NTRK fusion detection across multiple assays and 33,997 cases: diagnostic implications and pitfalls

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Abstract

With the FDA approval of larotrectinib, *NTRK* fusion assessment has recently become a standard part of management for patients with locally advanced or metastatic cancers. Unlike somatic mutation assessment, the detection of *NTRK* fusions is not straightforward, and various assays exist at the DNA, RNA, and protein level. Here, we investigate the performance of immunohistochemistry and DNA-based next-generation sequencing to indirectly or directly detect *NTRK* fusions relative to an RNA-based next-generation sequencing approach in the largest cohort of *NTRK* fusion positive solid tumors to date. A retrospective analysis of 38,095 samples from 33,997 patients sequenced by a targeted DNA-based next-generation sequencing panel (MSK-IMPACT), 2189 of which were also examined by an RNA-based sequencing assay (MSK-Fusion), identified 87 patients with oncogenic *NTRK1-3* fusions. All available institutional *NTRK* fusion positive cases were assessed by pan-Trk immunohistochemistry along with a cohort of control cases negative for *NTRK* fusions by next-generation sequencing. DNA-based sequencing showed an overall sensitivity and specificity of 81.1% and 99.9%, respectively, for the detection of *NTRK* fusions when compared to RNA-based sequencing. False negatives occurred when fusions involved breakpoints not covered by the assay. Immunohistochemistry showed overall sensitivity of 87.9% and specificity of 81.1%, with high sensitivity for *NTRK1* (96%) and *NTRK2* (100%) fusions and lower sensitivity for *NTRK3* fusions (79%). Specificity was 100% for carcinomas of the colon, lung, thyroid, pancreas, and biliary tract. Decreased specificity was seen in breast and salivary gland carcinomas (82% and 52%, respectively), and positive staining was often seen in tumors with neural differentiation. Both sensitivity and specificity were poor in sarcomas. Selection of the appropriate assay for *NTRK* fusion detection therefore depends on tumor type and genes involved, as well as consideration of other factors such as available material, accessibility of various clinical assays, and whether comprehensive genomic testing is needed concurrently.

Introduction

Promising recent clinical trial data and the subsequent accelerated FDA approval of the selective Trk inhibitor, larotrectinib, for the treatment of solid tumors with an oncogenic *NTRK* fusion has created an immediate need for *NTRK* fusion testing in the oncology community. This need is reflected in the National Comprehensive Cancer Center management guidelines for several cancers [1]. Larotrectinib and entrectinib have shown a dramatic and durable activity against locally advanced and metastatic solid tumors with *NTRK* fusions [2, 3]. Notably, this remarkable activity was seen regardless of tumor site of origin, histologic classification, or *NTRK* fusion type [2].

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Oncogenic fusions occur when one of the three neurotrophic tyrosine receptor kinase genes *NTRK1*, *NTRK2*, and *NTRK3* (that encode TrkA, B, and C, respectively) fuses with any of a wide variety of fusion partners. Normally, physiologic binding of neurotrophin to the extracellular portion of the Trk receptor is required for receptor dimerization, transphosphorylation, and activation of downstream signaling cascades [4]. However, the oncogenic fusion of the C-terminal kinase domain with the N-terminal fusion partner causes constitutive activation of the Trk pathway and drives tumorigenesis [4–6]. While *NTRK* fusions were first described in colorectal carcinoma [7], the t(12;15)(p13;q25) translocation resulting in an *ETV6-NTRK3* fusion was later found to be characteristic of infantile fibrosarcoma [8, 9], mesoblastic nephroma [10], and secretory carcinomas of the breast and salivary gland [11–13]. More recently, however, *NTRK* fusions have also been identified at low frequencies across a variety of more common malignancies [14–24].

Because there are three different *NTRK* genes, a variety of potential fusion partners, and a few possible breakpoints at which different exons of the *NTRK* tyrosine kinase would join the fusion partner, screening for *NTRK* fusions is somewhat complex. Assays commonly used to identify *NTRK* fusions include DNA-level sequencing using large targeted cancer gene panels with inclusion of *NTRK* introns, RNA level sequencing, and pan-Trk immunohistochemical staining to identify protein-level expression. Here we report the sensitivity, specificity, and pitfalls of various clinically validated assays used to identify *NTRK* fusions in a cohort of 87 *NTRK* fusion positive, as well as *NTRK* fusion negative cancers of different subtypes.

Materials and methods

Case selection

After approval from our institutional review board, a retrospective review of the MSK-IMPACT (DNA sequencing) and MSK-Fusion panel (RNA sequencing) results from January 1st, 2014 to March 30th, 2019 was performed. All 38,095 tumor samples from 33,997 patients were formalin-fixed paraffin embedded tissue, and all testing was performed in CLIA approved laboratories and reported clinically.

DNA sequencing

DNA sequencing was performed using MSK-IMPACT, a hybridization capture based next-generation sequencing assay that interrogates all exons (including those of *NTRK1-3*) and select introns of 468 genes to identify mutations,

copy number changes, microsatellite instability status, and select structural variants in its current iteration (previous versions interrogated 341 and 410 genes) [25]. Sensitivity to detect *NTRK1-3* fusions is increased by the inclusion of capture probes for introns 3 and 7 through 12 in *NTRK1*, intron 15 in *NTRK2* and introns 4 and 5 in *ETV6*, the most common *NTRK3* fusion partner. Kinase domain introns in *NTRK3* are not captured due to a large size (introns 13, 14, and 15 where most of the fusion breakpoints occur span 193 kb), and additionally these intronic sequences are repetitive, rendering them not feasible to include. DNA-level structural variants involving *NTRK1-3* detected by MSK-IMPACT often predicted previously described fusions with known partners. Novel structural variants, including intrachromosomal gene rearrangements, that were not previously known to result in a transcribed fusion were further characterized at the RNA level with an RNA-based next-generation sequencing assay. If a novel structural variant did not result in a transcribed fusion, the structural variant that was detected by MSK-IMPACT was deemed a false positive.

RNA sequencing

Tumors were tested for RNA-level fusions using the MSK-Fusion panel, a custom RNA sequencing panel via a next generation sequencing platform that uses anchored multiplex PCR (via the Archer platform) as previously described [26, 27]. The custom panel covers fusions involving the kinase domains of the following genes: *ALK*, *BRAF*, *EGFR*, *ERBB2*, *ERBB4*, *FGFR1*, *FGFR2*, *FGFR3*, *KIT*, *MET*, *NTRK1*, *NTRK2*, *NTRK3*, *RET*, and *ROS1*. Tumors tested included cases where there was a high suspicion of fusion based on outside testing results or morphology (such as infantile fibrosarcoma, congenital mesoblastic nephroma, or secretory carcinomas of the breast or salivary gland), and cases involving select tumor types commonly enriched in MAPK driver alterations (lung adenocarcinoma, colon adenocarcinoma, pancreas adenocarcinoma) that were negative for MAPK driver alterations (i.e., *BRAF* p.V600E, *KRAS/NRAS/EGFR* hotspot) on MSK-IMPACT testing [28].

Definition of positive cases

Tumors positive for *NTRK1-3* fusions were defined as those with either a transcribed in-frame fusion involving the kinase domain of *NTRK* detected by the RNA-based MSK-Fusion panel or, if RNA sequencing was not possible, a previously described *NTRK* fusion involving the kinase domain of the *NTRK* gene and a known fusion partner detected by the DNA-based sequencing panel MSK-IMPACT. Novel structural variants involving *NTRK*

detected by MSK-IMPACT were always confirmed at the RNA level by MSK-Fusion. In our cohort, there were two exceptions to the above definition. There was one instance of a secretory carcinoma of the salivary gland with classic histologic features where fluorescence in situ hybridization using an *ETV6* breakapart probe was used to define this patient as *NTRK3* fusion positive. Additionally, a single melanoma case (cBioPortal ID: P-0009855) was excluded from the study. This case demonstrated a *GON4L-NTRK1* structural rearrangement on both DNA-based and RNA-based sequencing, but the tumor also harbored an *NRAS* p. Q61R driver mutation and whole gene amplification of *NTRK1*, and the patient was clinically determined to be indeterminate for *NTRK* fusion.

Immunohistochemistry

Pan-Trk immunohistochemical staining using clone EPR17341 (Abcam, Cambridge, MA) was performed to assess for protein expression as previously described [29]. This rabbit recombinant monoclonal antibody, used in clinical labs and a number of recent studies [29–31], reacts with a conserved proprietary peptide in the C-terminal portion of TrkA, B, and C. Positive staining was defined as staining above background in at least 1% of tumor cells in any pattern including membranous, cytoplasmic, perinuclear, or nuclear. *NTRK* fusion positive cases that were negative for pan-Trk immunohistochemistry were re-stained with an increased concentration using a 1:25 antibody dilution if additional material was present.

Material was available for 66 cases positive for *NTRK* fusions as defined above. Negative control cases included 16 cases where a novel *NTRK1-3* structural variant was

detected by DNA sequencing yet reflex RNA sequencing was negative, 13 cases where *NTRK1-3* amplification/copy number gain was detected by DNA sequencing yet RNA sequencing was negative, 241 cases of various tumor types negative for *NTRK* fusions by RNA sequencing, and 47 additional cases with strong MAPK pathway (*BRAF* p. V600E, *KRAS/NRAS* hotspot) drivers seen by DNA sequencing in the absence of *NTRK1-3* structural rearrangements. The latter were included as previous studies have shown that *NTRK1-3* fusions are mutually exclusive with the aforementioned mutations in the vast majority of cases [27].

Results

NTRK1-3 fusion prevalence

A total of 38,095 samples from 33,997 patients had MSK-IMPACT and/or MSK-Fusion testing at Memorial Sloan Kettering Cancer Center (MSKCC). The 87 tumors identified with oncogenic *NTRK1-3* fusions and the tumor types are described in Table 1. In some of the tumor categories listed, which are classified by primary site, there are histological classifications that are enriched for *NTRK* fusions, and in these cases, histologic features can be suggestive of *NTRK* fusions (e.g., secretory carcinomas of the salivary gland and breast or infantile fibrosarcoma, see Supplementary Fig. 1). In the other categories, however, histological features have not been identified to be indicative of the presence of *NTRK* fusions. In carcinomas of the lung, pancreas, biliary tract, and appendix, *NTRK* fusions are seen in our cohort at a prevalence ranging from 0.3% to 0.5%. In

Table 1 Prevalence of *NTRK* fusions in various tumor types

	Number of cases with <i>NTRK</i> fusions	Total patients for which molecular testing was performed	Percentage
Salivary gland carcinoma	13	256	5.08%
Thyroid carcinoma	13	571	2.28%
Sarcoma	13	1915	0.68%
Lung adenocarcinoma	9	3993	0.23%
Colorectal carcinoma	9	2929	0.31%
Glioma/neuroepithelial tumor	8	1465	0.55%
Breast carcinoma	6	4458	0.13%
Pancreatic adenocarcinoma	5	1492	0.34%
Melanoma	4	1125	0.36%
Inflammatory myofibroblastic tumor	3	17	17.7%
Cholangiocarcinoma	2	787	0.25%
Appendiceal adenocarcinoma	1	208	0.48%
Neuroendocrine tumor	1	322	0.31%

thyroid carcinoma, *NTRK* fusions appear to be more prevalent, seen in 2.3% of tumors, with one explanation for the increased prevalence being that *NTRK* fusions are associated with radiation history for this tumor type [32]. *NTRK* fusions are seen in 0.36% of melanomas, including both cutaneous and mucosal primary sites, and are seen in 0.55% of gliomas and other primary CNS tumors. The positive tumors with the specific *NTRK* translocation identified and detailed molecular results are in Supplementary Table 1. In this study, *NTRK* fusions were mutually exclusive with strong activating MAPK pathway alterations. No *KRAS*, *BRAF*, *NRAS*, *EGFR* hotspot mutations nor kinase fusions were detected in the *NTRK* fusion cohort in Trk-inhibitor naive cancers.

DNA-based next-generation sequencing by MSK-IMPACT

Through March 30, 2019, tumors from 33,997 patients had been tested by MSK-IMPACT, and the ability of this assay for detection of *NTRK* fusion positive cases as defined in the Materials and Methods are shown in Table 2. *NTRK1-3* structural variants with novel partners or breakpoints underwent subsequent RNA testing for confirmation of an *NTRK* fusion. There were 107 cases with *NTRK* structural variants identified by MSK-IMPACT, but 46 did not exhibit a transcribed *NTRK* fusion transcript when reflexed to RNA testing. In terms of sensitivity, IMPACT was not able to detect 14 *NTRK* fusions identified by the RNA-based MSK-Fusion panel because the breakpoints were likely outside the areas covered by MSK-IMPACT, as only selected introns in *NTRK1*, *NTRK2*, and *ETV6* are covered (see methods). It is worth noting that for *ETV6-NTRK3* fusions, all 29 were detected by IMPACT, while *NTRK3* fusions with other partners were only detected in 1 out of 10 cases. The single case that was positive by IMPACT was because the breakpoint was within 133 bp of intron 15-exon 16 boundary. The *NTRK* fusions that were missed by MSK-IMPACT were four cases with *ETV6-NTRK3*, two cases with *SQSTM1-NTRK3*, two cases with *RBPMS-NTRK3*, and one case each with *TPM4-NTRK3*, *TRAF2-NTRK2*, *BCR-NTRK2*, *KANK1-NTRK2*, *STRN-NTRK2*, and *EPS15-NTRK1*. However, this

last case, a lung adenocarcinoma with the *EPS15-NTRK1* fusion, had very low tumor content and no other alterations were seen by MSK-IMPACT, likely because the variant allele frequency was too low. It should be noted that an *EPS15-NTRK1* fusion was picked up in a different lung adenocarcinoma case with adequate tumor content.

While 14 *NTRK* fusion positive cases were not detected by MSK-IMPACT, copy number analysis in a few situations provided clues that led to further testing. In two of the cases that were falsely negative by IMPACT, (a *TRAF-NTRK2* and an *ETV6-NTRK3*) an intragenic gain of *NTRK* kinase domain exons was seen, suggestive of a structural variant. Further analysis at the RNA level by MSK-Fusion confirmed the fusion. Although for the purposes of this study, these cases were called a false negative by MSK-IMPACT since a structural variant was not called by the pipeline, the clinical reports mentioned the intragenic gain, and the pathologist sent the case for follow-up testing with MSK-Fusion at the time of signout.

Immunohistochemical testing

A total of 66 fusion positive cases and 317 fusion negative cases were tested by pan-Trk immunohistochemistry. The negative cases included 13 cases with *NTRK* amplification as this has been reported to cause Trk overexpression [33], 16 cases with DNA-level *NTRK* structural variants that did not result in an expressed fusion transcript by MSK-Fusion, a mixture of common tumor types negative by MSK-Fusion that mirrored the tumor types where *NTRK* fusions were detected (carcinomas of the salivary gland, colon, thyroid, breast, lung, pancreas, and biliary tract), as well as tumor types with smooth muscle or neural differentiation (sarcomas, gliomas, neuroendocrine tumors, and small round blue cell tumors), as this has been reported to lead to false positive pan-Trk immunohistochemistry results [29]. All of the cases tested along with their molecular testing and immunohistochemical staining results are listed in Supplementary Tables 1 and 2. For pan-Trk immunohistochemistry, sensitivity was stratified by *NTRK* gene or tumor type, and the specificity was stratified by tumor type (Table 3).

Immunohistochemistry sensitivity

The sensitivity of immunohistochemistry was lower for detecting *NTRK3* fusions (79.4%) as compared to that in *NTRK1* and *NTRK2* fusions (combined 96.9%). In some *NTRK3* fusion positive cases with pan-Trk expression, staining was weak and focal (in less than 5% of cells) (Fig. 1a, b). Therefore, any positive staining in at least 1% tumor cells was classified as positive. While sometimes focal, staining in *ETV6-NTRK3* fusion positive cancers was often nuclear, which was

Table 2 Sensitivity and specificity of DNA-based cancer gene panel next generation sequencing (MSK-IMPACT) for detecting *NTRK* fusions

	Sensitivity	Specificity
<i>NTRK1</i>	96.8% (30/31)	
<i>NTRK2</i>	0% (0/4)	
<i>NTRK3</i>	76.9% (30/39)	
Total	81.1% (60/74)	99.86% (33877/33923)

Table 3 Sensitivity and specificity of pan-Trk immunohistochemistry for detecting *NTRK* fusions

	Sensitivity	
<i>NTRK1</i>	96.2% (26/27)	
<i>NTRK2</i>	100% (5/5)	
<i>NTRK3</i>	79.4% (27/34)	

	Sensitivity	Specificity
Total	87.9% (58/66)	81.1% (257/317)
Colon	87.5% (7/8)	100% (25/25)
Lung	87.5% (7/8)	100% (24/24)
Thyroid	81.8% (9/11)	100% (27/27)
Salivary	88.9% (8/9)	52% (13/25)
Breast	80% (4/5)	82.1% (23/28)
Inflammatory myofibroblastic tumor	100% (3/3)	100% (5/5)
Sarcoma	80% (8/10)	74.4% (29/39)
Pancreas	(0/0) ^a	100% (20/20)
Appendix	100% (1/1)	100% (1/1)
Cholangio	100% (2/2)	100% (19/19)
Glioma	100% (6/6)	20.8% (5/24)
Melanoma	100% (3/3)	100% (17/17)
Neuroendocrine	(0/0) ^a	88.9% (8/9)
Small round cell tumor ^b	(0/0)	45.8% (11/24)
Other ^c	(0/0)	100% (30/30)

^aThe *NTRK* fusion positive pancreatic adenocarcinoma and pancreatic neuroendocrine tumor cases were detected on cytology specimens, and no material remained available for immunohistochemical studies

^bSmall round cell tumors included embryonal rhabdomyosarcoma (8), neuroblastoma (5), Ewing sarcoma (5), desmoplastic small round cell tumor (5), and *NUT* midline carcinoma (1)

^cOther tumor types included prostatic adenocarcinoma (9), high-grade serous ovarian carcinoma (7), endometrioid adenocarcinoma (4), ampullary carcinoma (2), esophageal adenocarcinoma (2), esophageal squamous cell carcinoma (1), uterine perivascular epithelioid cell tumor (1), histiocytosis (1), myoepithelial carcinoma (1), granular cell tumor (1), and cancer of unknown primary (1)

not observed in false positive cases. When stratified by tumor type, sensitivity ranged from 80% in sarcomas and breast carcinomas to 100% in inflammatory myofibroblastic tumor, appendiceal adenocarcinoma, cholangiocarcinoma, glioma, and melanoma. It was hypothesized that for some of the tumor types, the reduced sensitivity could be due to the overrepresentation of *NTRK3* fusions. Therefore, we compared the subgroup of cases where there could be a high suspicion of *NTRK* fusions due to histology (secretory carcinoma or infantile fibrosarcoma that could be alternatively confirmed by fluorescent in situ hybridization) and found that the sensitivity was 81.8% (9 of 11 cases) versus 89.1% (49 of 55 cases) in the remaining cases ($p = 0.50$, Chi-square test). The pattern of immunohistochemistry expression (cytoplasmic only, nuclear,

peri-nuclear, or membranous) varied with the fusion partner as previously described (Fig. 1c–f) [29].

Immunohistochemistry specificity

The overall specificity of pan-Trk immunohistochemistry in our cohort of MSK-Fusion negative cases was 81.1%. Some tumor types, most notably colon, lung, thyroid, pancreatobiliary, and melanoma showed 100% specificity in our cohort, while other selected tumor types showed a higher incidence of false positive staining (Table 3), which was always cytoplasmic (Fig. 2).

Since Trk is physiologically expressed in neural and muscle tissue, tumors derived from or involving such organ systems can show pan-Trk staining. Gliomas and neuroblastomas showed particularly poor specificity and strong background staining given the presence of neuropil (Fig. 2a, b). Occasionally focal cytoplasmic staining was also seen in carcinomas with neuroendocrine differentiation (Fig. 2c). Interestingly, two carcinoma types showed a higher rate of false positive expression: breast carcinomas showed false positive pan-Trk expression in 18% of cases, and salivary gland tumors showed false positive staining in 48% of cases. All 5 false positive breast carcinomas demonstrated weak and focal cytoplasmic staining (Fig. 2d). In the salivary gland, adenoid cystic carcinoma showed positive cytoplasmic staining in 8 of 12 cases and was occasionally moderate to strong intensity (Fig. 2e). The staining pattern was cytoplasmic and often in the outer layer of tumor cells. Although the differential diagnosis should not overlap with secretory carcinoma because of differential mamoglobin expression [34], even discounting these cases, the specificity was still only 69% (9/13) in other salivary gland tumors (Fig. 2f).

In sarcomas, pan-Trk staining often showed faint cytoplasmic positivity in tumor cells, while in rarer cases, the staining can be quite striking. Pan-Trk staining is often positive in tumors within the small round blue cell category, including 5 of 5 desmoplastic small round cell tumors (Fig. 2g), 1 of 5 Ewing sarcomas, and 1 of 7 rhabdomyosarcomas. Pan-Trk staining was also positive in 3 of 5 sarcomas with *BCOR* translocations (Fig. 2h). Notably, all false positive cases demonstrated cytoplasmic or membranous expression only, and no nuclear staining was seen in these false positive cases. It should be noted that in some cases, the tumor cells themselves can be negative, but the high levels of background staining can make interpretation extremely difficult (Fig. 2i).

Immunohistochemistry specificity in cases with *NTRK* amplifications and structural variants

Of the 13 cases that showed *NTRK* amplification, two showed cytoplasmic pan-Trk staining. Both of these cases

Fig. 1 Patterns of pan-Trk immunohistochemistry expression in *NTRK* fusion positive cancers. **a** Strong cytoplasmic and nuclear staining in this secretory carcinoma of the salivary gland with canonical *ETV6-NTRK3* fusion. **b** Staining is occasionally weak and focal, seen in approximately 1% of tumor cells, as in this secretory carcinoma of the salivary gland. **c** Cytoplasmic staining is seen in this Lipofibromatosis-

like Neural Tumor with a *TPM3-NTRK1* fusion. **d** Cytoplasmic and perinuclear staining is seen in this colonic adenocarcinoma with a *LMNA-NTRK1* fusion. **e** Cytoplasmic and nuclear staining is seen in this inflammatory myofibroblastic tumor with an *ETV6-NTRK3* fusion. **f** Membranous staining is seen in this intrahepatic cholangiocarcinoma with a *PLEKHA6-NTRK1* fusion

occurred in tumor types where physiological expression is often seen: one neuroblastoma (Fig. 2a) and one undifferentiated pleomorphic sarcoma. Of the 16 cases that showed *NTRK* structural variants that did not result in an expressed fusion transcript, three showed pan-Trk staining. All 3 of the cases occurred in tumor types where physiologic expression is seen, including 1 oligodendroglioma (Fig. 2b), 1 anaplastic astrocytoma, and 1 gastrointestinal stromal tumor. Overall, the specificity of pan-Trk staining in cases with *NTRK* amplifications and structural variants is not significantly different from the specificity in the entire cohort. In the cases that were interpreted as positive, the false positive staining can be attributed to neural differentiation or to the fact that nonspecific staining is often seen in sarcomas, and it is therefore not due to the genomic alterations involving *NTRK*.

Discussion

This study represents the largest cohort of *NTRK* fusion positive solid tumors stained with pan-Trk immunohistochemistry, and it is the first to detail the sensitivity and specificity of a targeted DNA-based next-generation sequencing panel for the detection of *NTRK1-3* fusions. Pan-Trk immunohistochemistry demonstrated an overall sensitivity of 88%, with *NTRK3* fusions comprising the vast majority of false negatives.

Similarly, DNA-based sequencing had a sensitivity of 81%, with *NTRK2* and *NTRK3* fusions comprising the majority of false negatives. The specificity was over 99% for DNA-based sequencing, while the specificity for immunohistochemistry was largely dependent on the type of tumor tested.

The immunohistochemistry findings in the current study are in line with previous studies [23, 30, 31], with false positives occurring mainly in tumors with smooth muscle or neural differentiation and false negatives occurring in *NTRK3* fusion positive cancers. A positive predictive value of 100% was seen when used for examining carcinomas of the colon, lung, thyroid, pancreas and biliary tract, and for melanomas. In these tumors, therefore, one could theoretically use immunohistochemistry to definitively rule in an *NTRK* fusion, but it cannot rule out an *NTRK* fusion due to less than 100% sensitivity. Although our study did not show immunostaining in *NTRK* amplified tumors that could not be explained by other factors, such as neural differentiation, previous studies have suggested the opposite [33]. Therefore, if a positive immunohistochemistry result is obtained in these tumors, further workup with other ancillary testing should be performed.

Specificity of immunohistochemistry was worse in breast and salivary gland carcinomas (82% and 52%, respectively), which is a novel finding to our knowledge. Therefore, in breast and salivary gland tumors, caution should be exercised, and other methods of fusion detection may be more cost effective and tissue conservative. Finally, in

Fig. 2 Pan-Trk immunohistochemistry expression in *NTRK* wild type carcinomas. **a, b** This pitfall can be seen in neural derived tumors such as neuroblastoma (**a**) and oligodendroglioma (**b**). **c** Occasionally focal cytoplasmic staining can be seen in carcinoma with neuroendocrine differentiation. **d** Weak cytoplasmic staining is seen in a minority of breast invasive ductal carcinomas. **e** Adenoid cystic carcinoma often shows moderate to strong cytoplasmic staining. **f** Cytoplasmic and membranous staining is seen in this atypical pleomorphic adenoma.

The myoepithelial cells show particularly strong staining. **g, h** There is often staining in sarcomas, particularly those with neural or smooth muscle differentiation or those with other translocations, such as this desmoplastic small round cell tumor (**g**) and this sarcoma with *BCOR-CCNB3* translocation (**h**). **i** In this papillary thyroid carcinoma, there is nonspecific staining of the colloid, but the tumor cells themselves show no staining, and the stain is therefore interpreted as negative

sarcomas, both sensitivity and specificity are poor. If a fusion is suspected, a DNA or RNA based assay should be considered. Considering these results, pan-Trk immunohistochemistry is a time and tissue efficient fusion screen in several specific primary tumor types in which *NTRK1-3* fusions have been identified and are rare.

In this study, we also show that *NTRK1-3* fusion testing is feasible with relatively high sensitivity and specificity with a targeted panel DNA based sequencing assay, MSK-IMPACT. The DNA-based next generation sequencing cancer gene panel MSK-IMPACT showed very high sensitivity for detecting *NTRK1* and *ETV6-NTRK3* fusions (97% and 100%, respectively) because the *NTRK1* and *ETV6* introns are covered by the panel. However, rarer *NTRK2* and *NTRK3* fusions involving partners other than

ETV6 were missed. The challenge of detecting these latter fusions are not unique to MSK-IMPACT, as other large targeted DNA-based next-generation sequencing panels such as FoundationONE CDx do not cover these introns either. In some *NTRK* fusion positive cases without structural variants detected by MSK-IMPACT, intragenic gains of the kinase domain were seen by copy number analysis. This finding prompted further investigation by RNA testing, which was often positive for an *NTRK* fusion where the breakpoints were not covered by MSK-IMPACT. Although these intragenic gains are seen in a minority of cases, an algorithm in a bioinformatic pipeline to highlight such alterations could be beneficial to suggest further testing in targeted panel DNA-based assays to increase sensitivity, especially for *NTRK3* fusions not involving *ETV6*.

In the current study, we use the detection of a transcribed *NTRK* fusion by RNA-based sequencing to define an *NTRK* fusion positive cancer, which reflects our clinical practice. RNA-based sequencing appears to be the optimal way to approach *NTRK* fusions because the splicing out of introns simplifies the technical requirements of adequate coverage and because detection of RNA-level fusions provides direct evidence of functional transcription. However, even though it provides many advantages, RNA-based testing is not perfect. Our MSK-Fusion assay is a custom design that examines the most likely breakpoints but does not interrogate every exon within the *NTRK* genes. It is therefore possible that an event may be missed that could be detected by a more comprehensive targeted panel or whole transcriptome sequencing. Further, it is possible that some of the cases that were called false positive by immunohistochemistry or DNA-based sequencing in this study could actually be a false negative by MSK-Fusion. Other significant drawbacks to RNA sequencing more generally include its cost, relative unavailability, and labile nature of RNA compared to other methodologies. Therefore, other assays described here clearly have a role to play. Overall, the development of a testing algorithm for identification of *NTRK* fusions requires consideration of accessibility of testing modalities and economic implications. Consideration of MAP kinase driver status can be used to triage specimens for further workup, given that in this cohort, the presence of a conventional MAP kinase driver was mutually exclusive with *NTRK* fusions. For sarcoma cases, immunohistochemistry should be eschewed for more specific testing methods, such as RNA-based sequencing, particularly if there is neural or smooth muscle differentiation, as this would not only be able to definitively determine *NTRK* fusion status but may identify other fusions characteristic of certain sarcomas.

Our study has several limitations. The site of the current study was a major site for the phase I larotrectinib clinical trial [2], and a major referral center in general. We thus have referral bias that may result in a slight over-representation of *NTRK* fusions in advanced cancers. We also did not investigate the specificity of pan-Trk immunohistochemistry in every single tumor type. Tumor types without any *NTRK1-3* fusion positive cases (e.g., renal cell carcinoma) were not retrospectively investigated with pan-Trk immunohistochemistry due to the extremely low likelihood of finding an *NTRK* fusion in clinical practice.

In conclusion, newer methods of screening for *NTRK* fusions include immunohistochemistry and DNA-based next-generation sequencing. Each assay has advantages and disadvantages and works best in selected circumstances. Thus, selection of the appropriate assay depends on the tumor type, differentiation, and suspected fusion, as

well as whether comprehensive genomic testing is needed concurrently.

Compliance with ethical standards

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