**NTRK fusion positive colorectal cancer is a unique subset of CRC with high TMB and microsatellite instability**

Hui Wang¹ | Zhi-Wei Li² | Qiuxiang Ou³ | Xue Wu³ | Misako Nagasaka⁴ | Yang Shao³,⁵ | Sai-Hong Ignatius Ou⁶ | Yu Yang⁷

¹Department of Medical Oncology, Beijing Hospital, National Center of Gerontology, Institute of Geriatric Medicine, Chinese Academy of Medical Sciences, Beijing, China
²Department of Internal Medicine, Harbin Medical University Cancer Hospital, Harbin, China
³Geneseeq Research Institute, Nanjing Geneseeq Technology Inc., Nanjing, Jiangsu, China
⁴Karmanos Cancer Institute, Wayne State University, Detroit, Michigan, USA
⁵School of Public Health, Nanjing Medical University, Nanjing, Jiangsu, China
⁶Chao Family Comprehensive Cancer Center, University of California Irvine School of Medicine, Orange, California, USA
⁷Department of Oncology, the Second Affiliated Hospital of Harbin Medical University, Harbin Medical University, Harbin, China

**Abstract**

TRK fusions are rare but targetable mutations which occur across a wide variety of cancer types. We report the prevalence of approximately 0.7% for NTRK-positive colorectal cancer (CRC) by genetically profiling 2519 colonic and rectal tumors. The aberrations of APC and TP53 frequently co-occurred with NTRK gene fusions, whereas RAS/BRAF oncogenic alterations and NTRK fusions were almost always mutually exclusive. NTRK-driven colorectal cancer patients demonstrated increased TMB (median = 53 mut/MB, 95% CI: 36.8–68.0 mut/MB), high microsatellite instability, and an enrichment for POLE/POLD1 mutations when compared to molecularly unstratified colorectal cancer population. These data shed light on possible future approach of multimodality treatment regimen including TRK-targeted therapy and immune checkpoint inhibitor therapy in NTRK-positive CRCs.

**Keywords**

colorectal cancer, gene fusions, microsatellite instability, NTRK, POLE/POLD1, tumor mutation burden

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1 | INTRODUCTION

The NTRK genes (NTRK1/2/3) encode tropomyosin receptor kinase (trk) proteins (TrkA/B/C) which are mainly involved in neural development and homeostasis. TRK fusions are rare but targetable mutations which occur in both adults and children. Studies have shown that TRK inhibitors were able to produce durable responses in TRK fusion-positive cancer patients. Currently, two first generation (1G) NTRK TKIs (larotrectinib, entrectinib) have been approved by the US Food and Drug Administration (FDA) for the treatment of both adult and pediatric cancers in a tumor-agnostic manner. Furthermore, a number of next-generation NTRK TKIs (selitrectinib [LOXO-195], repotrectinib, and taletrectinib) that can overcome acquired on-target NTRK resistance mutations especially solvent-front mutation to first-generation NTRK TKIs are in clinical development.

There were previous reports that NTRK+ colorectal cancer may represent a unique subset of CRC with high tumor mutation burden (TMB) and are more likely to be microsatellite unstable. In this study, we analyzed the clinicopathologic and molecular characteristics of a large cohort of Chinese CRC patients through comprehensive genomic profiling using next-generation sequencing from either tumor or blood samples, and identified the frequency, and clinicopathologic and genetic features, including tumor mutation burden (TMB) and microsatellite instability status (MSI), of NTRK-driven colorectal cancers with the ultimate goal of further informing diagnostic and treatment decisions.

2 | MATERIALS AND METHODS

2.1 | Patients and samples

A series of 2519 consecutive colorectal cancer clinical cases were analyzed using comprehensive genomic profiling (CGP) in a Clinical Laboratory Improvement Amendments-certified, College of American Pathologists accredited laboratory (422-gene panel – GeneseeqOne™; 425-gene panel – GeneseeqPrime™; Nanjing Geneseeq Technology, Jiangsu, China), as previously described. Detailed panel gene lists are provided in Table S1. While both panels could detect NTRK1 fusions, GeneseeqPrime™ had the additional capacity of detecting NTRK2/3 fusions, in which all exons (including flanking intronic regions) of NTRK1/2/3 plus selected introns including NTRK1 (introns 4, 7–13), NTRK2 intron 12, and NTRK3 introns 12–14 were covered. Furthermore, ETV6 introns 4–6 were included for the detection of ETV6-NTRK3 fusions. We identified patients with NTRK+ fusions by searching using natural language search tool in the Laboratory Information Management System (LIMS) database. Relevant demographic and clinical data were extracted from the database for these cases, including age, gender, date of diagnosis, histology type, pathological stage, and evaluation of treatment response per reports by clinical investigators.

For tumor tissue samples, the pathologic diagnosis and tumor content of each case was confirmed by pathologists. Peripheral blood of 8–10 ml was collected in EDTA-coated tubes (BD Biosciences) and centrifuged at 1800 g for 10 min within 2 h of collection to separate the plasma for circulating tumor DNA (ctDNA) extraction and white blood cells for genomic DNA extraction as germline control. In accord with the Declaration of Helsinki, written informed consent was collected from each patient prior to sample collection. This study was approved by the ethics committee of the Second Affiliated Hospital of Harbin Medical University, Harbin, China.

2.2 | DNA extraction and targeted enrichment

Genomic DNA from the white blood cells were extracted using the DNeasy Blood & Tissue Kit (Qiagen), while genomic DNA of fresh or formalin-fixed paraffin-embedded (FFPE) tumor specimens was purified using the QIAamp DNA FFPE Tissue Kit (Qiagen). All DNA was quantified using the dsDNA HS Assay Kit on a Qubit Fluorometer (Life Technologies). Sequencing libraries were prepared using the KAPA Hyper Prep Kit (Roche), as described previously. Indexed DNA libraries were pooled together for probe-based hybridization capture of the targeted gene regions covered by different gene panels.

2.3 | Sequencing data processing

Sequencing was performed on the Illumina HiSeq4000 platform (150 bp paired end sequencing) followed by data analysis as previously described.
FIGURE 1  NTRK fusions in colorectal cancer. (A). Colon tumor site. (B). Venn diagram of the relationships between NTRK+ colorectal cancer (CRC), high tumor mutational burden (TMB), and positive microsatellite instability status (MSI). (C). The comparison of TMB between NTRK+ CRC, molecularly unstratified CRC, NTRK+ non-CRC, and CRC that carried other kinase fusions.

TABLE 1  Patient overview

| Characteristics | TRK+ CRC (N = 17) | NTRK1+ subset (N = 14) |
|-----------------|------------------|------------------------|
| Age of onset, median, years | 65 (range: 38–76) | 67 (range: 52–76) |
| Sex, n (%) | | |
| Female | 9 (52.9%) | 8 (57.1%) |
| Male | 8 (47.1%) | 6 (42.9%) |
| Stage, n (%) | | |
| III-IV | 5 (29.4%) | 4 (28.6%) |
| n.d. | 12 (70.6%) | 10 (71.4%) |
| NTRK kinases, n (%) | | |
| NTRK1 | 14 (82.3%) | 14 (100%) |
| NTRK3 | 3 (17.7%) | — |
| Tumor site, n (%) | | |
| Colon | | |
| Right-sided | 11 (64.7%) | 9 (71.5%) |
| n.d. | 5 (29.4%) | 4 (28.5%) |
| Rectum | 1 (5.8%) | 0 |
| TMB, median, mut/MB | 53 (range: 2–108) | 53 (range: 2–108) |
| MSI-positive, n (%) | 13 (76.5%) | 12 (85.7%) |

Abbreviation: n.d., not determined.
sequencing coverage and quality statistics of patients’
tumor or plasma specimens are summarized in Table S2.
The corresponding whole blood control samples were
sequenced to a median depth of 240X (range: 177X–
384X). Specifically, sequencing data were analyzed by
Trimmomatic\textsuperscript{15} to remove low-quality (quality <15) or
N bases, and then mapped to the human reference ge-
nome hg19 using the Burrows-Wheeler Aligner (https://
github.com/lh3/bwa/tree/master/bwakit). PCR dup-
licates were removed by Picard (available at: https://
broadinstitute.github.io/picard/). The Genome Analysis
Toolkit (GATK) (https://software.broadinstitute.org/
gatk/) was used to perform local realignments around
indels and base quality reassurance. SNPs and indels
were analyzed by VarScan\textsuperscript{16} and HaplotypeCaller/
UnifiedGenotyper in GATK, with the mutant allele fre-
quency (MAF) cutoff as 0.5% for tumor tissue/FFPE
samples, 0.1% for plasma cfDNA samples, and a mini-
mum of three unique mutant reads. Common SNPs
were excluded if they were present in >1% population
frequency in the 1000 Genomes Project or the Exome
Aggregation Consortium (ExAC) 65,000 exomes data-
base. The resulting mutation list was further filtered by
an in-house list of recurrent artifacts based on a normal
pool of whole blood samples. Gene fusions were identi-
fied by FACTERA.\textsuperscript{17}

Tumor mutation burden (TMB) was calculated based
on the number of non-synonymous somatic mutations in
the coding region per megabase.\textsuperscript{13} Microsatellite (MS)
status of tumor sample was determined on the overall sta-
bility of MS loci tested in the panel. A sample was reported
as microsatellite instable (“MSI”) if ≥40% of the MS loci
display instability, or as “MSS” if <40% of the MS loci dis-
play instability.
from 2519 patients were successfully evaluated with comprehensive genomic profiling using next-generation sequencing. Among them, a total of 17 NTRK+ colorectal cancer patients were identified, including 14 cases of NTRK1+ CRCs and three cases of NTRK3+ CRCs (Figure 1A). The overall incidence of NTRK+ fusion positive CRC was thus approximately 0.7% (17/2519). The characteristics of the patients are summarized in Table 1, and a detailed description of each patient's demographic and clinical information are provided in Table 2. The median age of diagnosis was 65 years (range: 38–76 years, Table 1). The cohort had 16 cases of colon cancer and one case of rectal cancer, and more than half (58.8%) were confirmed of right-sided tumors (ascending colon) (Figure 1A). As provided in Table 2, TPM3 was the most common fusion partner (11/14) of NTRK1, and the other detected partners included LMNA (n = 2) and TRP (n = 1). NTRK1 rearrangements most frequently occurred in NTRK1 introns.

### 2.4 PD-L1 staining

PD-L1 staining was performed using the monoclonal mouse antihuman PD-L1 antibody (clone 22C3, Cat No. M3653; Dako). A minimum of 100 viable tumor cells must be present in the specimen slide for the PD-L1 expression to be calculated with complete or partial membrane staining. PD-L1 assay results were interpreted according to the scoring guidelines as previously described.18

### 3 RESULTS

#### 3.1 Incidence of NTRK-positive colorectal cancer and fusion partners

From April 2016 to May 2020, a total of 2940 unique clinical colorectal cancer fresh or FFPE tumor samples derived

| Breakpoint1 | Breakpoint2 | Allele frequency (P, plasma; F, FFPE | Sample type | Molecular assay | TMB\(^a\) (mut/MB) | MSI status | PD-L1 (TPS, CPS) |
|-------------|-------------|--------------------------------|-------------|----------------|-------------------|------------|-----------------|
| 1:154139441 | 1:156843913 | 12.20%                          | Tissue      | 425 gene panel | 56                | MSI        | <1%, 2%         |
| 1:156843713 | 1:154132660 | 16.50%                          | FFPE        | 425 gene panel | 53                | MSI        | NA              |
| 1:154132662 | 1:15684371512.30% |                      | FFPE        | 425 gene panel | 60                | MSI        | NA              |
| 1:154130478 | 1:156843696 | 23.57%                          | Tissue      | 425 gene panel | 75                | MSI        | 2%, 7%          |
| 1:156106224 | 1:156844785 | 32.27%                          | FFPE        | 425 gene panel | 2                 | MSS        | NA              |
| 1:154134168 | 1:156844322 | 27.80%                          | FFPE        | 425 gene panel | 108               | MSI        | NA              |
| 1:154138026 | 1:156843608 | 30.30%                          | FFPE        | 425 gene panel | 49                | MSI        | NA              |
| 1:154139680 | 1:156844015 | 9.53%                           | FFPE        | 425 gene panel | 80                | MSI        | NA              |
| 1:154134245 | 1:156843862 | 22.75%                          | FFPE        | 425 gene panel | 45                | MSI        | NA              |
| 1:156844130 | 1:154134285 | 26.44%                          | FFPE        | 425 gene panel | 60                | MSI        | NA              |
| 21:36258226 | 15:88668341 | 8.90%                           | FFPE        | 425 gene panel | 11                | MSS        | NA              |
| 1:156106765 | 1:156844901 | 38.60%                          | FFPE        | 425 gene panel | 51                | MSI        | NA              |
| 1:154138749 | 1:156845155 | 3.4% (P), 9.7% (F)              | FFPE & Plasma| 425 gene panel | 45 (F)            | MSI        | NA              |
| 1:154138750 | 1:156845151 | 1.8% (P), 11% (F)               | FFPE & Plasma| 425 gene panel | 12 (F)           | MSI        | NA              |
| 1:154134718 | 1:15683508  | 0.45% (P), 64.5% (F)            | FFPE & Plasma| 425 gene panel | 73                | MSI        | NA              |
| 12:12035081 | 15:8848921  | 27.80%                          | FFPE        | 425 gene panel | 10.40%           | NA         | NA              |
| NTRK3:exon13-ETV6:exon6 | 15:8848917 | 12:12035083                     | FFPE        | 425 gene panel | 23.94%            | Plasma     | 4 (P)           |
| 1:156844343 | 1:186317771 | 3.04%                           | Plasma      | 425 gene panel | 10 (P)           | MSS        | NA              |
| 1:154134066 | 1:156843950 | 21.37%                          | FFPE        | 425 gene panel | 67 (F)           | MSI        | 25%, 30%        |
| 1:154134066 | 1:156843952 | 1.13%                           | Plasma      | 425 gene panel | 1.13%            | Plasma     | 10 (P)          |
| 15:64624388 | 15:88486523 | 5.00%                           | Plasma      | 425 gene panel | 5.00%            | Plasma     | 10 (P)          |

| Molecular assay | TMB (mut/MB) | MSI status | PD-L1 (TPS, CPS) |
|-----------------|--------------|------------|-----------------|
| 425 gene panel  | 56           | MSI        | <1%, 2%         |
| 425 gene panel  | 53           | MSI        | NA              |
| 425 gene panel  | 75           | MSI        | 2%, 7%          |
| 425 gene panel  | 2            | MSS        | NA              |
| 425 gene panel  | 108          | MSI        | NA              |
| 425 gene panel  | 49           | MSI        | NA              |
| 425 gene panel  | 80           | MSI        | NA              |
| 425 gene panel  | 45           | MSI        | NA              |
| 425 gene panel  | 60           | MSI        | NA              |
| 425 gene panel  | 11           | MSS        | NA              |
| 425 gene panel  | 51           | MSI        | NA              |
| 425 gene panel  | 45 (F)       | MSI        | NA              |
| 425 gene panel  | 73           | MSI        | NA              |
| 425 gene panel  | 12 (F)       | MSI        | NA              |
| 425 gene panel  | 3            | MSI        | NA              |
| 425 gene panel  | 4 (P)        | MSS        | NA              |
| 425 gene panel  | 10 (P)       | MSS        | NA              |
NTRK3+ fusions accounted for the remaining three NTRK+ CRC, in all cases that NTRK3 (exon 14) was fused to ETV6, RUNX1 (Figure S1A), and CSNK1G1 (Figure S1B), respectively. Neither RUNX1-NTRK3 nor CSNK1G1-NTRK3 fusions were previously reported in CRC or any other cancer types. The patient P10, who was detected of RUNX1-NTRK3 (MAF: 8.9%), also carried a KRAS Q61R point mutation (Figure 2A). The patient P18 harbored a novel CSNK1G1-NTRK3 fusion at a MAF of 2.7% with concurrent deleterious mutations of TP53 and APC (Figure 2A), although no canonical driver mutations were identified.

Four patients (P2, P9, P10, and P16) received first-line chemotherapy, six patients were treated with first-line surgery, while the remaining seven cases were treatment-naïve (Table 2). None of the patients received targeted therapy or immune checkpoint inhibitor therapy. All samples being analyzed by NGS were treatment-naïve except in the cases of P2, P9, and P16 (Table 2).

### 3.2 Microsatellite instability status

Seventy-six percent of the NTRK+ CRC cohort was MSI-positive tumors (microsatellite unstable) (Table 1), a rate much higher than that of the molecularly unstratified Chinese CRC population according to our database (8%, unpublished). Among the CRC samples that were microsatellite unstable (MSI), 6% was NTRK fusion positive (Figure 1B), in comparison to a rate 0.17% of NTRK+ in the microsatellite stable (MSS) sub-population. Furthermore, mutations of POLE or POLD1 were detected in approximately 47% (8/17) of the NTRK+ CRC subset and more than half of the patients (5/8) carried concurrent POLE/POLD1 mutations including

**FIGURE 2** Genomic features observed in NTRK+ colorectal cancers. (A) Co-mutation plot illustrating alterations with the occurrence of at least one third of the NTRK+ cohort. Each column represents a NTRK-fusion positive patient. Alteration types are color-coded shown on the right panel. Patient’s clinicopathological features and tumor mutation burden were shown on top of the co-mutation plot. (B) The lollipop plot mapping identified mutations of POLD1 or POLE to protein sequences.
missense and truncating variants (Figure 2B). Of note, all POLE+/POLD1+ tumors were microsatellite unstable.

3.3 DNA mismatch repair genes (MMR) status

Six patients were identified with somatic missense or frameshift aberrations of DNA mismatch repair (MMR) genes including MLH1, MSH2, MSH6, and PMS2 (Table 2). Patient P1 and two additional patients (P6 and P12) also carried germline mutations of MMR genes (Table 2). All eight patients (47%, 8/17) who contained germline or somatic alterations of MMR genes were microsatellite unstable.

3.4 Tumor mutation burden (TMB)

NTRK+ colorectal cancer patients had significantly higher tumor mutation burden (median 53 mutations per megabase [mut/MB], 95% CI: 36.8–68.0 mut/MB, Figure 1C) in comparison to that of the overall colorectal cancer population (median: 7.7 mut/MB, 95% CI: 11.8–14.2 mut/MB, p < 0.0001), NTRK+ non-CRC solid tumors (lung cancer) (median: 4 mut/MB, 95% CI: 2.4–7.7 mut/MB, p < 0.0001), or CRC samples harboring other onco- genetic fusions including ALK, ROS1, and FGFR fusions (median: 6.6 mut/MB, 95% CI: 5.5–13 mut/MB, p < 0.0001, Figure 1C). All microsatellite unstable tumors had TMB of more than 10 mutations per megabase (TMB-H) (Table 2). The patient P10 was MSS but had a TMB of 11 mutations per megabase (Table 2). Importantly, among all CRC samples that were TMB-H (≥10 mut/MB), approximately 1.6% was NTRK fusion positive (Figure 1B).

3.5 PD-L1 expression

In addition, we have also evaluated the PD-L1 expression levels of three patients whose original samples were retrieved and remained adequate for testing (Figure S2). Both tumor proportion score (TPS) and combined positive score (CPS) were calculated (Table 2). All three patients were microsatellite unstable and had TMB of ≥10 mutations per megabase as well as CPS of 1 or higher, although the TPS appeared to be less than 1% in P1 (Table 2).

3.6 Genetic co-alterations

RNF43 was the most frequently mutated gene (71%) in NTRK+ patients (Figure 2A), followed by ARID1A (53%), TP53 (53%), and KMT2B (47%). The frequency of TP53 (53%) or APC (35%) mutations, was relatively lower in the NTRK+ cohort compared to that of the total CRC population (75% and 65%, respectively, unpublished). Notably, mutations of RNF43 and ARID1 were significantly enriched in NTRK+ MSI-positive tumors when compared to the NTRK+ MSS counterparts (p values = 0.002 and 0.02, respectively, Fisher’s exact test, Figure 2A). Mutated APC was identified in six out of 17 patients (35%) including missense, frameshift, in-frame insertion, and truncations. Oncogenic RAS/BRAF aberrations were almost absent in the NTRK+ CRC subset. The majority of NTRK+ patients (15/17) were RAS/BRAF wildtype, except that a KRAS Q61R (mutant allele frequency [MAF]: 13.57%) was detected in P10 and a BRAF frameshift variant (A404Cfs*9, MAF: 22.65%) was identified in P9 (Table 2), although the clinical significance of the latter remained uncharacterized.

4 DISCUSSION

We demonstrated that CRC harboring NTRK fusion is rare with an approximate incidence of 0.7%. The NTRK-positive cohort primarily consisted of NTRK1 fusions. Three out of 17 NTRK+ CRC were NTRK3 fusions including two novel NTRK3 fusions. No NTRK2 fusions were identified. This is not due to insufficient “baiting” of NTRK2 as probes to all kinase domain encoding exons of NTRK2 as well as intron 12 were used and we have successfully identified NTRK2 fusions from other tumor types in our database. Of note, while the aberrations of APC and TP53 frequently co-occurred with NTRK fusions, these fusions rarely co-existed with other activating driver mutations, consistent with what was previously reported for the NTRK rearrangement in a pan-cancer setting by Rosen et al.19

The significance of our findings is that NTRK+ CRC represents a unique molecular subtype of CRC with very high TMB (median 53 mut/MB, range 2–108 mut/MB) and were more likely to be microsatellite unstable. A total of eight patients (47% of the NTRK+ CRC subset) harbored germline or somatic alterations of MMR genes. This dual molecular signature is not only unique to CRC, but also unique among other NTRK+ solid tumors where the median TMB is 4 mut/MB for NTRK+ lung cancer.

There is also important clinical implication of these dual molecular signature in NTRK+ CRC is that there are two NTRK inhibitors (larotrectinib and entrectinib) approved in the US with several next-generation TKIs being developed (selitrectinib, repotrectinib, and talrectinib) to overcome the on-target acquired resistance NTRK mutations in particularly the solvent-front mutations. Additionally, the immune checkpoint inhibitor (ICI)
pembrolizumab has now been approved for use first in a tumor-agnostic manner in tumors that are microsatellite unstable or mismatch repair deficient that have progressed following prior treatment on May 23, 2017 and on June 29, 2020 approved for use as first-line treatment of MSI-high or MMR-deficient CRC. Pembrolizumab was approved on June 27, 2020 in another tumor-agnostic manner in tumors with high TMB (≥10 mut/MB). Thus, not only will most patients with this subset NTRK+ CRC benefit from the current approved NTRK TKIs, but may also potentially benefit from ICIs. Notably, a prior study by Zou et al. reported that enriched CD8+ tumor-infiltration T cells, quantified by using a DNA methylation-based method, was associated with MSI-H tumors in CRC cohorts and predicted better survival. However, it will require further investigation as to whether two molecular signatures (TMB and MSI) being positive, the response to pembrolizumab will be higher (additive or synergistic effect) than just having one molecular signature. Given the rarity of these NTRK+ CRC, none of the 17 NTRK+ colorectal cancer patients have been treated with pembrolizumab or any other ICIs.

At last, this study has a few limitations. First, we report an approximate frequency of 0.7% of NTRK fusions in colorectal cancer. Although this study was based on a large CRC population, it lacked a particular attention to potential accrual biases at different research sites owing to the study’s real-world and retrospective nature. Second, a more comprehensive diagnostic evaluation of the NTRK gene family is warranted. The current data can be supplemented by results of alternative diagnostic approaches, including targeted RNA testing, pan-NTRK immunohistochemical (IHC) staining, and DNA methylation analysis, which could particularly be useful in an scenario in which a novel rearrangement needs to be validated. In addition, a close follow-up of patient’s response to the following treatment is required, including TKI treatment and immunotherapy, if applicable.

5 | CONCLUSIONS

NTRK fusions positive colorectal cancer are rare (0.7% of colorectal cancer). In addition to the absence of other known actionable driver mutations, NTRK+ CRC tumors harbor very high tumor mutation burden (median 53 mut/MB), with most of them being microsatellite instability-high (MSI-H), and an enrichment of POLE/POLD1 mutations. Of the 17 NTRK+ colorectal cancer identified, 14 cases had NTRK1-rearranged events with TPM3 being the most frequent fusion partner, and the remaining three cases were NTRK3+ fusion cases. These data may be informative in guiding molecularly driven treatment including targeted therapy and immunotherapy for treating NTRK+ CRC patients. Patients with MSI-H or high TMB CRC should also be screened for NTRK fusions.

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CONFLICT OF INTEREST

QO and XW are the employees of Nanjing Geneseeq Technology Inc., Nanjing, Jiangsu, China. MN received honorarium from Astra Zeneca and Tempus. YS is an employee and shareholder of Nanjing Geneseeq Technology Inc., Nanjing, Jiangsu, China. SHIO has received speaking/advisory honorarium from Pfizer, Merck, Roche/Genentech, Takeda/ARIAD, and AstraZeneca. SHIO is a stock owner and former member of the scientific advisory board of Turning Point Therapeutics, Inc. The remaining authors have no conflict of interest to declare.

AUTHOR CONTRIBUTIONS

HW and ZL conceived and designed the study. QO analyzed the data. XW reviewed the data and revised the manuscript. YS provided the resources for the study. YY supervised the study. HW, ZL, and QO wrote the manuscript. MN and SHIO critically reviewed and revised the manuscript. All authors read and approved the final manuscript.

ETHICS STATEMENT

In accord with the Declaration of Helsinki, written informed consent was collected from each patient prior to sample collection. This study was approved by the ethics committee of the Second Affiliated Hospital of Harbin Medical University, Harbin, China.

DATA AVAILABILITY STATEMENT

Mutations identified in the 17 NTRK+ colorectal cancer patients are provided in Table S3. Other data that supports the findings of this study are available from the corresponding author upon request.

ORCID

Qiuxiang Ou https://orcid.org/0000-0002-2961-2057
Misako Nagasaka https://orcid.org/0000-0001-5308-615X
Sai-Hong Ignatius Ou https://orcid.org/0000-0002-1764-4975

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SUPPORTING INFORMATION
Additional supporting information may be found in the online version of the article at the publisher’s website.

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