Original Article

Real-world challenges in undertaking *NTRK* fusion testing in non-small cell lung cancer

Ashleigh Poh[1,2], Abdelaziz Sammour[3], Jared Mathai[3], Joanne Peverall[4], Chris Van Vliet[5], Khashayar Asadi[6], Sagun Parakh[1,2,3]

[1]Olivia Newton-John Cancer Wellness and Research Centre, Heidelberg, Victoria, Australia; [2]La Trobe University School of Cancer Medicine, Bundoora, Victoria, Australia; [3]Department of Medical Oncology, Austin Hospital, Heidelberg, Victoria, Australia; [4]PathWest, Department of Diagnostic Genomics, QEII Medical Centre, Nedlands, Western Australia, Australia; [5]PathWest, Department of Anatomical Pathology, QEII Medical Centre, Nedlands, Western Australia, Australia; [6]Department of Pathology, Austin Hospital, Heidelberg, Victoria, Australia

Contributions: (I) Conception and design: A Poh, K Asadi, S Parakh; (II) Administrative support: A Poh; (III) Provision of study materials or patients: K Asadi, S Parakh; (IV) Collection and assembly of data: A Sammour, J Mathai, K Asadi, S Parakh; (V) Data analysis and interpretation: A Sammour, J Peverall, C Van Vliet, K Asadi, S Parakh; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

Correspondence to: Dr. Sagun Parakh, BSc, MBChB, FRACP, PhD. Olivia Newton-John Cancer Wellness and Research Centre, Heidelberg, Victoria, Australia; La Trobe University School of Cancer Medicine, Bundoora, Victoria, Australia; Department of Medical Oncology, Austin Hospital, 145 Studley Rd, Heidelberg, VIC 3084, Melbourne, Australia. Email: Sagun.parakh@onjcri.org.au.

Background: We performed a retrospective analysis to determine the incidence of neurotrophic tropomyosin-receptor kinase (*NTRK)* fusion in non-small cell lung cancer (NSCLC).

Methods: Archival NSCLC tissues between 2018–2020 were screened by immunohistochemistry (IHC) with IHC-positive cases undergoing confirmatory molecular analysis. Correlative clinicopathologic parameters were collected.

Results: Of 289 samples analyzed, 10 (3.5%) cases had NTRK expression on IHC. The median age of patients with NTRK-positivity on IHC was 74.9 (range, 44–88) years and 70% had a smoking history. The cohort included seven adenocarcinomas and one each squamous cell carcinoma, large-cell neuroendocrine and not otherwise specified histologies. PDL1 expression was ≤50% in five cases. Concurrent *EGFR* mutations were detected in three cases, with two cases also showing a *PIK3CA E542K* mutation and *MET* amplification, respectively. Due to insufficient tumor material, RNA-sequencing was undertaken in only one IHC-positive case, with the other nine cases analyzed by Fluorescent in-situ Hybridisation. A *NTRK* fusion, *EML4-NTRK3* gene fusion was detected in one patient, a frequency of 0.35%.

Conclusions: *NTRK* fusions in NSCLC are rare. This study highlights real world diagnostic challenges regarding NTRK testing, such as requirements of adequate tumor tissue and appropriate testing methodologies.

Keywords: Neurotrophic tropomyosin-receptor kinase (*NTRK*); non-small cell lung cancer (NSCLC); immunohistochemistry (IHC); incidence; fluorescence in situ hybridization (FISH)

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Introduction

Treatments targeting oncogenic mutations have demonstrated high response rates and improved outcomes in patients with non-small cell lung cancer (NSCLC) (1). The tropomyosin-receptor kinase (TRK) receptor family plays an essential role in the development and function of the nervous system and comprise of three transmembrane proteins: TRKA, TRKB, and TRKC, encoded by the neurotrophic tropomyosin-receptor kinase (*NTRK)1*, *NTRK2*, and *NTRK3* genes, respectively (2). Chromosomal rearrangements of these genes cause activation and/or

overexpression of TRK receptors resulting in activation of downstream oncogenic pathways, establishing NTRK as a major target for therapy (2). *NTRK* gene fusions have been reported across a wide range of solid tumour types as the primary oncogenic driver, however their frequency is low in more common cancers (3). Larotrectinib, a specific TRK inhibitor, and entrectinib, a multi-kinase TRK inhibitor, have been approved by the US Food and Drug Administration and European Medicines Agency as cancer agnostic drugs for patients with solid tumours harbouring a *NTRK* fusion. Both these agents have demonstrated impressive overall response rates and tolerability in large basket studies that enrolled different types of *NTRK* fusion-positive tumors (4,5).

Despite the excellent treatment outcomes, the challenge for *NTRK* rearrangements remains a diagnostic one, due to the rarity of the alteration and the multiple approaches developed to identify *NTRK* rearrangements. While RNA-based next-generation sequencing (NGS) is the diagnostic tool of choice, turn-around time, cost and pathologist expertise are some of the challenges which need to be considered. In this study we retrospectively evaluated the incidence of *NTRK* fusions on a lung carcinoma cohort by screening with immunohistochemistry and followed by molecular analysis of all positive samples. The study highlights some of the challenges faced in a real-world setting in screening for these alterations. We present this article in accordance with the STROBE reporting checklist (available at https://jtd.amegroups.com/article/view/10.21037/jtd-23-113/rc).

Highlight box

Key findings

- NTRK fusions are rare genomic alterations in NSCLC that pose a diagnostic challenge relating to insufficient "tissue" and "tools".

What is known and what is new?

- In this study we found *NRTK* fusions are a rare occurrence in NSCLC consistent with other studies. Screening with IHC followed by confirmation of positivity with sequencing is recommended.
- With the discovery of increasing number of targetable genomic alterations, this real-world study highlights some of the challenges experienced with often only small tumor samples available for analysis as well as determining the most appropriate methodology.

What is the implication, and what should change now?

- Testing methodologies and algorithms need to be developed based on local resourcing while also considering the limited amount of tissue available for analysis.

Methods

This is a retrospective, single-site study in which archival formalin-fixed paraffin embedded (FFPE) tissue from histologically diagnosed NSCLC between 2018 and 2020 were sectioned and screened by immunohistochemistry (IHC) for *NTRK* rearrangements using the VENTANA® pan-TRK (EPR17341) assay. IHC-positive cases were analyzed by fluorescent in-situ hybridisation (FISH) to confirm the presence of the fusion.

For immunohistochemistry, four-micron sections of the FFPE block were cut and prepared for staining. A senior pathologist (KA) reviewed tumor histology and reported on NTRK staining. Immunohistochemistry was performed on the Benchmark Ultra platform (Ventana Medical Systems, Tucson, AZ) using the VENTANA® pan-TRK (EPR17341) assay as per manufacturer's instructions. The staining patterns, intensity (none/0, weak/1+, moderate/2+, strong/3+) and percentage of stained tumor cells were evaluated. Samples with 2+ or 3+ immunoreactivity in at least 1% of tumour cells was considered as positive.

Specimens scored positive were further analyzed by FISH to confirm the presence of a *NTRK* fusion. Unstained tissue sections were dewaxed in Hurstsol (Hurstchem, Australia), then treated in 10 mM sodium citrate buffer at 98 ℃ then incubated with pepsin (2.5 mg/mL) at 37 ℃. The sections were hybridized overnight at 37 ℃ separately with the NTRK1 (Z-2167-200), NTRK2 (Z-2205-200) and NTRK3 (Z-2206-200) probes (Zytovision, Germany) and analyzed using Olympus BX53 microscope and the Cytovision Software. A positive disruption was noted when there was a separation of the orange and green signals of at least one signal dot diameter and in greater than 10% of nuclei. However, due to the high occurrence of intrachromosomal translocation involving *NTRK1* or *NTRK*3, an additional criterion was applied in that only one orange-green signal set show the split while the other orange-green signal should not appear separated.

As per routine standard of care, all patients during the study period underwent reflex testing for at least an epidermal growth factor receptor *(EGFR)* mutation, *ALK* and *ROS1* rearrangement and PDL1. Correlative clinicopathologic parameters were also collected. Data collected included: baseline patient demographics, smoking history, tumor histology and stage, PDL1 and oncogene status and treatment history.

Table 1 Clinical characteristics of IHC-positive NTRK cases

Baseline characteristics	N=10
Age, year, median age [range]	74.9 [44–88]
Sex	
Male	7
Female	3
Ethnicity	
Caucasian/White	7
Asian	3
History of tobacco use	
Never	2
Current or previous	7
Not known	1
Stage at diagnosis	
II	1
III	1
IV	8
Histological subtype	
Adenocarcinoma	7
Squamous cell carcinoma	1
Non-small cell lung carcinoma not otherwise specified	1
Large cell neuroendocrine carcinoma	1
Lines of treatment	
No prior lines	2
≥1 prior lines	2
≥2 prior lines	5
Not known	1

IHC, immunohistochemistry; NTRK, neurotrophic tropomyosin-receptor kinase.

Statistical analysis

Patient characteristics and IHC outcomes were summarised using mean, standard deviation (or medians and ranges where appropriate), range if continuous, or using percentages if categorical.

Ethical statement

The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Austin Hospital Research and Ethics committee (No. H2006/02394) and individual consent for this retrospective analysis was waived.

Results

Clinical-pathological features

A total of 289 samples were analyzed and included 81 cytology cell blocks, 30 resection specimens and 178 biopsies. For the entire cohort, the median age was 70.9 (range, 35–93) years, with 42% [121] female and 77% [223] with a current or prior smoking history. The majority of patients had stage IV disease (83%) and tumors of adenocarcinoma histology (69%).

Of the samples analyzed, 10 (3.5%) cases had NTRK expression on IHC. The median age of patients with NTRK-expression on IHC was 74.9 (range, 44–88) years, majority male (70%) and 70% were current or former smokers. Of the tumors with NTRK expression, seven (70%) were of adenocarcinoma histology, and one each of squamous cell carcinoma, large-cell neuroendocrine and not otherwise specified histologies (*Table 1*). PDL1 expression was ≤50% in five cases (50%). Concurrent *EGFR* mutations were detected in three samples (30%), with two cases also showing a *PIK3CA E542K* mutation and *MET* amplification respectively (*Table 2*).

Immunohistochemistry analysis

Altogether six cases demonstrated moderate staining (score 2+) and four cases (40%) showed strong cytoplasmic and membranous staining (score 3+) (*Figure 1*). In nearly all cases staining was cytoplasmic, with one case demonstrating paranuclear dot-like staining. In the one patient found to have a *NTRK* fusion on molecular analysis the percentage of positive tumour cells in the evaluated sections demonstrated diffuse and homogenous positivity in all cancer cells, i.e., 100% staining (*Table 2*).

Molecular analysis

The cases expressing NTRK were sent for molecular analysis to confirm the presence of a fusion. Of these, one case (patient 10) had a *NTRK* fusion, *EML4-NTRK3* gene fusion, detected by NGS (Trailblaze Pharos, Ignyta, San Diego, CA, USA) on the STARTRK2 clinical trial (ClinicalTrials.gov number NCT02568267). No tissue was left over from this case for FISH analysis. The additional

Table 2 Molecular characteristics of IHC-positive NTRK cases

Patient	Histology	Stage	Tumor tissue	Concurrent mutations	PD-L1 (%)	IHC staining pattern	IHC staining intensity	NTRK FISH	Nucleic acid testing
1	Adeno	III	FNA	EGFR ex19 del[±] PIK3CA E542K	0	Cytoplasmic	100% 2+	Negative*	Not done*
2	LCNE	IV	Core biopsy	Pan WT	1	Cytoplasmic	20% 2+, 40% 1+	Negative*	Not done*
3	Adeno	IV	Core biopsy	Pan WT	80	Cytoplasmic	10% 2+, 60% 1+	Negative*	Not done*
4	Adeno	IV	Core biopsy	Pan WT	0	Paranuclear	30% 3+	Negative*	Not done*
5	Adeno	IV	Core biopsy	EGFR L858R[±]	NA	Cytoplasmic	40% 2+, 40% 1+	Negative*	Not done*
6	Adeno	IV	Core biopsy	Pan WT	90	Cytoplasmic	30% 2+, 50% 1+	Negative*	Not done*
7	SqCC	II	Core biopsy	Pan WT	20	Cytoplasmic	<5% 3+, 90% 2+	Negative*	Not done*
8	Adeno	IV	Core biopsy	EGFR L858R[±] MET amplification	NA	Cytoplasmic	10% 2+, 70% 1+	Negative*	Not done*
9	NOS	IV	Resection	Pan WT	80	Cytoplasmic	5% 3+, 20% 2+, 15% 1+	Negative*	Not done*
10	Adeno	IV	Core biopsy	Pan WT	2	Cytoplasmic	100% 3+	Not done*	EML4-NTRK fusion

*, insufficient tissue; [±], T790M mutation not detected. IHC, immunohistochemistry; NTRK, neurotrophic tropomyosin-receptor kinase; PD-L1, programmed death-ligand 1; FISH, fluorescence in situ hybridization; Adeno, adenocarcinoma; FNA, fine needle aspirate; EGFR, epidermal growth factor receptor; LCNE, large cell neuroendocrine; WT, wild-type; SqCC, squamous cell carcinoma; NA, data not available; NOS, not otherwise specified.

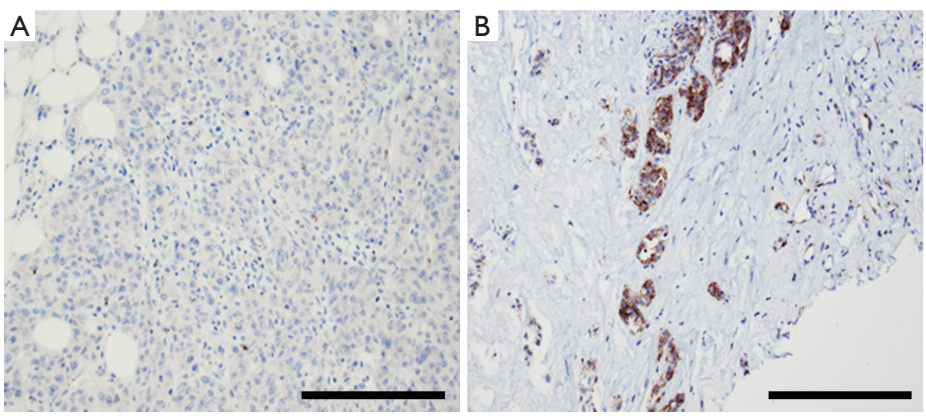

Figure 1 Representative immunohistochemical staining of lung adenocarcinoma with a pan-TRK antibody showing (A) no NTRK staining and (B) strong (3+) cytoplasmic staining (×200 magnification). NTRK, neurotrophic tropomyosin-receptor kinase.

nine cases had insufficient tumor material for nucleic acid sequencing and therefore FISH was performed instead. In all these cases no *NTRK* rearrangements were detected (*Figure 2*).

Clinical history

Patient 10, is of Asian descent and a life-long non-smoker, who presented with a cough. A CT guided biopsy of a metastatic liver lesion confirmed an adenocarcinoma of lung origin. The patient was commenced on platinum-doublet chemotherapy but unfortunately developed disease progress after two cycles of treatment. Further analysis of the tumor tissue by NGS on the STARTRK2 clinical trial (ClinicalTrials.gov number NCT02568267) demonstrated

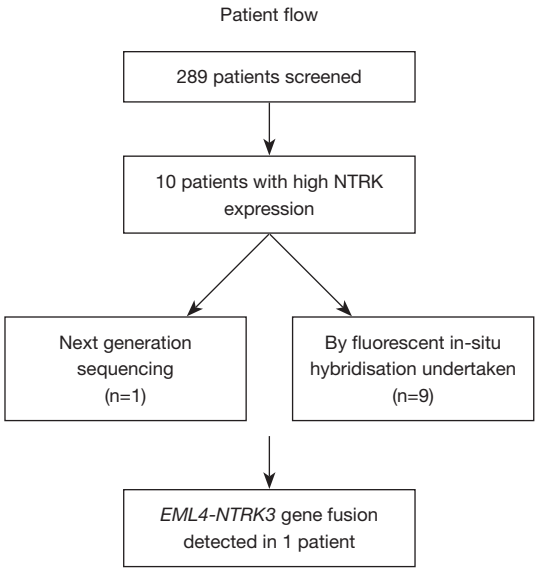

Figure 2 Patient flow. NTRK, neurotrophic tropomyosin-receptor kinase.

a *EML4-NTRK3* gene fusion. The patient was commenced on entrectinib, which he took for 12-month at which point further disease progression was seen in the liver. A repeat liver biopsy demonstrated a $NTRK^{G623R}$ compound mutation. The patient was enrolled onto the clinical trial Trident-1 (ClinicalTrials.gov number NCT03093116) where he received repotrectinib for almost two years. The patient is now off study and receiving chemoimmunotherapy with bevacizumab.

Discussion

In this study we found *NRTK* fusions are extremely rare in NSCLC, which is in concordance with other studies (2,6). Despite identifying a number of cases with high protein expression (3.5%), only one case of *NTRK* gene fusion using molecular techniques was detected. This highlights the requirement of confirmation of IHC positivity with genomic sequencing to determine fusion partners and precise breakpoints (7). In this study high NRTK expression did not correlate with sex, age, smoking history or tumour histology. We did observe a numerically higher rate of NTRK expression in tumours with low PDL1 expression; which has also been previously reported (8).

Several testing methodologies to identify *NTRK* fusions are available each with their own advantages and disadvantages and are utilised based on available resources. While nucleic acid-based sequencing, in particular NGS, is the diagnostic of choice, pan-TRK IHC has shown to have high sensitivity and specificity particularly for *NTRK*1 and *NTRK*2 fusions in NSCLC (9,10), in addition to being cheap, widely accessible and quick. Screening with IHC is in line with international recommendations (11-13), and can be particularly useful in cancers with low prevalence of *NTRK* gene fusions and in laboratories where molecular sequencing methods are not readily available or *NTRK* testing is not part of routine workflow. Currently, two monoclonal antibodies are used for detecting specific proteins include EPR17341 (Ventana® and Abcam®; used in this study) and A7H6R (Cell Signaling®) with comparable performance (14). In this study, the majority of IHC positive cases were adenocarcinomas and staining typically cytoplasmic with a heterogenous pattern. With exception of the case in which a *NTRK* fusion was detected by NGS, all other cases rarely demonstrated positive staining in more than 80% of tumor cells. We undertook FISH analysis for nine out of 10 cases as there was insufficient tumor quantity to perform RNA sequencing in these cases. While FISH is highly sensitive for fusions with canonical breakpoints, certain *NTRK*1 fusions due to small inversion or deletions may be missed by interphase FISH analysis. In addition, a positive FISH result does not provide information on the functional significance nor fusion partner and a false negative FISH result may occur as some of the *NTRK*1 and *NTRK*3 fusion partners are intrachromosomal. As all tumors included in this study were not analyzed using RNA-sequencing the rate of IHC false-negative cases is not known, especially involving *NTRK*3 (7). Furthermore, the NTRK antibody binds both the wild-type as well as the fusion protein, therefore, strong staining may indicate either expression of the wild-type protein or the presence of a TRK fusion protein (15).

NTRK fusions are typically present in a mutually exclusive manner with other oncogenic mutations and fusions (3). In this study, concurrent EGFR sensitising mutations were identified in almost a third of cases with high NTRK expression including one patient with a concurrent *EGFR* L858R mutation and *MET* amplification. Whether EGFR mutations or indeed other driver mutations lead to false positive NTRK IHC staining is not known. Tumors harboring *EGFR* mutations have shown to express *ROS1* mRNA at levels comparable to those of tumors with *ROS1* rearrangement leading to false positive *ROS1* IHC (16). RTK fusions, including TPM3-*NTRK*1 fusion, have been identified as resistance mechanisms to first, second and

third generation EGFR tyrosine kinase inhibitors (TKIs) (2). Combination treatments using EGFR TKIs with other kinase inhibitors such as osimertinib with alectinib or osimertinib and pralsetinib have shown to be successful in overcoming acquired resistance of *ALK* and *RET* fusions to EGFR TKIs (17).

This study has limitations in being a single-centre retrospective study and our ability to perform appropriate confirmatory molecular analysis was restricted by tumor quantity, which reflects some of the real world challenges faced with *NTRK* fusion testing. Despite the relative low number of cases examined, this study included a range of histological subtypes and tumor stages and highlights some of the challenges with *NRTK* testing in a real-world setting where small tumor samples (biopsies and cytology specimens) are most commonly available.

Conclusions

In conclusion, *NTRK* fusions are rare but targetable genomic alterations that pose a diagnostic rather than therapeutic challenge and require testing methodologies and algorithms developed based on local resourcing. While screening with IHC followed by confirmation of positivity with sequencing is a potential strategy, this study as well as others have shown high protein expression does not imply the presence of *NTRK*1–3 gene fusions.

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Footnote

Reporting Checklist: The authors have completed the STROBE reporting checklist. Available at https://jtd.amegroups.com/article/view/10.21037/jtd-23-113/rc

Data Sharing Statement: Available at https://jtd.amegroups.com/article/view/10.21037/jtd-23-113/dss

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://jtd.amegroups.com/article/view/10.21037/jtd-23-113/coif). SP served on the advisory board of Astra Zeneca, received speaking honoraria from Astra Zeneca, Roche and MSD, and received research funding from Roche outside the submitted work. The other authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Austin Hospital Research and Ethics committee (No. H2006/02394) and individual consent for this retrospective analysis was waived.

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