHC, in order to make a well-considered choice on the use of pan-TRK IHC as a screening tool for *NTRK* fusions in solid tumours in the clinical setting.

2. Materials and methods

2.1. Case selection

We retrospectively analysed all solid tumours that routinely underwent anchored multiplex (AMP)-based targeted RNA NGS among others for *NTRK1*, *NTRK2*, and *NTRK3* gene fusions in the Leiden University Medical Center (LUMC), Leiden, the Netherlands, between 2008 and 2021. All solid tumour types were eligible for inclusion, irrespective of malignant, borderline malignant, or benign diagnosis. Cases in which RNA NGS analyses were incomplete or failed, e.g., due to insufficient tissue, were excluded. There is an overrepresentation of radioactive iodine-insensitive thyroid carcinomas and driver-negative lung and colorectal carcinomas, since these cases were more frequently submitted for RNA NGS, due to a high quantity of referrals of these cancers to the LUMC. Cases were screened for therapeutic reasons (*NTRK* TKI treatment), diagnostic reasons (e.g. differential diagnosis of Spitz tumours with spitzoid melanoma), or both.

The study was performed according to the Dutch FEDERA Code for Proper Use of Human Tissue. A waiver of consent was given by the Leiden-the Hague-Delft Medical Ethical Committee (B20.017). Cases were anonymised completely before processing, omitting the need to obtain informed consent from the included patients.

2.2. Fusion analysis

For *NTRK* fusion analysis, RNA was isolated from formalin-fixed paraffin-embedded (FFPE) tissue by

microdissection using five 10 μm slides and extracted using a tissue preparation system robot (Siemens). AMP-based-targeted RNA NGS was performed with the ArcherDx assay, with either the Comprehensive Thyroid and Lung panel, the Solid Tumors panel, or the Sarcoma v2 panel, which all cover the complete *NTRK1*, *NTRK2*, and *NTRK3* genes and are validated according to the NEN-EN-ISO15189 guidelines. This method is capable of detecting fusions with either a novel or unknown fusion partner by using gene-specific primers in conjunction with molecular barcoded adapters. The generated libraries were sequenced on the IonTorrent S5 platform (Thermo Fisher Scientific, Canada). Analysis was performed using a local installation of the Archer Analysis software. Different versions (ranging from version 5.1.7 to version 6.2.3) were used. NGS library generation, analysis, and reporting were performed under ISO15189 accreditation in the molecular diagnostics section of the pathology department (LUMC).

2.3. Immunohistochemistry

For the purpose of this study, pan-TRK IHC was performed on cases with a confirmed *NTRK* fusion by RNA NGS. For IHC, 4-μm-thick slides were cut from the FFPE tissue blocks of histological biopsies or resection specimens and automatically stained with the pan-TRK monoclonal antibody clone EPR17341 (Abcam, Cambridge, MA) on the Dako Omnis stainer, in a 1:50 dilution [4]. A subset of cases was stained manually, with the same antibody in a 1:150 dilution. Cases with insufficient FFPE tissue were excluded.

The pan-TRK IHC was independently scored by two pathologist (DC and AS) and discordant cases were discussed until consensus was reached. Cases were considered positive when staining of any pattern and intensity was seen in more than 1% of the tumour cells. In addition, for each positive case, the staining pattern was determined: cytoplasmatic, nuclear, membranous, or a combination of $\geq$2 patterns. In case of multiple staining patterns in the same slide, the case was included in both staining categories.

2.4. Meta-analysis

In addition, we performed a meta-analysis of the literature to evaluate the sensitivity and false-negative rate of pan-TRK IHC as a screening method for *NTRK* fusions, using the PRISMA criteria [25]. We searched PubMed on June 19, 2021, with the search term included in Supplement 1. First selection existed of title and abstract screening by LH. Second selection existed of full text screening of the resulting articles by LH.

All studies comprising five or more unique cases of solid tumours that were evaluated with pan-TRK IHC with the monoclonal antibody clone EPR17341 as well as a molecular diagnostics test with high sensitivity for the detection of *NTRK* fusions were included in our analysis. Suitable molecular techniques included targeted RNA NGS, whole genome sequencing (WGS), DNA-based NGS panels with good coverage of the *NTRK1*, *NTRK2*, and *NTRK3* introns and fluorescence *in situ* hybridisation (FISH) for the *NTRK1*, *NTRK2*, and *NTRK3* genes, as these molecular tests are known to have high sensitivity and specificity [26,27]. Additionally, studies in which cases were prescreened with Nanostring and, when positive, confirmed with one of the aforementioned molecular techniques, were also included. Studies in which pan-TRK IHC was used as a screening tool to select cases for molecular analysis were excluded, as these studies might introduce a selection bias with regard to the sensitivity and false-negative rate. Studies written in another language than English and harmonisation studies were excluded.

Based on the included studies, we constructed a database for each case listing the diagnosis, type of molecular analysis used, molecular analysis results, fused *NTRK* gene and breakpoint (when available), fusion partner and breakpoint (when available), and pan-TRK IHC result and staining pattern (when

Table 1
Overview of all included solid tumour types including the *NTRK*-fusion prevalence.

Diagnosis group or tractus	Included cases	*NTRK* fusions	*NTRK1*	*NTRK2*	*NTRK3*	Sensitivity
Lung and thorax	738	2 (0.3%)	0	0	2	50%
Thyroid	190	12 (6.3%)	2	0	10	75%
Digestive tract	82	2 (2.4%)	1	0	1	100%
Sarcoma	68	2 (2.9%)	1	0	1	100%
Carcinoma of unknown primary	65	0	0	0	0	–
Head and neck	66	4 (6.1%)	0	0	4	100%
Central nerve system	52	0	0	0	0	–
Skin	32	4 (12.5%)	0	1	3	75%
Urogenital tract	18	0	0	0	0	–
Breast	9	1 (11.1%)	0	0	1	100%
Other	9	0	0	0	0	–
Total	**1329**	**27 (2.0%)**	**4**	**1**	**22**	**79%**

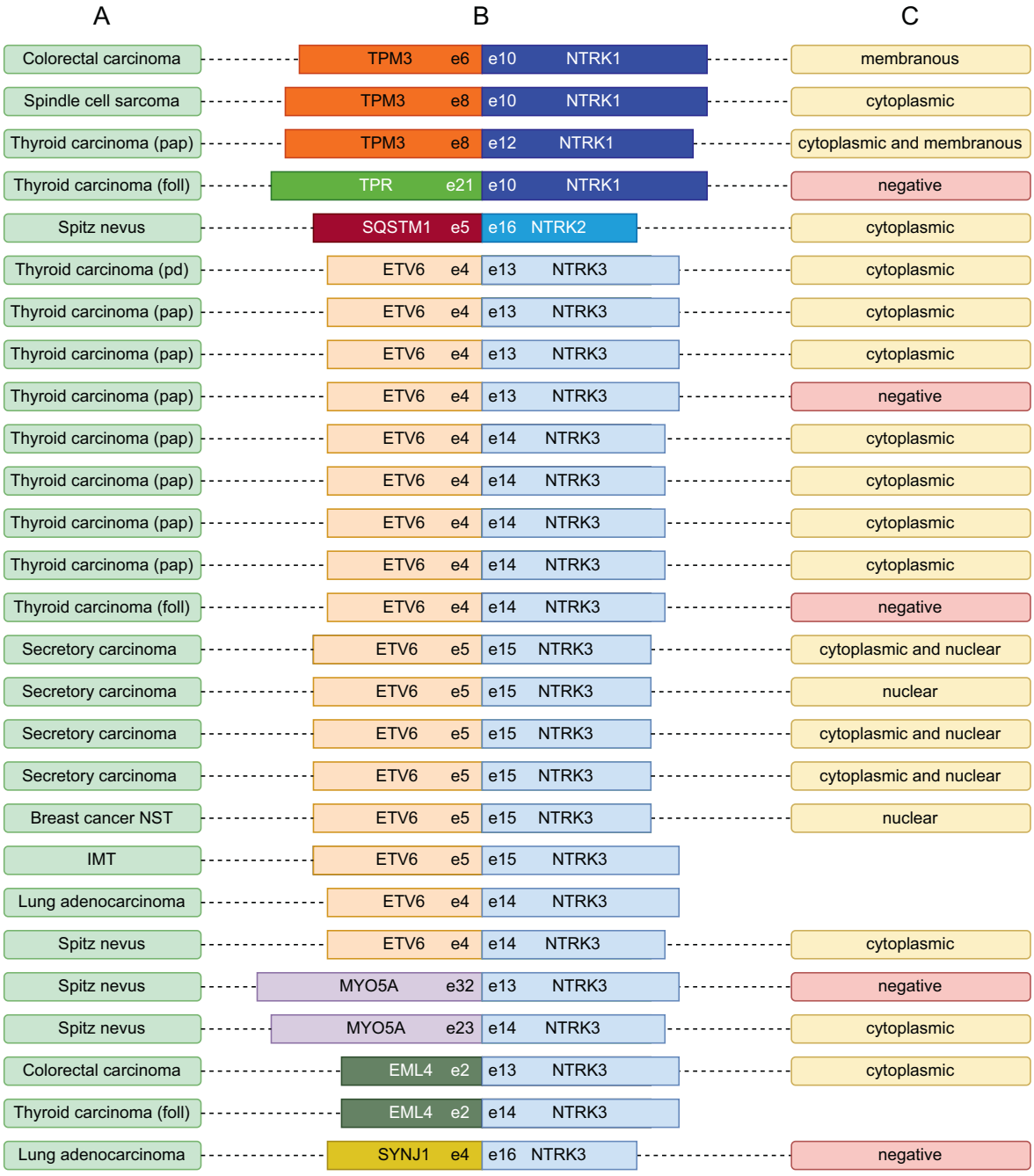

Fig. 1. Overview of all *NTRK*-fused cases in our cohort, arranged by diagnosis (A), fusion product including breakpoints (B), and pan-TRK immunohistochemical staining pattern (C). In three cases, immunohistochemistry was not performed due to tissue unavailability. Breast cancer NST: breast cancer no special type, IMT: inflammatory myofibroblastic tumour, pap: papillary type, foll: follicular type, pd: poorly differentiated type.

available). Our own cohort of NTRK-fused cases was added to this database.

2.5. Statistics

Statistical analysis was performed using IBM SPSS Statistical software, version 26. Statistical significance was set at a P-value of <0.05.

3. Results

3.1. Case characteristics

In total, we included 1329 cases on which RNA NGS was routinely performed. This cohort included 738 lung and thoracic tumours, 190 thyroid carcinomas, 82 digestive tract tumours, 68 bone and soft tissue tumours,

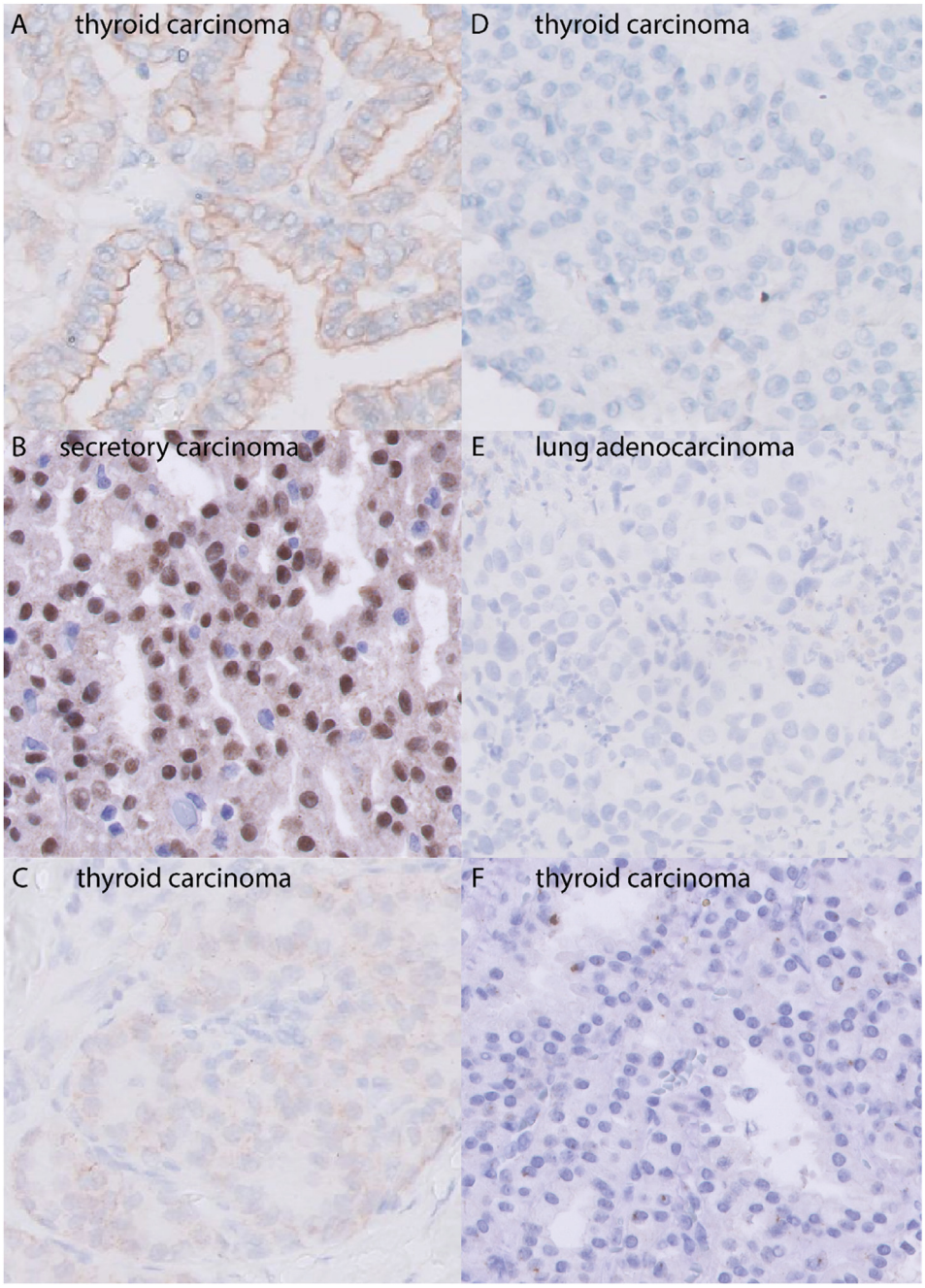

Fig. 2. Pan-TRK immunohistochemistry with positive (A, B, C) and negative staining (D, E, F). Membranous positivity in thyroid carcinoma with *NTRK1* fusion (A), nuclear positivity in a secretory carcinoma in the parotid gland with *NTRK3* fusion (B), cytoplasmic positivity in a thyroid carcinoma with *NTRK3* fusion (C), pan-TRK-negative thyroid carcinoma with *NTRK3* fusion (D), pan-TRK-negative lung adenocarcinoma with *NTRK3* fusion (E), and pan-TRK-negative thyroid carcinoma with *NTRK1* fusion (F).

65 carcinomas of unknown primary, 66 head and neck tumours, 52 central nerve system tumours, 32 melanocytic tumours, 18 urogenital tumours, nine breast cancers, and nine other lesions (Table 1). RNA NGS was performed for diagnostic purposes in 69 cases, therapeutic purposes in 960 cases, and for both diagnostic and therapeutic purposes in 300 cases. In 751 cases that were analysed for therapeutic purposes, mainly colorectal carcinomas and lung adenocarcinomas, previous DNA NGS was performed without identification of a driver mutation. In 347 cases, a strong driver mutation, such as KRAS or EGFR, was identified in DNA NGS. In all other cases, DNA NGS was not performed.

3.2. Fusion analysis

Using RNA NGS, 27 of the 1329 (2%) cases demonstrated an *NTRK* gene fusion. These cases comprised the

NTRK1 gene in four (15%) cases, the *NTRK2* gene in one (4%) case, and the *NTRK3* gene in 22 (81%) cases (Fig. 1). *NTRK1* was fused with *TPM3* in three cases (mismatch repair deficient colorectal carcinoma, spindle cell sarcoma, and thyroid carcinoma) and with *TPR* in one case (thyroid carcinoma). The *NTRK2* fusion occurred in a Spitz nevus with *SQSTM1* as the fusion partner. The *NTRK3* fusions most frequently involved *ETV6* (17 cases: nine thyroid cancers, four secretory carcinomas, one breast cancer NST, one inflammatory myofibroblastic tumour, one lung adenocarcinoma, and one Spitz nevus). Additionally, we observed two *MYO5A*:*NTRK3* fusions (Spitz nevi), two *EML4*:*NTRK3* fusions (mismatch repair proficient colorectal carcinoma and thyroid carcinoma), and one *SYNJ1*:*NTRK3* fusion (lung adenocarcinoma).

3.3. Immunohistochemistry

In 24 of the 27 (89%) *NTRK*-fused cases, pan-TRK IHC was successfully performed. In three cases, insufficient tissue was available for pan-TRK IHC. Nineteen (79%) of the successfully stained cases scored positive. Positive staining consisted of cytoplasmatic staining in 12 (63%) cases, nuclear staining in two (11%) cases, membranous staining in one (5%) case, combined cytoplasmatic and nuclear staining in three (16%) cases, and combined cytoplasmatic and membranous staining in one (6%) case (Fig. 2). The two observers were concordant in 100% of the cases. Benign neural tissue stained positive for pan-TRK as well as some macrophages.

Five (21%) of the 24 *NTRK*-fused cases were completely negative with pan-TRK IHC (Fig. 2). This false negativity was observed in 4 of 19 (21%) *NTRK3*-fused cases and in one of four (25%) *NTRK1*-fused cases. The negative cases included a lung carcinoma with a *SYNJ1*:*NTRK3* fusion, a Spitz nevus with a *MYO5A*:*NTRK3* fusion, and three thyroid carcinomas, two with *ETV6*:*NTRK3* fusions, and one with a *TPR*:*NTRK1* fusion. Overall, the sensitivity of pan-TRK IHC in our cohort was 79%, and the false-negative rate was 21%. This indicates that when using pan-TRK IHC as a screening method, 21% of *NTRK*-fused cases in our cohort would have been missed.

3.4. Meta-analysis

A literature search in PubMed on June 19th 2021 (search strategy in Supplement 1) yielded 580 articles. After first selection, which included removal of duplicates and screening of the title and abstract, 54 articles remained. After second selection, which included assessment of the full-texts of the 54 potentially relevant articles, a total of 13 eligible studies were included in our analysis [11–21,23,28]. A flow chart of the selection process of the meta-analysis is presented in Fig. 3. An overview of the characteristics of the included studies is provided in Supplement 2. The complete dataset is provided in Supplement 3.

The 13 included studies describe the *NTRK* fusion status of 6609 solid tumours. In 200 (3%) of these cases, an *NTRK* fusion was detected. When combining these 200 cases with our cohort, the total number of *NTRK*-fused cases is 224, including 83 (37%) *NTRK1* fusions, 21 (9%) *NTRK2* fusions, and 120 (54%) *NTRK3* fusions. Overall, pan-TRK IHC was positive in 184 of 224 (82%) cases, resulting in a sensitivity of pan-TRK IHC for the detection of *NTRK* fusions of 82% (Table 2). In 40 of 224 (18%) of the *NTRK*-fused cases, however, pan-TRK IHC was false negative. The highest percentage of false negativity with pan-TRK IHC was seen in *NTRK3* fusions (27%) compared with *NTRK1* (6%) and *NTRK2* (14%), which is statistically significant (χ^2 test, p-value <0.001).

In the meta-analysis and our own cohort, *NTRK* fusions did not co-occur with other driver mutations (such as *BRAF* V600E, *KRAS* G12C, etc.). The mutual exclusivity of driver mutations in TKI-treatment naive tumours is in line with the literature [29].

Regarding the staining patterns of pan-TRK IHC, membranous staining was significantly more common in *NTRK2*-fused cases (χ^2 test, P-value <0.001), while nuclear staining was associated with *NTRK3* fusions (χ^2 test, P-value <0.001), and perinuclear staining was only seen in *NTRK1* fusions (χ^2 test, P-value <0.001). An overview of the staining patterns is provided in Table 2.

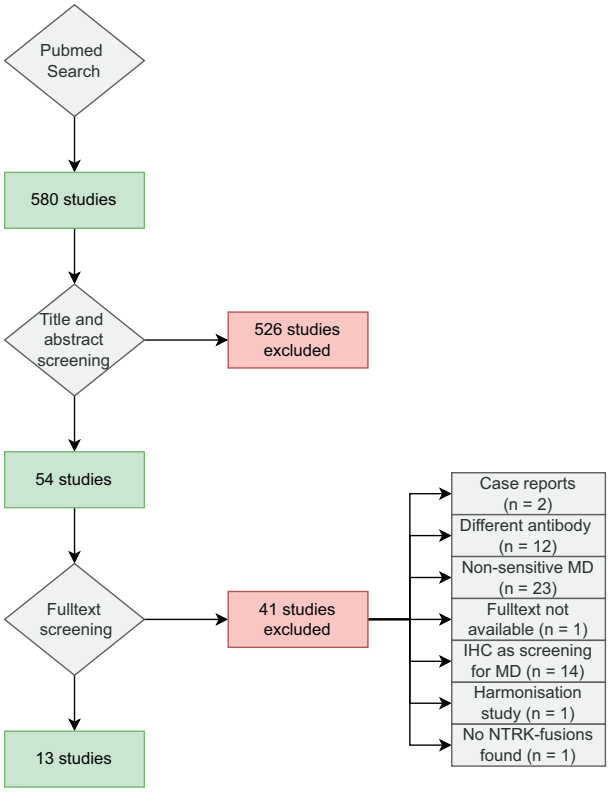

Fig. 3. Meta-analysis workflow and results.

Table 2
Immunohistochemistry results versus molecular diagnostics for 224 *NTRK*-rearranged cases. Pan-TRK IHC had an overall sensitivity of 82% and a false-negative rate of 18%. Staining patterns were significantly different across *NTRK* genes. Studies that did not address the scoring patterns outlined in this table were excluded from part of this table. Some cases displayed multiple staining patterns in the same slide and were scored in both staining categories. P-values are calculated with χ^2 test.

	NTRK1 fusion (n = 83)	*NTRK2* fusion (n = 21)	*NTRK3* fusion (n = 120)	P-value	All *NTRK* fusions (n = 224)
Pan-TRK IHC				0.0006	
Negative	5 (6%)	3 (14%)	32 (27%)		40 (18%)
Positive	78 (94%)	18 (86%)	88 (73%)		184 (82%)
Staining patterns					
Cytoplasmic (n = 100)	32 (80%)	5 (71%)	37 (70%)	0.53	74 (74%)
Membranous (n = 131)	14 (29%)	4 (44%)	4 (5%)	0.0002	22 (17%)
Nuclear (n = 168)	8 (12%)	1 (8%)	45 (51%)	0.0000004	54 (32%)
Perinuclear (n = 122)	12 (24%)	0	0	0.0001	12 (10%)

4. Discussion

This study describes the sensitivity and false-negative rate of pan-TRK IHC for the detection of *NTRK* fusions in solid tumours, based on our cohort of 24 *NTRK*-fused cases combined with a meta-analysis of literature comprising another 200 *NTRK*-fused cases, in order to make a well-considered choice on the use of pan-TRK IHC as a screening tool for *NTRK* fusions in solid tumours the clinical setting.

This study demonstrates a sensitivity of 82% and a false-negative rate of 18% for pan-TRK IHC with the monoclonal antibody clone EPR17341 (Abcam) to detect *NTRK* fusions in solid tumours. Therefore, using IHC as a screening method and confirming IHC-positive cases with molecular methods (the IHC-first approach) will result in missing 18% of the *NTRK*-fused cases. In the authors opinion, a 'miss rate' of 18% is high, especially in the clinical setting of *NTRK* as a therapeutic target, considering the substantial clinical benefit of treatment with *NTRK* TKIs. The molecular-only approach (omitting IHC) is more sensitive and comprehensive, but will result in a substantial logistical and financial burden for most laboratories, especially in tumour types with a low a priori chance of finding *NTRK* fusions. Pathologists and molecular biologists will need to consider the 18% miss rate and weigh it against the chance of finding an *NTRK* fusion and the burden of broad molecular testing in their specific laboratory circumstances, to come to the most optimal *NTRK* fusion testing for their patient population. The IHC-first approach does miss 18% of targetable fusions but might be a defensible alternative in specific circumstances. Testing is not useful for cases with a known driver mutation, such as *KRAS* G12C or *BRAF* V600E, as those are mutually exclusive with *NTRK* fusions.

False negativity for pan-TRK IHC was correlated to the fused *NTRK* gene, as it was significantly more common in *NTRK3*-fused cases (27%) compared with *NTRK1* (6%) and *NTRK2* (14%) (χ^2 test, P-value $<$ 0.001). The reason for these significant differences between the *NTRK* genes is unknown and might be a subject for further research. In order to rule out false negativity in our cohort because of the pan-TRK antibody titration (1:150), we performed a titration experiment on one of the two false-negative *ETV6*:*NTRK3*-fused thyroid carcinomas. The neoplastic cells finally stained positive at a dilution of 1:10 but so did the surrounding normal tissue (Supplement 4).

The clinical relevance of pan-TRK IHC-negative *NTRK*-fused solid tumours with regard to NTRK TKIs is currently unknown. Patients with *NTRK*-fused tumours who lack IHC expression might have a diminished response compared with IHC-positive patients as a result of limited *NTRK* gene expression. In our cohort and meta-analysis, follow-up and response data were not available. Therefore, this study is unable to determine whether IHC-negative patients have the same benefit of TKI treatment as IHC-positive patients and this should be a topic of future research.

Despite the analysis of the world-wide literature, a limitation of this study is the relatively small number of confirmed *NTRK*-fused cases, reducing the power of the meta-analysis. In total, 224 of 7938 (3%) solid tumours with an *NTRK* fusion were identified in literature and our cohort, demonstrating the overall rarity of this genetic alteration in solid tumours. As several cohorts in this meta-analysis, including our own, might be enriched for *NTRK*-fused cases, e.g. due to selection of cases based on the absence of a driver mutation by DNA NGS, specific morphology of the tumour, or the inclusion of therapy-resistant tumours, our study might already overrepresent *NTRK*-fused cases. Another limitation is heterogeneity of the included studies for inclusion criteria and techniques used for pan-TRK IHC and molecular diagnostics for *NTRK* fusions. Despite the fact that all studies used a cut-off of 1% staining with

pan-TRK IHC for a case to be considered positive, there was substantial variation in the dilution used for pan-TRK IHC.

Currently, *NTRK* is the only pan-cancer treatment target, but this is likely to change in the near future. Novel therapies for alternative targets are discovered each year, and several of these treatments are already available in experimental settings, via early access, or compassionate use programs, greatly increasing the number of treatment options of late stage cancer patients. In addition, the diagnostic setting not only requires screening of the *NTRK* genes but also of other fusion genes, e.g. *RET*, *ROS1*, and *ALK*. Therefore, for both therapeutic and diagnostic purposes, a multi-target analysis of *NTRK* in combination with other genes of interest will become more and more clinically relevant, preferring comprehensive molecular analysis, such as RNA NGS and WGS, over single-target assays, such as IHC and FISH [30].

In conclusion, our study demonstrates a sensitivity of 82% and a false-negative rate of 18% for pan-TRK IHC as a screening method for the detection of *NTRK* fusions in solid tumours. These data should be considered when choosing a strategy to screen for *NTRK* fusions in the clinical setting.

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CRediT statement

L.M. Hondelink – Conceptualisation, Methodology, Formal analysis, Investigation, Data curation, Writing – Original Draft, Writing – Review & Editing, Visualisation, A.M.R. Schrader – Conceptualisation, Methodology, Resources, Writing – Original Draft, Writing – Review & Editing, Visualisation, Supervision, Project administration, G. Asri Aghmuni – Conceptualisation, Methodology, Formal analysis, Investigation, Data curation, Writing – Original draft, Writing – Review & editing, N. Solleveld-Westerink – Resources, Data curation, Writing – Review & editing, A.M. Cleton-Jansen – Resources, Data curation, Writing – Review & editing, D. van Egmond – Resources, Data curation, Writing – Review & editing, A. Boot – Resources, Investigation, Writing – Review & editing, S. Ouahoud – Resources, Investigation, Writing – Review & editing, M.N. Khalifa – Resources, Investigation, Writing – Review & editing, S.W. Lam – Resources, Data Curation, Writing – Review & editing, H. Morreau – Resources, Data Curation, Writing – Review & editing, J.V.G.M. Bovee – Resources, Data Curation, Writing – Review & editing, T. van Wezel – Conceptualisation, Methodology, Resources, Data Curation, Writing – Review & editing, Supervision, Project administration, Funding acquisition, D. Cohen – Conceptualisation, Methodology, Resources, Data Curation, Writing – Original draft, Writing – Review & editing, Supervision, Project administration, Funding acquisition.

Conflict of interest statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejca.2022.06.030.

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