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ARTICLE

NTRK gene rearrangements are highly enriched in MLH1/PMS2 deficient, *BRAF* wild-type colorectal carcinomas—a study of 4569 cases

Angela Chou[1,2,3] · Tamara Fraser[1,3,4] · Mahsa Ahadi[1,2,3] · Talia Fuchs[1,2,3] · Loretta Sioson[1,3] · Adele Clarkson[1,3] · Amy Sheen[1,3] · Nisha Singh[3] · Christopher L. Corless[5] · Anthony J. Gill[1,2,3]

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Abstract

NTRK gene rearrangements are important to identify as predictors of response to targeted therapy in many malignancies. Only 0.16–0.3% of colorectal carcinomas (CRCs) harbor these fusions making universal screening difficult. We therefore investigated whether pan-Trk immunohistochemistry (IHC), mismatch repair deficiency (MMRd), and *BRAFV600E* mutation status could be used to triage molecular testing for *NTRK* gene rearrangements in CRC. CRCs from 4569 unselected patients underwent IHC in TMA format with two different anti-pan-Trk rabbit monoclonal antibodies. All positive cases were confirmed on whole sections and underwent RNA-sequencing. Pan-Trk IHC was positive in 0.2% of CRCs (9/4569). Both antibodies demonstrated similar staining characteristics with diffuse positive staining in all neoplastic cells. Of note 8/9 (89%) IHC positive cases were both MMRd (all showing MLH1/PMS2 loss) and lacked *BRAFV600E* mutation. That is, IHC was positive in 5.3% (8/152) MLH1/PMS2/*BRAFV600E* triple negative CRCs, but only 0.02% (1/4417) not showing this phenotype. All nine IHC positive CRCs demonstrated gene rearrangements (*LMNA-NTRK1* in 5 CRCs, *TPR-NTRK1*, *STRM-NTRK1*, *MUC2-NTRK2*, and *NTRK1* with an unknown partner in one each), suggesting close to 100% specificity for IHC in this sub-population. *NTRK* fusions were associated with right sided ($p = 0.02$), larger tumors ($p = 0.029$) with infiltrative growth ($p = 0.021$). As a part of universal Lynch syndrome screening many institutions routinely test all CRCs for MMRd, and then proceed to reflex *BRAFV600E* mutation testing in MLH1/PMS2 negative CRCs. We conclude that performing pan-Trk IHC on this preselected subgroup of MLH1/PMS2/*BRAFV600E* triple negative CRCs (only 3.3% of all CRC patients) is a resource effective approach to identify the overwhelming majority of CRC patients with *NTRK* gene fusions.

Introduction

The *neurotrophic tyrosine receptor kinase* (*NTRK*) family of genes (*NTRK1* located at 1q23.1, *NTRK2* at 9q21.33, and *NTRK3* at 15q25.3) encode three closely related tropomyosin receptor kinases TrkA, TrkB, and TrkC, respectively [1]. When activated by binding to neurotropins, these tyrosine kinases contribute to neuronal development, function, and proliferation [2, 3]. Most oncogenic events involving the *NTRK* genes require the fusion of the 3′ end of an *NTRK* gene, which contains the kinase domain, to the 5′ end of another gene resulting in constitutive overexpression and ligand-independent activation of a chimeric Trk protein. This drives proliferation via downstream signaling of the mitogen-activated protein kinase (MAPK) pathway [2].

ETV6-NTRK3 fusions drive the great majority of certain specific rare neoplasms—infantile fibrosarcoma, cellular, and

✉ Anthony J. Gill
affgill@med.usyd.edu.au

1 Cancer Diagnosis and Pathology Group, Kolling Institute of Medical Research, Royal North Shore Hospital, St Leonards, NSW 2065, Australia

2 University of Sydney, Sydney, NSW 2006, Australia

3 NSW Health Pathology, Royal North Shore Hospital, St Leonards, NSW 2065, Australia

4 School of Life Sciences, University of Technology Sydney, Ultimo, NSW 2007, Australia

5 Department of Pathology and Knight Cancer Institute, Oregon Health & Science University, Portland, OR 97239, USA

mixed congenital mesoblastic nephroma and secretory carcinoma of the breast and salivary glands [4–8]. However, oncogenic *NTRK* fusions with many other partners also occur at a very low incidence in a wide range of malignancies. To date, published data on the incidences of these fusions may be subject to a referral bias towards cases with advanced disease undergoing extensive molecular testing, but current best estimates of the incidences in different malignancies include glial/neuroepithelial tumors 0.55–1.4%, lung adenocarcinoma 0.07–0.23%, pancreatic adenocarcinoma 0.34%, melanoma 0.36%, and cholangiocarcinoma 0.25% [4, 9].

There are three different *NTRK* genes; fusions may be either intrachromosomal or interchromosomal; and fusions may involve a wide range of partners including *AFAP1F1*, *ARHGEF11*, *BCR*, *CTRC*, *DIAPH1*, *EML4*, *EPS15*, *ETV6*, *IRFBP2*, *KANK1*, *KIF21B*, *LMNA*, *PEAR1*, *PLEKHA6*, *QKI*, *RBPMS*, *STRN*, *SNHG26*, *SQSTM1*, *TFG*, *TPM3*, *TPR*, *TRAF2, TRIM63*, and *ZBTB7B* [2, 4–6]. Therefore, detection of these gene rearrangements by multiplexed next-generation sequencing assays (NGS) or fluorescence in-situ hybridization assays (FISH) studies can be complex, expensive, and difficult to deploy in the routine clinical setting.

In November 2018, larotrectinib became the first in a new class of rationally designed Trk inhibitors to receive accelerated approval for solid cancers with *NTRK* fusions in the advanced/metastatic setting, or where other treatment options are not feasible, regardless of histological classification [10, 11]. Larotrectinib and other Trk inhibitors have continued to demonstrate tremendous promise in this setting [10–12]. This has driven a demand for *NTRK* fusion testing in routine surgical pathology laboratories. Whilst the expense of these assays can be justified in the small number of advanced or resistant malignancies with a very high incidence of these rearrangements such as secretory carcinoma, infantile fibrosarcoma, and mesoblastic nephroma, universal screening can be difficult to achieve in a resource effective manner in the large numbers of common malignancies with very low incidences of *NTRK* fusions.

Similar to other malignancies, dramatic responses to larotrectinib have been reported in patients with metastatic colorectal carcinoma (CRC) harboring *NTRK* fusions [11]. In some well-resourced institutions most patients with advanced CRC already undergoing NGS or advanced molecular testing, which may include NTRK fusion testing as part of a broad panel. However, CRC is one of the most common malignancies, with an estimated 1.8 million new cases report worldwide each year [13], and *NTRK* fusions occur in only 0.16–0.31% of CRC [4, 9]. Therefore, despite the potential benefit for a small number of patients, in many centres it is considered difficult to justify the cost of routine molecular testing for *NTRK* fusions in all patients with CRC.

Pan-Trk immunohistochemistry (IHC) is emerging as a promising but not flawless surrogate marker for the detection of *NTRK* fusions in a range of malignancies [1, 4, 14]. Recently it has also been demonstrated that CRCs which are microsatellite unstable (MSI) due to MLH1 promoter hypermethylation are highly enriched for targetable tyrosine kinase fusions including *NTRK* fusions [15] and that such fusions are mutually exclusive with *BRAFV600E* and *RAS* hotspot mutations (which also activate the MAPK pathway) [15]. As a part of Lynch syndrome screening programs, most institutions now perform either MSI or mismatch repair deficiency (MMRd) testing on all CRCs, with cascade molecular or immunohistochemical testing for *BRAFV600E* mutation in CRCs that demonstrate dual PMS2 and MLH1 loss of expression [16].

We therefore sought to investigate whether the combination of pan-Trk IHC, MMRd, and *BRAFV600E* mutation status could be used to triage molecular testing for *NTRK* gene rearrangements in all patients with CRC.

Methods

Patients

We developed a cohort of unselected patients undergoing surgical resection for CRC by searching the computerized database of the Department of Anatomical Pathology, Royal North Shore Hospital, Sydney, Australia for all cases between June 1998 and 31 December 2017. Exclusion criteria included extracolonic and appendiceal location, tumors undergoing biopsy alone or treated endoluminally, and histological type other than adenocarcinoma and its variants as defined by the World Health Organization 2019 classification [17]. A tissue microarray (TMA) was created containing duplicate 1 mm cores from formalin-fixed paraffin-embedded (FFPE) tumor blocks. The entire cohort was annotated for clinicopathological details including stage, grade, MMR status, and *BRAFV600E* mutation status. Detailed methods for MMR and *BRAFV600E* detection have been previously described [18]. Overall survival was obtained from medical records and publicly available death notices and defined as the duration alive from the time of surgical resection until 1 August 2019.

Immunohistochemistry (IHC)

IHC for Trk was performed on TMA sections using the Leica-Bond III automated staining platform (Leica Micro systems, Mount Waverley, Victoria, Australia). Two different rabbit monoclonal anti-NTRK antibodies were employed on all cases and sections – clone EPR17341 (Abcam, Cambridge, MA) and clone A7H6R (Cell Signaling Technology, Danvers, MA). Both antibodies were used at a dilution of 1:50 after heat-induced epitope retrieval

for either 90 min (EPR17341) or 60 min (A7H6R) at 97 °C in the manufacturer's alkaline retrieval solution ER2 (VBS part no: AR9640).

The results of Trk IHC were interpreted independently by two pathologists (AG, AC) who were blinded to all clinical and pathological data. Cases were scored as positive if there was unequivocal staining in any percentage of tumor cells in any pattern (nuclear, cytoplasmic, and/or membrane) locations. The absence of any staining was scored as negative. If there was any doubt (for example if there was weak non-specific staining possibly in mucus only) on the TMA sections, cases were interpreted as equivocal. All cases that were considered positive or equivocal on TMA sections underwent repeat IHC on whole sections, which were again scored blinded to all clinical and pathological details.

Molecular testing

All Trk IHC whole section positive cases underwent NGS of FFPE tumor tissue. This was performed in a CLIA-certified laboratory (Knight Diagnostic Laboratories, OHSU) using a QIAseq amplicon based (Qiagen) RNA-sequencing assay (GeneTrails® Solid Tumor Fusion Gene Panel) which covers 21 target genes including *NTRK1, NTRK2, NTRK3, AKT3, ALK, BRAF, EGFR, ERBB4, ERG, FGFR1, FGFR2, FGFR3, MET, NOTCH1, NOTCH2, NRG1, NUTM1, PDGFRA, RAF1, RET*, and *ROS1* [19]. This assay is fusion partner agnostic and requires a minimum of 100,000 unique mapped reads for analysis.

FISH testing

Any case for which a definitive diagnostic result was not obtained by RNA-sequencing underwent FISH testing for *NTRK1* gene rearrangements using the ZytoLight SPEC NTRK1 Dual Color Break Apart Probe (cat no Z-2167-50, ZytoVision, Bremerhaven, Germany). FISH was performed and interpreted according to the manufacturer's instructions. A threshold of 15% nuclei positive for a break apart signal, or the same percentage with a red only signal (indicating a preserved 3′ end containing the tyrosine kinase domain with a disrupted 5′ end) was considered positive for gene rearrangement [20].

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics v23 on OSX and P values of <0.05 were considered as statistically significant. Mean overall survival was estimated using Kaplan–Meier methods and the significance of the differences was tested using the log-rank test. Clinicopathological characteristics between pan-TRK positive and pan-TRK negative tumors were compared using Fisher's exact test. A p value of <0.05 was considered significant. This study was approved by the Northern Sydney Local Health District Human Research Ethics Committee—ref: LNR 1312-417 M.

Results

Patient characteristics

A total of 4569 patients with CRC diagnosed between June 1998 and December 2017 had material in TMA sections and comprised the study cohort. The clinicopathological characteristics and overall survival are summarized in Table 1. As expected, male gender ($p = 0.026$), older age ($p = 0.0001$), right-sided location ($p = 0.0001$), larger size ($p = 0.0001$), apical node involvement ($p = 0.0001$), high pT stage ($p = 0.0001$), high pN stage ($p = 0.0001$), high pM stage ($p = 0.0001$), high overall stage ($p = 0.0001$), high histological grade ($p = 0.0001$), infiltrative pattern of growth ($p = 0.0001$), small vessel invasion ($p = 0.0001$), extramural venous invasion ($p = 0.0001$), discontinuous tumor nodules ($p = 0.0001$), positive margin involvement ($p = 0.0001$), and *BRAFV600E* mutation ($p = 0.001$) were all associated with worse overall survival.

Characteristic of NTRK positive CRC

Trk IHC was diffusely positive in all neoplastic cells in both the TMAs and whole sections in tumors from 9 of 4569 (0.2%) CRCs – Fig. 1. An additional four cases had demonstrated possible very focal non-specific staining confined to mucus on TMA sections but were definitively negative when staining was repeated on whole sections. The clinicopathological characteristics of the nine Trk IHC positive cases are summarized in Table 2. Briefly, all nine cases were negative for *BRAFV600E* mutation. Eight of the nine cases were MMRd characterised by MLH1 and PMS2 loss of expression with positive staining for MSH2 and MSH6. The one case that was mismatch repair proficient (MMRp) was also confirmed to be microsatellite stable on formal molecular testing.

When restricted to MMRd tumors, positive Trk IHC expression was found in 0.9% of the CRCs, and when restricted to both MLH1/PMS2-ve and *BRAFV600E* wild-type tumors, Trk IHC expression was found in 5.3% of cases. Trk IHC positive CRCs were associated with location in the right colon ($p = 0.02$), larger tumor size ($p = 0.029$), and MMRd ($p = 0.0001$)—summarized in Table 3. When examined in MMRd tumors only, Trk IHC positive CRC was associated with *BRAFV600E* wild type ($p = 0.0001$) and infiltrative pattern of growth ($p = 0.021$). Other than

Table 1 Clinicopathological characteristics and survival data of CRC cohort ($n = 4569$ patients)

	$n = 4569$	mean survival (months)	*P* value*
Gender			
Male	2245	115	**0.026**
Female	2324	123	
Age			
<72 years	2010	152	**0.0001**
≥72 years	2559	91	
Tumor location			
Right colon	2208	110	**0.0001**
Left colon	2361	127	
Tumor size			
<40 mm	2630	125	**0.0001**
≥40 mm	1901	111	
Apical node involvement			
Present	343	58	**0.0001**
Absent	4212	125	
T stage			
T1	318	155	**0.0001**
T2	752	156	
T3	2366	123	
T4a	944	79	
T4b	187	57	
N stage			
N0	2515	135	**0.0001**
N1a	591	134	
N1b	585	103	
N1c	170	85	
N2a	387	81	
N2b	321	55	
M stage			
M0	4350	123	**0.0001**
M1a	119	50	
M1b	100	23	
AJCCC Stage 8th ed			
I	854	155	**0.0001**
IIA	1317	131	
IIB	255	116	
IIC	63	90	
IIIA	191	175	
IIIB	1229	110	
IIIC	440	55	
IVA	115	52	
IVB	104	22	
Histological grade			
Low grade	3308	126	**0.0001**
High grade	945	95	
Pattern of growth			
Pushing	1953	134	**0.0001**
Infiltrative	1558	103	
Lymphovascular invasion (small vessels)			
Present	1186	97	**0.0001**
Absent	2137	136	
Extramural venous invasion			
Present	648	81	**0.0001**
Absent	2642	131	
Discontinuous tumor nodules			
Present	688	78	**0.0001**
Absent	2487	120	
Margin involvement			
Present (<1 mm)	218	77	**0.0001**
Absent	2857	129	
Mismatch repair protein expression			
Mismatch repair deficient (MMRd)	869	123	0.073
Mismatch repair proficient (MMRp)	3649	118	
BRAFV600E mutation			
Present	1094	109	**0.001**
Absent	3383	122	
NTRK rearrangement			
Present	9	83	0.976
Absent	4560	119	

*Kaplan-Meier analysis compared using log-rank

Bold values indicate statistical significance *p*-values

features that are well reported to be associated with MMRd, including areas of mucinous differentiation ($n = 3$ cases), a tendency to a solid-cribriform growth pattern ($n = 3$) and prominent tumor infiltrating lymphocytes, there were no specific histological features of Trk IHC positive CRCs. Four patients underwent MLH1 promoter methylation studies and all were found to be somatically hypermethylated. In view of a significant family history one of these patients also underwent genetic testing for Lynch syndrome and was found to lack germline *MLH1* and *PMS2* mutations. No other patients were tested for Lynch syndrome, and none were known to have Lynch syndrome.

Survival analysis of the entire cohort demonstrated that pan-Trk positive CRCs were associated with a trend toward shortened mean overall survival, however the difference did not reach statistical significance (83 vs 119 months, $p = 0.976$). This finding of a non-statistically significant trend towards shortened survival was similar when restricted to MMRd tumors ($p = 0.732$) and MLH1/PMS2/*BRAFV600E* triple negative tumors ($p = 0.582$).

RNA-sequencing

All nine pan-TRK positive tumors were submitted for RNA-sequencing. One case failed RNA-sequencing (repeated on two separate blocks) due to poor RNA yield. The other eight cases all showed gene rearrangement involving either the *NTRK1* (7/8) or *NTRK2* (1/8) genes. *LMNA-NTRK1* was found in 62.5% (5/8) of the cases. The other rearrangements comprised *TPR-NTRK1, STRM-NTRK1,* and *MUC2-NTRK2* fusions—Table 2.

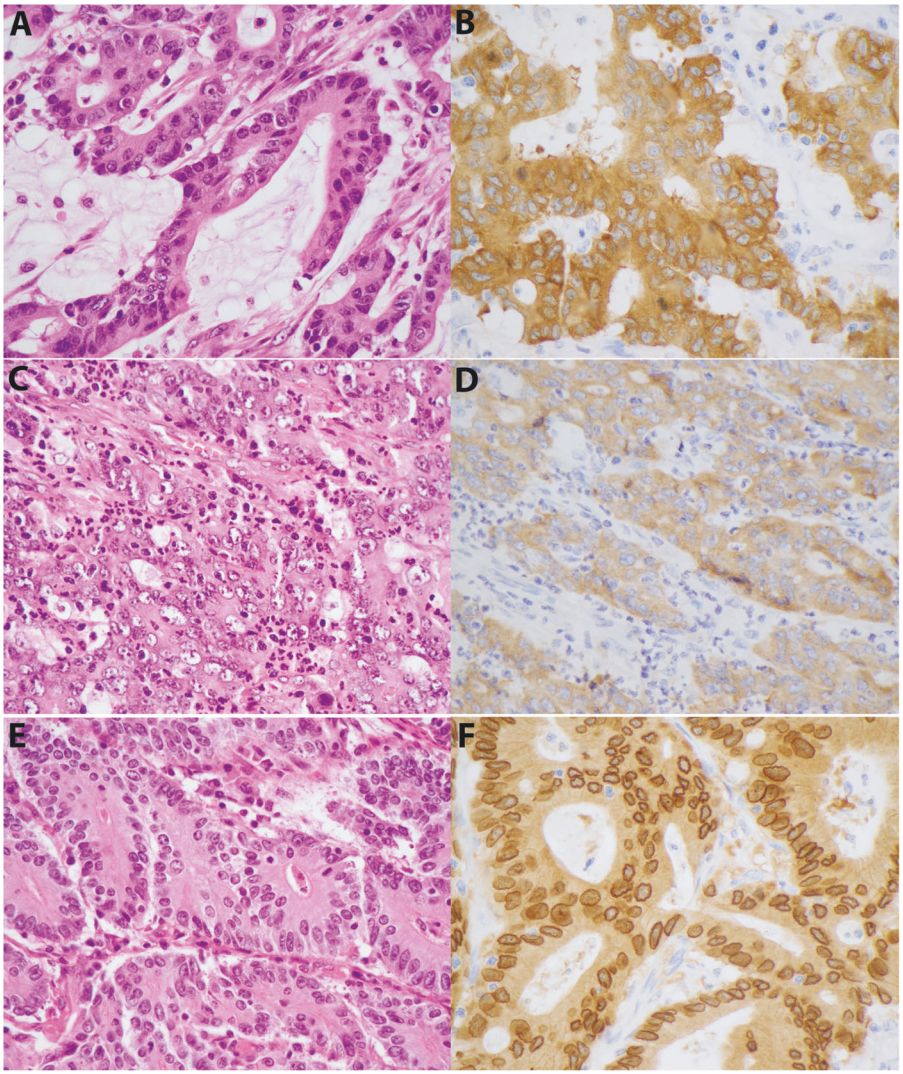

Fig. 1 Morphology and IHC of *NTRK* rearranged colorectal carcinomas. Serial H&E (**a**, **c**, **e**) and Trk IHC (**b**, **d**, **e**) stained sections. **a**, **b** Case 3 showing some mucinous differentiation and cytoplasmic only Trk staining (*MUC2-NTRK2* fusion). **c**, **d** Case 4 showing a solid-cribriform growth pattern and cytoplasmic only staining (*STRM-NTRK1* fusion). **e**, **f** Case 9 pan-TRK IHC showing nuclear, cytoplasmic, and nuclear membrane staining (*LMNA-NTRK1* fusion). [Original magnifications ×400]

Fluorescence in-situ hybridization

We performed *NTRK1* FISH on case 7, which was positive for pan-Trk IHC in a nuclear, nuclear membrane, and cytoplasmic pattern but failed RNA-sequencing. FISH studies confirmed the presence of a gene rearrangement with a red only signal pattern, which has previously been reported in an orthogonally confirmed *NTRK1* rearranged lung adenocarcinoma [20]—Fig. 2.

IHC staining pattern

Both rabbit monoclonal anti-Trk antibodies demonstrated similar staining characteristics. Although we noted that clone EPR17341 was more prone to focal non-specific uptake in extracellular mucin (explaining the four cases which were equivocal on TMA but definitively negative with both antibodies on whole sections), there was complete concordance between the two antibodies on whole sections. There was also complete concordance between the two observers in interpreting both antibodies.

On whole and TMA sections all nine Trk IHC positive cases showed diffuse strong cytoplasmic staining in all neoplastic cells. Positive staining was also noted in the adenomatous and in-situ components when present in whole sections ($n = 3$). In the three cases with adenomatous components, all were conventional adenomas (two tubulovillous and one villous). None of the precursor lesions had a discernible serrated component. There was no uptake in non-neoplastic epithelium, but staining was noted in ganglion cells and nerves in the myenteric plexus. Details of the staining patterns are summarized in Table 2 and Fig. 1. In addition to cytoplasmic staining, three cases with *LMNA-NTRK1* fusions (case 1, case 6, and case 9) and case 7 (failed RNA-seq, but *NTRK1* rearranged by FISH) also showed nuclear and nuclear membrane staining (Fig. 1f). The case with *MUC2-NTRK2* fusion (case 3), and one case with *LMNA-NTRK1*

Table 2 Clinicopathological and molecular characteristics of NTRK fusion-positive CRCs ($n = 9$)

Case no	Sex	Age (years)	Location	Size (mm)	pT stage	pN stage	pM stage	Combined AJCC stage	Grade	Pattern of growth	Follow up (months)	Survival status	pan-TRK IHC pattern			Fusion type			
													Nuclear	Nuclear membrane	Cytoplasmic	*NTRK* gene	Breakpoint	Partner	Breakpoint
1	M	82	Right	80	4b	2a	0	3c	High	Infiltrating	15.1	Alive	Patchy	Patchy	+++	*NTRK1*	Exon 15	*LMNA*	Exon 10
2	M	80	Right	75	3	0	0	2a	Low	Pushing	92.6	Dead	–	–	+++	*NTRK1*	Exon 15	*TPR*	Exon 21
3	F	93	Right	45	3	1a	0	3b	Low	Pushing	83.3	Dead	–	++	+++	*NTRK2*	Exon 9	*MUC2*	Exon 31
4	F	72	Right	35	3	0	0	2a	High	Infiltrating	73.5	Dead	–	–	+++	*NTRK1*	Exon 10	*STRM*	Exon 3
5	F	52	Right	65	3	0	0	2a	High	Infiltrating	25.2	Alive	–	–	+++	*NTRK1*	Exon 12	*LMNA*	Exon 6
6	M	69	Right	75	3	1b	0	3b	Low	Pushing	29.2	Alive	++	++	+++	*NTRK1*	Exon 12	*LMNA*	Exon 7
7	F	58	Right	50	3	2a	0	3b	High	Infiltrating	39	Alive	++	++	+++	Failed[a]	failed	failed	failed
8	F	65	Right	42	3	1a	0	3b	Low	Pushing	20	Alive	–	++	+++	*NTRK1*	Exon 11	*LMNA*	Exon 4
9	F	77	Left	30	4	0	0	2b	Low	Infiltrating	7.2	Alive	++	++	+++	*NTRK1*	Exon 12	*LMNA*	Exon 7

[a]RNA-sequencing failed on case 7, but FISH studies demonstrated *NTRK1* gene rearrangement

Table 3 Comparison of Trk IHC and fusion positive ($n = 9$) and Trk IHC and fusion negative ($n = 4569$) CRCs

Variables	NTRK positive $n = 9$	NTRK negative $n = 4560$	*P* value*
Sex			
Male	3	2242	0.343
Female	6	2318	
Age			
<72 years	4	2006	0.978
≥72 years	5	2554	
Tumor location			
Right colon	8	2286	**0.02**
Left colon	1	2274	
Other synchronous colorectal carcinoma(s)			
Absent	9	4259	0.425
Present	0	301	
Tumor size			
<40 mm	2	2628	**0.029**
≥40 mm	7	1894	
Apical node involvement			
Present	0	343	0.391
Absent	9	4203	
T stage			
T1–2	0	1070	0.097
T3–4	9	3488	
N stage			
N0	4	2511	0.522
N1–2	5	2049	
M stage			
M0	9	4341	0.5
M1	0	219	
AJCCC stage 8th Ed			
I–II	4	2485	0.545
III–IV	5	2075	
Histological grade			
Low grade	5	3303	0.108
High grade	4	941	
Pattern of growth			
Pushing	4	1949	0.499
Infiltrative	5	1553	
Lymphovascular invasion (small vessels)			
Present	5	1181	0.213
Absent	4	2133	
Extramural venous invasion			
Present	0	648	0.137
Absent	9	2633	
Discontinuous tumor nodules			
Present	1	687	0.441
Absent	8	2479	
Margin involvement			
Present (<1 mm)	0	218	0.407
Absent	9	2848	
Mismatch repair proteins expression			
Mismatch repair deficient (MMRd)	8	861	**0.0001**
Mismatch repair proficient (MMRp)	1	3648	
BRAFV600E mutation			
Present	0	1094	0.088
Absent	9	3374	

*Chi-square test

Bold values indicate statistical significance *p*-values

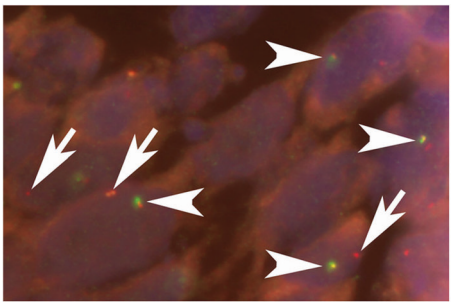

Fig. 2 *NTRK1* FISH studies from case 7 which failed RNA-sequencing. There are individual 3′ (red only) probe signals (arrows) indicating a preserved 3′ end containing the tyrosine kinase domain along with normal paired green and red signals (arrowheads) that represent non-rearranged alleles

(case 8) demonstrated nuclear membrane staining without nuclear staining.

Discussion

In our large unselected cohort of 4569 surgically resected CRCs, we found that pan-Trk IHC is 100% specific for the presence of *NTRK* gene rearrangements with 8 of 9 IHC positive cases confirmed rearranged by RNA-sequencing and the remaining case which failed this approach confirmed rearranged by FISH. That is, providing IHC is validated in the local setting, IHC expression in CRC as determined by either of the Trk antibodies we tested can be considered very strong presumptive evidence of *NTRK* gene rearrangement.

However, we note that in the unselected cohort only 9 of 4569 (0.2%) CRCs were positive and thus universal Trk IHC testing is a very low yield approach. Therefore it is emphasized that Trk IHC was positive in 5.3% (8/152) CRCs demonstrating the PMS2/MLH1/*BRAFV600E* triple negative phenotype but only 0.02% (1/4417) lacking this phenotype. Given that many laboratories already perform either MSI or MMR testing on all CRCs at first biopsy or resection and then perform *BRAFV600E* mutation testing or mutation specific IHC in PMS2/MLH1 dual negative cases as part of Lynch syndrome screening programs [16, 21], CRCs with this PMS2/MLH1/*BRAFV600E* triple negative phenotype are already identified in routine clinical practice. We propose that by performing Trk IHC in this highly enriched preselected cohort, this marker then becomes high yield (5.3% of cases positive) and identifies the great majority (89%) of Trk IHC positive CRCs.

We do not routinely perform *RAS* mutation testing at first diagnosis, but do perform this testing at the time of recurrence to guide anti-EGFR therapy. In addition to being highly enriched in CRCs which are MMRd and *BRAF* wild type, there is now emerging evidence that *NTRK* gene rearrangements are also highly enriched in tumors that lack *RAS* mutations (and other abnormalities that affect the MAPK pathway) [15, 22, 23]. Although two of the *NTRK1* gene rearranged CRCs in our study were known to be *KRAS* wild type, we do not have detailed data on the *RAS* mutation status in our cohort. It is known that approximately 30% of *MLH1*-hypermethylated *BRAF* wild-type CRCs harbor *KRAS* mutations [24]. Therefore, the yield of Trk IHC could be further increased by restricting testing to CRCs which are MLH1/PMS2/*BRAFV600E*/*RAS* quadruple negative. This would have added advantages in planning treatment, given that there is now emerging evidence that *NTRK* rearranged CRCs are likely to be resistant to anti-EGFR therapy despite being *RAS* wild type [22, 23].

We designed this study to assess the practicalities of restricting reflex Trk IHC to *MLH1*/*PMS2*/*BRAFV600E* triple negative CRC. However, a significant weakness of this study is that we did not assess the sensitivity of Trk IHC for *NTRK* fusions as we did not perform *NTRK* fusion testing on the IHC negative cases. This is an important limitation of the study because Trk IHC is an imperfect screening test for *NTRK* rearrangements. In one study [14], 4 of 5 (80%) molecularly confirmed *NTRK* rearranged CRCs were Trk IHC positive, but IHC did not identify one case that was *ETV6-NTRK3* gene rearranged. In a follow up study from the same group, presumably in an overlapping cohort, Trk IHC identified NTRK rearrangements in 7 of 8 (87.5%) CRCs [4].

Two consistent themes are emerging on the sensitivity and specificity of Trk IHC in the pan-malignancy setting and mirror our experience [3]. Firstly, it appears that Trk IHC is highly specific (close to 100%) for *NTRK* rearrangements in certain tumors such as colon, lung, thyroid and pancreatobiliary, but at high risk of false positive staining in certain other malignancies including some salivary gland neoplasms, sarcomas, gliomas, and tumors with neurogenic differentiation [4, 25]. Secondly, it appears that Trk IHC is highly sensitive for *NTRK1* and *NTRK2* gene rearrangements with reported sensitivities in the pan-malignancy setting of 87.5–96.2% for *NTRK1* and 89–100% for NTRK2 [4, 9]. However, the sensitivity for *NTRK3* rearrangements is much lower—ranging from just 55–79.4% [4, 9]. Therefore, it is possible that our IHC stain may have been falsely negative in some CRCs, and in particular may have missed *NTRK3* rearranged CRCs. However, *NTRK3* rearrangements are relatively rare in CRC [4, 9]. Furthermore, the reported incidences of *NTRK* fusions in CRCs range from 2 of 1272 (0.16%) [4] to 9 of 2929 (0.31%) [9] and our finding of rearrangements in 9 out of 4569 (0.20%) CRCs in this study is certainly within the expected incidence of rearrangements in this population. That is, although Trk IHC may have missed some rearranged cases in this cohort, it is unlikely that we missed many cases.

However we fully accept that some *NTRK* (particularly *NTRK3*) gene rearranged CRCs may be negative for Trk IHC. Therefore the fact that we did not directly assess the sensitivity of Trk IHC by screening large numbers of IHC negative CRCs, but rather presumed it is likely to have good sensitivity based on similar incidences in molecularly screened populations, remains a weakness of this study.

Despite the cost advantages of restricting Trk IHC to MLH1/PMS2/*BRAFV600E* triple negative or MLH1/PMS2/*BRAFV600E*/*RAS* quadruple negative CRCs where systemic therapy is being considered, we emphasize that we still consider Trk IHC to be a triage/screening test. We note that this approach will identify the overwhelming majority of TrK IHC positive CRCs (89%), but it will certainly not identify all IHC positive CRCs and, as discussed above, based on current knowledge it may be that not all *NTRK* gene rearranged CRCs will be positive for TrK IHC. Therefore, if resources permit, molecular testing for *NTRK* gene rearrangements may still be reasonable on very low risk CRCs (those that lack the MLH1/PMS2/*BRAFV600E* triple negative phenotype) or extremely low risk CRCs (those that lack this phenotype and are TrK IHC negative).

Another potential weakness of this study is that screening was first performed on TMA rather than whole sections and it is possible that it may have missed cases with focal expression on whole sections (which would still be considered positive). However, we note that in all Trk IHC positive CRCs, the protein was expressed diffusely through the carcinomas (including in the adenomatous and in-situ components). Therefore this is less likely to be a confounding factor.

The most common *NTRK* rearrangements found in the present study involved *NTRK1* ($n = 8$), partnered with *LMNA* ($n = 5$), *TPR* ($n = 1$), *STRN* ($n = 1$), or an unknown partner ($n = 1$). One case involved *NTRK2* partnered with *MUC2*. To date, reported *NTRK* fusion partners in CRC include *LMNA*, *TPM3*, *EML4*, *SCYL3, TPR,* and *ETV6* [14, 26, 27] and fusions involving all three *NTRK* genes have shown good response to larotrectinib in a recent basket trial [28]. Single case reports of good responses to NTRK inhibition have also been in reported in CRCs with *LMNA-NTRK1* and *TPM3-NTRK1* fusions [7, 29]. However, the *MUC2-NTRK2* fusion which we identified has not previously been described in any malignancy, and the *STRN-NTRK1* fusion has not previously been reported in CRC.

Recently Lasota et al. [30] reported their experience of NTRK IHC in a cohort of 7008 CRCs also screened first by IHC in TMAs or multi-tumor blocks with subsequent molecular testing on positive cases. Similar to us, they found that 0.23% (16 cases) demonstrated positive IHC expression for NTRK and *NTRK* fusions were confirmed in all 15 of these cases with sufficient RNA for testing—*TPM3-NTRK1* ($n = 9$), *LMNA-NTRK1* ($n = 3$), *TPR-NTRK1* ($n = 2$), and *EML4-NTRK3* ($n = 1$). They also found a predisposition for right-sided involvement (75% compared with our 89%), female predominance (4.3:1 vs our 2:1), frequent solid growth pattern, mucinous differentiation, and high tumor infiltrating lymphocytes, and that the majority (81% versus our 89%) were MMRd (MLH1/PMS2 deficient) with no *BRAF*, *K-RAS*, *N-RAS,* or *PIK3CA* mutations found in any of the ten CRCs tested.

In conclusion, pathogenic *NTRK* fusions occur in only a small minority of CRCs—estimated at 0.20% in this study with previously reported incidences of 0.16–0.31%. Because of their rarity, *NTRK* fusions can be difficult and expensive to identify in the routine clinical setting. This study, although not intended to address sensitivity, demonstrates that Trk IHC is close to 100% specific for the presence of *NTRK* rearrangements in CRC. Furthermore, given that universal MMRd/MSI screening with cascade *BRAFV600E* mutation testing or mutation specific IHC in PMS2-ve/MLH-ve CRCs is established as part of routine clinical care in most laboratories, we propose that the addition of Trk IHC to all patients with MLH1/PMS2/*BRAFV600E* triple negative CRCs in whom systemic therapy is being considered represents a rational and cost-effective approach to identify the great majority of patients with CRC who would benefit from this novel targeted therapy.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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