Pathology & Oncology Research (2020) 26:1385–1399
https://doi.org/10.1007/s12253-019-00685-2

REVIEW

Methods for Identifying Patients with Tropomyosin Receptor Kinase (TRK) Fusion Cancer

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Received: 14 December 2018 / Accepted: 11 June 2019 / Published online: 29 June 2019

Abstract

NTRK gene fusions affecting the tropomyosin receptor kinase (TRK) protein family have been found to be oncogenic drivers in a broad range of cancers. Small molecule inhibitors targeting TRK activity, such as the recently Food and Drug Administration-approved agent larotrectinib (Vitrakvi®), have shown promising efficacy and safety data in the treatment of patients with TRK fusion cancers. *NTRK* gene fusions can be detected using several different approaches, including fluorescent in situ hybridization, reverse transcription polymerase chain reaction, immunohistochemistry, next-generation sequencing, and ribonucleic acid-based multiplexed assays. Identifying patients with cancers that harbor *NTRK* gene fusions will optimize treatment outcomes by providing targeted precision therapy.

Keywords *NTRK* gene fusions · TRK fusions · TRK inhibitors · Next-generation sequencing · NGS

Introduction

TRK Receptor Family and Signaling

The tropomyosin receptor kinase (TRK) family is a group of three neurotrophic receptor tyrosine kinase proteins (TRKA, TRKB, and TRKC) encoded by the *NTRK1, NTRK2,* and *NTRK3* genes located on chromosomes 1q23.1, 9q21.33, and 15q25.3, respectively. These receptors are normally expressed in neuronal tissues and have high affinity for and are activated by neurotrophins. Activation of a TRK protein and subsequent signal transduction requires homo-dimerization of TRK membrane receptors following ligand binding [1]. Developmentally, TRK proteins are important for the differentiation and maturation of the central and peripheral nervous system through activation of the phosphoinositide 3-kinase/protein kinase B (PI3K-AKT) and mitogen-activated protein kinase (MAPK) signaling cascades [2–5] (Fig. 1).

NTRK Gene Fusions

Gene fusions involving the TRK protein family typically involve intra- or inter-chromosomal rearrangements of the 5′ end of a fusion partner containing a dimerization/oligomerization domain with the 3′ region of an *NTRK* gene encoding the tyrosine kinase domain. The resulting fusion gene leads to the expression of a chimeric protein that lacks the TRK ligand-binding domain but retains the tyrosine kinase domain. This fusion protein harbors oncogenic and transforming potential through overexpression and constitutive activation of the TRK kinase domain due to the presence of a dimerization domain derived from the fusion partner [5–8] (Fig. 2a). Historically, the first *NTRK* gene fusion was isolated from a human colon carcinoma by classical deoxyribonucleic acid (DNA) transformation assays [10]. The *ETV6-NTRK3* gene fusion is the most extensively studied *NTRK* gene fusion. Recurrent *NTRK* gene fusions involving *ETV6* and *NTRK3* (Fig. 2b) were first identified in infantile (or congenital) fibrosarcoma, a malignant tumor of fibroblasts that occur in patients aged 2 years or younger [11], and then shortly after in congenital mesoblastic nephroma, the renal counterpart of infantile fibrosarcoma [12, 13]. Since then, *ETV6-NTRK3* fusions have

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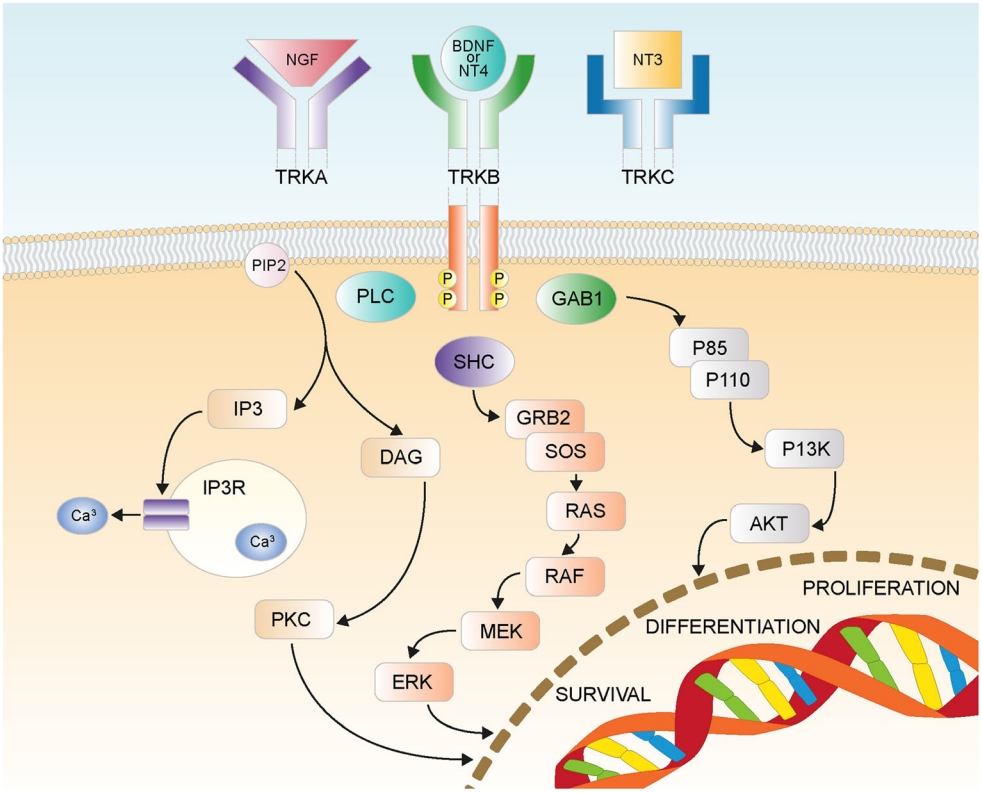

Fig. 1 Tropomyosin receptor kinase (TRK) receptor signaling [5]. AKT, v-akt murine thymoma viral oncogene homolog; BDGF, brain-derived growth factor; DAG, diacylglycerol; ERK, extracellular signal-regulated kinase; GAB1, GRB2-associated-binding protein 1; GRB2, growth factor receptor-bound protein 2; IP3, inositol trisphosphate; MEK, mitogen-activated protein kinase; NGF, nerve growth factor; NTF-3, neurotrophin 3; PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase; PIP2, phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; PLC, phospholipase C; RAF, rapidly accelerated fibrosarcoma kinase; RAS, rat sarcoma kinase; SHC, Src homology 2 domain containing. Reproduced with permission from Amatu A, Sartore-Bianchi A, Siena S. ESMO Open 2016;1(2):e000023

been identified in numerous other cancer types, including secretory breast carcinoma [14], acute myeloid leukemia [15], radiation-associated thyroid cancer [16], pediatric high-grade glioma [17], Philadelphia chromosome-like ALL, and other tumor types (Table 1).

Studies investigating the mechanism of *ETV6-NTRK3* transformation in NIH 3T3 cells and other fibroblasts have revealed that autophosphorylation of this chimeric protein results in the dual activation of RAS-ERK1/2 and PI3K-AKT signaling, and is dependent on homo- and hetero-dimerization mediated by the dimerization domain of *ETV6* [7, 50, 51]. Expression of the *ETV6-NTRK3* fusion in mammary tissues of mice has also identified early breast progenitor cells rather than stem cells as the direct targets of transformation and has provided valuable models for preclinical studies [52]. Interestingly, the protein encoded by the *ETV6-NTRK3* fusion has also been found to interact with and be dependent on the activity of insulin-like growth factor 1 receptor (IGF1R) for both stability and transformation, which may provide another clinical avenue for future treatments [53–55].

Although the *ETV6-NTRK3* fusion is the most extensively studied *NTRK* gene fusion, fusion events involving all three of the *NTRK* genes and over 50 different 5′ fusion partners have been identified (Table 1). *NTRK* gene fusions have been found in over 20 different cancer types and in up to 1% of all solid tumor malignancies, suggesting that *NTRK* gene fusions may be oncogenic drivers regardless of the tumor type [8].

Clinical Data for TRK Inhibitors

Fusions involving TRK proteins lead to constitutive activation of the kinase domain similarly to many other oncogenic drivers such as *BCR-ABL* translocation and *EGFR* amplification/mutation. One strategy in targeting these kinds of oncogenic drivers has been to develop small molecule inhibitors to block the downstream signaling pathways that are activated and drive the cancer. Currently, there are several small molecular inhibitors targeting TRK in phase II clinical trials; the most notable are larotrectinib, a highly selective TRK inhibitor (TRKA/B/C), and entrectinib, a broader tyrosine kinase inhibitor (*TRKA/B/C, ROS1, ALK*) [5, 56]. Both have demonstrated the ability to cross the blood-brain barrier, making them suitable agents for central nervous system (CNS) tumors that harbor TRK fusions [57, 58]. Clinical basket trials using larotrectinib (NCT02122913,

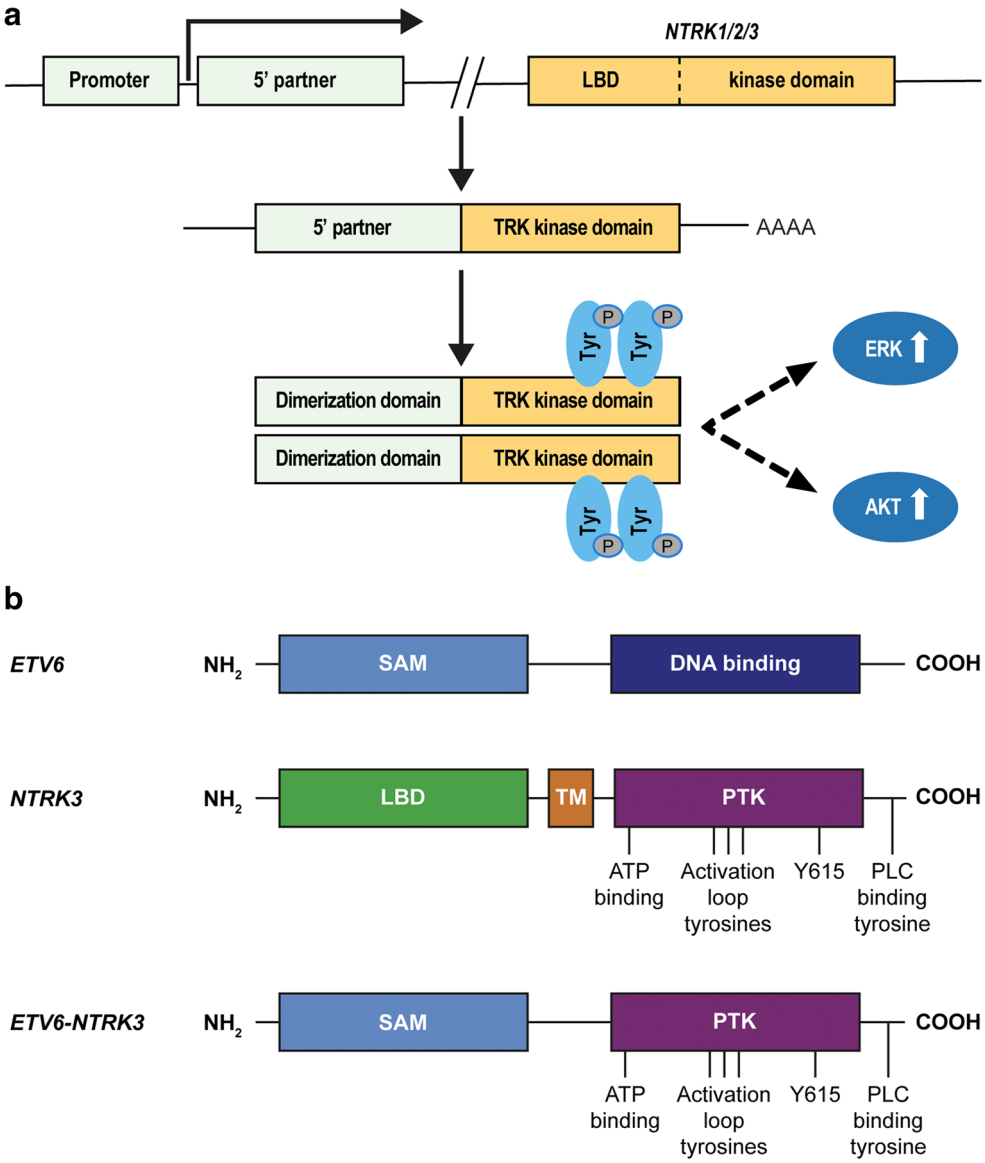

Fig. 2 *NTRK* gene fusions. (**a**) Mechanism of *NTRK1/2/3* gene fusions; (**b**) *ETV6-NTRK3* gene fusion [9]. DNA, deoxyribonucleic acid; LBD, ligand-binding domain; PTK, tyrosine kinase; TRK, tropomyosin receptor kinase; TM, transmembrane; SAM, sterile alpha motif. Figure 2b reproduced with permission from Triche TJ, Hicks MJ, Sorensen PH. Diagnostic Pathology of Pediatric Malignancies. In: Pizzo PA, Poplack DG, editors. Principles and Practice of Pediatric Oncology, 7th Edition: Wolters Kluwer Health; 2015

NCT02637687, NCT02576431) have shown durable overall response rates of 93% in a pediatric phase I/II trial and 75% in a combined adult and pediatric phase I/II trial [23, 59]. Adverse events were predominantly grade 1 or grade 2 with no grade 3 or grade 4 adverse events attributable to larotrectinib seen in more than 5% of the patients regardless of tumor type or fusion partner [23, 59]. These data clearly demonstrate the potency of larotrectinib as a therapeutic option for patients that harbor *NTRK* gene fusions. Similarly, phase I/IIa clinical trials for entrectinib demonstrated low toxicity with reversal of side-effects following dose monitoring [22]. Larotrectinib has been recently approved for use in the United States for patients with solid tumors that harbor an *NTRK* fusion gene [60]. Clinical trials for both drugs are still currently on-going and further support the utility of identifying TRK fusion cancers in order to provide effective and durable clinical therapeutic options to patients. In this review, we summarize the methods available for detecting *NTRK* gene fusions in cancer.

Methods to Identify Patients with TRK Fusion Cancer

Several different approaches have been used to detect the presence of *NTRK* gene fusions at the DNA and ribonucleic acid (RNA) level and TRK expression. These methods include traditional clinical assays such as fluorescent in situ hybridization (FISH), reverse transcription polymerase chain reaction (RT-PCR), immunohistochemistry (IHC), and newer, emerging technologies such as next-generation sequencing (NGS) and RNA-based multiplexed assays (Nanostring). Each technique is associated with advantages and disadvantages (Table 2).

Table 1 Summary of *NTRK* gene fusions by detection method and tumor type

Detection/validation method(s)[a]	Tumor type (*NTRK* gene fusion)	Study
FISH	Acute myeloid leukemia (*ETV6-NTRK3*)	Eguchi et al. [15]
	Congenital mesoblastic nephroma (*ETV6-NTRK3*)	El Demellawy et al. [18]
RT-PCR	Congenital mesoblastic nephroma (*ETV6-NTRK3*) Infantile fibrosarcoma (*ETV6-NTRK3*)	Knezevich et al. [12]
RT-PCR and FISH	Congenital mesoblastic nephroma (*ETV6-NTRK3*)	Rubin et al. [13]
	MASC (*ETV6-NTRK3*)	Skalova et al. [19]
RT-PCR and IHC	Infantile fibrosarcoma (*ETV6-NTRK3*)	Bourgeois et al. [20]
IHC and FISH	Colorectal cancer (*LMNA-NTRK1*)	Sartore-Bianchi et al. [21]
NGS (DNA- or RNA-seq), FISH, or IHC	MASC (*ETV6-NTRK3*) Colorectal cancer (*LMNA-NTRK1*)	Drilon et al. [22]
	Glioneuronal tumor (*BCAN-NTRK1*)	
	Lung cancer (*SQSTM1-NTRK1*)	
NGS[b] or FISH	Lung cancer (*IRF2BP2-NTRK1*) Melanoma (*GON4L-NTRK1, TRIM63-NTRK1*)	Drilon et al. [23];
	Pancreatic cancer (*CTRC-NTRK1*)	
	Thyroid cancer (*IRF2BP2-NTRK1, PPL-NTRK1, TPM3-NTRK1*)	
	Soft tissue sarcoma (*TPM3-NTRK1, TPM4-NTRK3*) Appendiceal cancer (*LMNA-NTRK1*)	Kummar and Lassen [24]
	Breast cancer (*GATAD2B-NTRK1, LMNA-NTRK1, TPM3-NTRK1*)	
	Cholangiocarcinoma (*LMNA-NTRK1, TPM3-NTRK1*)	
	Colon cancer (*PLEKHA6-NTRK1*)	
	Infantile fibrosarcoma (*SQSTM1-NTRK1, TPM3-NTRK1*)	
NGS (DNA-seq) and IHC	Melanoma (*GON4L-NTRK1, TRIM63-NTRK1, TRAF2-NTRK2, DDR2-NTRK1)*	Lezcano et al. [25]
NGS (RNA-seq) and array-comparative genome hybridization	Ganglioglioma (*TLE4-NTRK2*)	Prabhakaran et al. [26]
NGS (DNA-seq and/or RNA-seq)	Fibrous tumor (*TFG-NTRK3*) Infantile fibrosarcoma (*SQSTM1-NTRK1*)	Chmielecki et al. [27]
NGS (DNA- or RNA-seq) and IHC	Lung cancer (*IRF2BP2-NTRK1, MRPL24-NTRK1, P2RY8- NTRK1*) Melanoma (*TRIM63-NTRK1, TRAF2-NTRK2*)	Hechtman et al. [28]
	Soft tissue sarcoma (*TPM4-NTRK3*)	
	Colorectal cancer (*LMNA-NTRK1, ETV6-NTRK3*)	
	Glioblastoma (*BCR-NTRK2, ZNF710-NTRK3*)	
NGS (RNA-seq), IHC, and FISH	Colorectal cancer (*SCYL3-NTRK1*)	Milione et al. [29]
NGS (RNA-seq)	Pancreatic cancer (*CEL-NTRK1*)	Edgren et al. [30]
	Glioblastoma (*BCAN-NTRK1, NFASC-NTRK1*)	Kim et al. [31]

Table 1 (continued)

Detection/validation method(s)[a]	Tumor type (*NTRK* gene fusion)	Study
	Neuroendocrine cancer (*ETV6-NTRK3*)	Sigal et al. [32]
	Lung cancer (*IRF2BP2-NTRK1, TRIM24-NTRK2*) Thyroid cancer (*IRF2BP2-NTRK1, TFG-NTRK1, RBPMS-NTRK3*)	Stransky et al. [33]
	Squamous cell cancer of the head and neck (*PAN3-NTRK2, ETV6-NTRK3*)	
	Sarcoma (*TPM3-NTRK1*)	
	Glioma (*AFAP1-NTRK2, SQSTM1-NTRK2*)	
	Glioblastoma (*NFASC-NTRK1*)	
NGS (RNA-seq) and FISH	Congenital mesoblastic nephroma (*EML4-NTRK3*) Infantile fibrosarcoma (*EML4-NTRK3*)	Church et al. [34]
NGS (RNA-seq), IHC, and FISH	Colorectal cancer (*TPM3-NTRK1*)	Lee et al. [35]
NGS (RNA-seq), FISH, and RT-PCR	GIST (*ETV6-NTRK3*)	Brenca et al. [36]
NGS (whole-genome sequencing and RNA-seq)	Glioma (*AKAP13-NTRK3*)	Yoshihara et al. [37]
	Astrocytoma (*NACC2-NTRK2, QK1-NTRK2*)	Jones et al. [38]
	Glioma (*TPM3-NTRK1, AGBL4-NTRK2, VCL-NTRK2, BTBD1- NTRK3*)	Wu et al. [17]
NGS (whole-exome sequencing, whole-genome sequencing, and/or RNA-seq)	Acute lymphoblastic leukemia (*ETV6-NTRK3*)	Roberts et al. [39]
	Large cell neuroendocrine cancer (*COP1-NTRK1*)	George et al. [40]
NGS (whole-genome sequencing and RNA-seq)	Thyroid cancer (*TPM3-NTRK1*)	Ronsley et al. [41]
Targeted NGS (DNA-seq)	Uterine endometrial cancer (*LRRC71-NTRK1*) Lung cancer (*GRIPAP1-NTRK1*)	Hartmaier et al. [42]
	Intrahepatic cholangiocarcinoma (*RABGAP1L-NTRK1*)	Ross et al. [43]
	GIST (*ETV6-NTRK3*)	Shi et al. [44]
	Spitzoid neoplasm (*TP53-NTRK1, LMNA-NTRK1*)	Wiesner et al. [45]
	Lung cancer (*TPM3-NTRK1*) Thyroid cancer (*PPL-NTRK1*) Glioblastoma (*ARHGEF2-NTRK1, CHTOP-NTRK1*)	Zheng et al. [46]
Targeted NGS (DNA- and RNA-seq)	Thyroid cancer (*EML4-NTRK3, SQSTM1-NTRK3, IRF2BP2- NTRK1*)	Liang et al. [47]
Targeted NGS (DNA-seq) or FISH	Lung cancer (*CD74-NTRK1, MPRIP-NTRK1*)	Vaishnavi et al. [48]
Targeted NGS (DNA- or RNA-seq) and/or FISH	Uterine sarcoma (*LMNA-NTRK1*, *TPM3-NTRK1*, *RBPMS-NTRK3*)	Chiang et al. [49]

[a] Detection/validation method(s) used in study to identify *NTRK* gene fusion(s)

[b] Specific NGS method used in study not specified. DNA, deoxyribonucleic acid; FISH, fluorescent in situ hybridization; IHC, immunohistochemistry; MASC, mammary analog secretory carcinoma; NGS, next-generation sequencing; RNA, ribonucleic acid; RT-PCR, reverse transcription polymerase chain reaction

Table 2 Advantages and disadvantages of methodologies for detecting tropomyosin receptor kinase (TRK) fusion cancer

	Fluorescence in situ hybridization (FISH)	Reverse transcription polymerase chain reaction (RT-PCR)	Pan-TRK immunohistochemistry (IHC)	Next-generation sequencing (NGS)
Advantages	• Location of the target within the cell can be detected [61]	• High sensitivity and specificity [20, 62]	• Inexpensive [63, 64] • Decentralized, available in most laboratories [20]	• Detection of novel fusion partners [34] and fusions expressed at RNA level [65]
	• High sensitivity and specificity [63]	• Assays detect fusions expressed at the RNA level [62]	• Established reimbursement codes [66]	• Ability to test multiple actionable targets simultaneously [34]
	• Several fluorophores can be used at once to detect different targets in one sample [67]	• Inexpensive [68]	• Turnaround time: ~2 days [64]	• Plays key role in diagnostic work-up of TRK fusion cancer [23]
				• Relevance of NGS increases as number of actionable targets grows [69]
				• High sensitivity and specificity potential [63]
Disadvantages	• Requires fluorescence microscopy [61]	• Target sequences must be known; unable to detect novel fusion partners [34, 68]	• Cannot differentiate between fusion and wild-type TRK expression [28]	• Turnaround time: ~1–3 weeks [69]
				• Technically complex and costly [70]
	• Target sequence must be known; unable to detect novel fusion partners unless break-apart probes are used [34]	• Development of separate tests required for each *NTRK* gene [71]	• Scoring algorithms are not standardized [20]	• Requires highly centralized testing model [34] and bioinformatics infrastructure [72]
	• Development of separate tests required for each *NTRK* gene [71]		• Additional testing required to determine course of action [28]	• Reimbursement currently restricted [73]
	• Cannot demonstrate that functional protein has been generated [34, 67]			• Sensitivity and specificity of NGS assays vary widely [63, 74]

FISH

Historically, FISH has been the gold standard for the clinical detection of gene fusions (e.g. *BCR-ABL* rearrangements in chronic myeloid leukemia) [75]. FISH uses fluorescently labeled RNA or DNA probes that bind to complementary sequences on formalin-fixed paraffin embedded (FFPE) tumor samples. For gene fusions typical of certain malignancies (e.g. *ETV6-NTRK3* in infantile fibrosarcoma), dual color FISH probes can be used. One major advantage of FISH analysis is the ability to detect the presence of a fusion event involving a target gene without prior knowledge of the fusion partner by utilizing "break-apart" probes, where each probe is directed to the 5′ and 3′ ends of the target gene, respectively (Fig. 3a and b). An intact *NTRK* gene would result in overlapping probes and produce yellow fluorescence whereas a translocation event would result in the probes "breaking apart" to produce two individual probes (red and green) indicating a break in the gene most likely arising from a chromosomal translocation. An example is the detection of a *LMNA-NTRK1* fusion using break-apart probes in a soft-tissue sarcoma [76] (Fig. 3c). Although this method is useful when the fusion partner is unknown, individual FISH analysis must be performed for each of the three *NTRK* genes due to the sequence specificity of the probes; this can be labor- and cost-intensive. Lastly, due to the detection of gene rearrangements at the DNA level, FISH does not provide any information as to whether an oncogenic fusion protein is produced.

RT-PCR

Tumor RNA from fresh frozen or FFPE samples can be extracted and converted to complementary DNA (cDNA) sequences using reverse transcription. The cDNA is then amplified using polymerase chain reaction (PCR) primers that are located on either side of the fusion breakpoint, resulting in a PCR amplification product only when that specific fusion is present. The amplification products can then be visualized using intercalating dyes that bind to double-stranded DNA or a fluorescent reporter-quencher system that allows for multiplexing of multiple primer sets [77]. RT-PCR provides a highly specific, rapid, economical, and sensitive testing method, even at low transcript levels, with quick turnaround time and multiplexing capabilities [78] compared with FISH analysis. However, RT-PCR requires prior knowledge of the

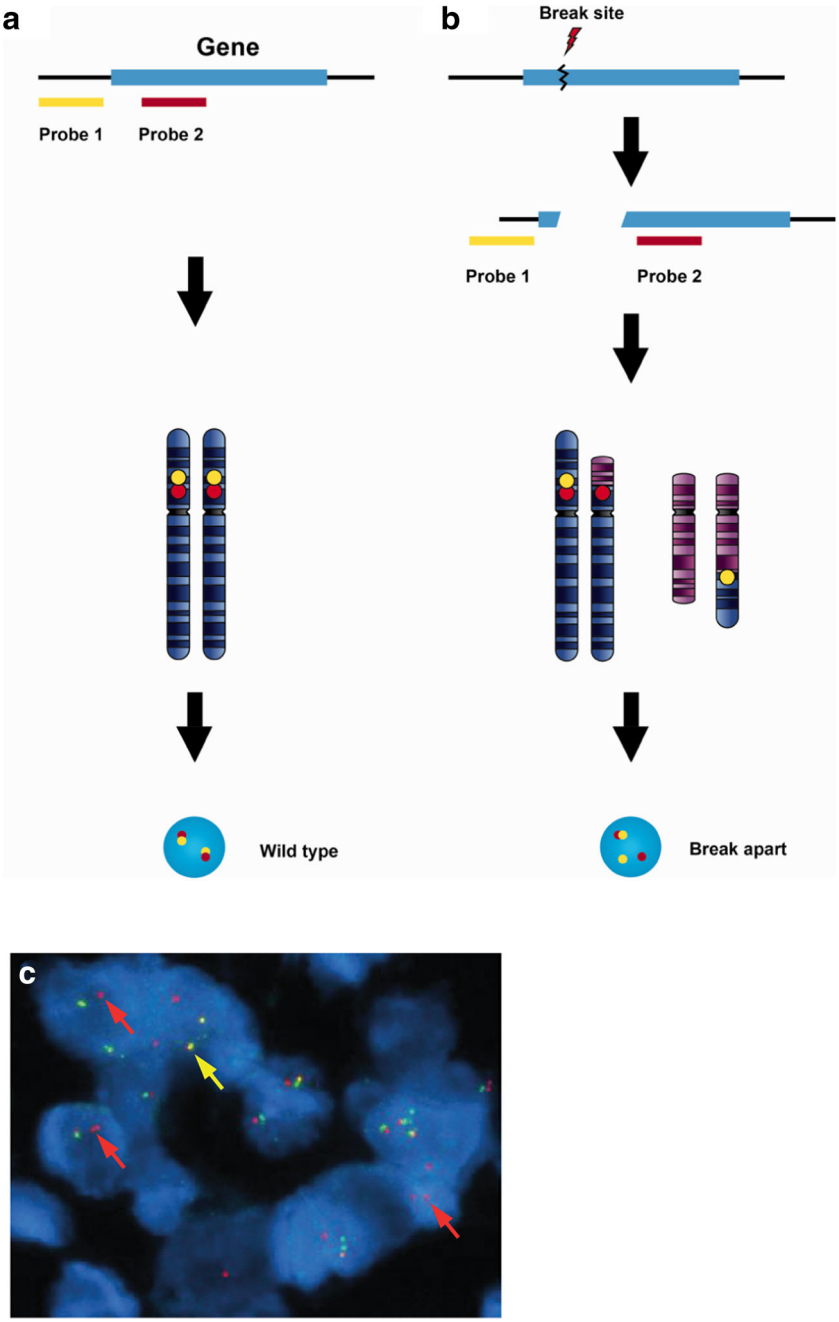

Fig. 3 Break-apart fluorescent in situ hybridization (FISH). (**a**) The wildtype pattern shows two pairs of closely situated or fused signals. (**b**) In break-apart FISH, a set of probes specific for the target gene is used. When translocation occurs involving a breakpoint between the two probe sites, the loci split apart. (**c**) An example of break-apart FISH testing results in a patient with soft-tissue sarcoma and an *LMNA-NTRK1* gene fusion [76]. *NTRK1* break-apart FISH demonstrates both paired green (5′ *NTRK1*) and red (3′ *NTRK1*) signals corresponding to the normal *NTRK1* gene (yellow arrow). Isolated red signals (red arrows) are observed in tumor nuclei (stained blue with DAPI) indicative of a chromosomal deletion leading to an *NTRK1* gene fusion. DAPI, 4′,6-diamidino-2-phenylindole. Figures 3a and b reproduced with permission from Cheng L, Zhang S, Wang L, MacLennan GT, Davidson DD. J Pathol Clin Res 2017;3(2):73–99. Figure 3c reproduced with permission from Doebele RC, Davis LE, Vaishnavi A, Le AT, Estrada-Bernal A, Keysar S, et al. Cancer Discov 2015;5(10):1049–57

fusion partners. A variation of RT-PCR that can detect the presence of a fusion with an unknown partner has been developed [79]. Although RT-PCR has a quick turnaround time once established, the design and validation of each primer set is labor intensive, more so when multiplexing multiple primer sets, which introduces the potential for cross-interactions. Robust detection by RT-PCR also relies on the quality of the RNA extracted, which can vary greatly due to the unstable nature of RNA. An example of the detection of an *ETV6-NTRK3* fusion in a mammary analog secretory carcinoma using RT-PCR is presented in Fig. 4a [80].

IHC

While FISH and RT-PCR are used to detect fusions at the DNA and RNA level, respectively, IHC can be used to survey the protein expression of your target of interest using antibodies tagged with a colorimetric label. In contrast to FISH, the availability of a pan-TRK antibody eliminates the need to perform individual assays for each TRK protein. The use of a pan-TRK monoclonal antibody has been shown to be sensitive and reliable, identifying TRK expression in 20/21 cases in one study [28] and 21/28 cases in another study [81]. In the second study, pan-TRK IHC was less effective in detecting

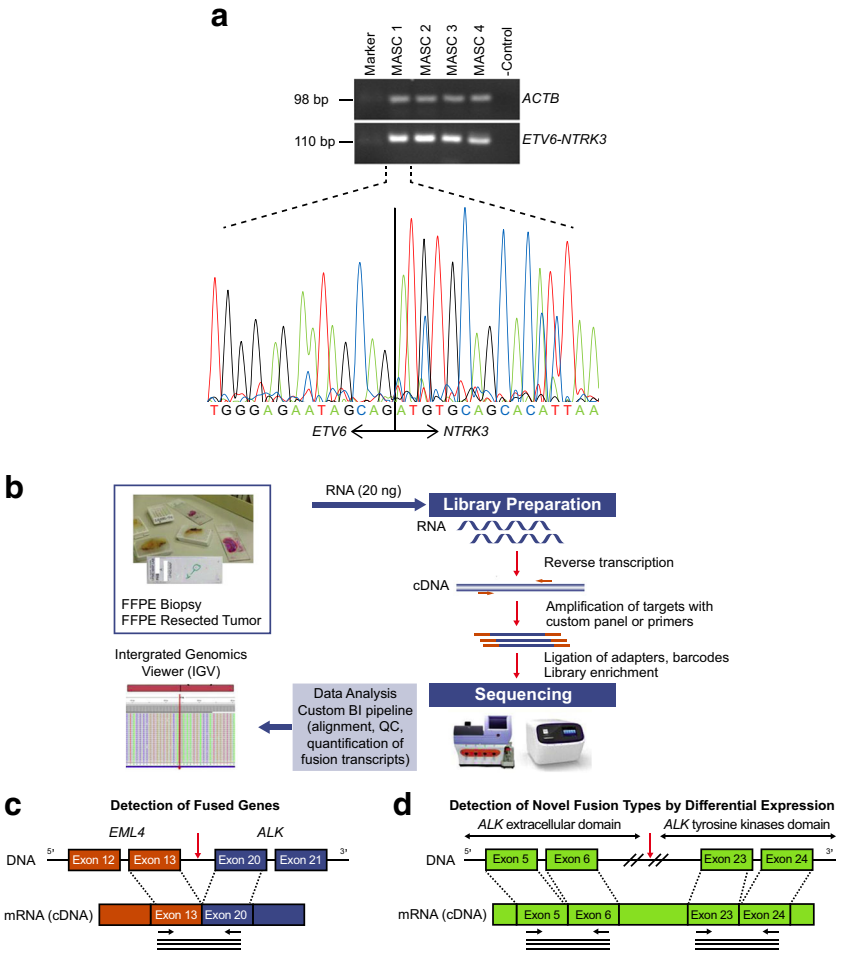

Fig. 4 Reverse transcription polymerase chain reaction (RT-PCR) and next-generation sequencing (NGS). (**a**) Example results for reverse transcription polymerase chain reaction (RT-PCR) testing [80]. RT-PCR for *ETV6-NTRK3* fusion transcripts in mammary analogue secretory carcinoma (MASC) tumors. ACTB, ß-actin, MASC 1, MASC 2, MASC 3 and MASC 4, tumor samples from Case 1, Case 2, Case 3, and Case 4, respectively. (**b**–**d**) Summary of NGS [79]. *(B) RNA* is extracted from formalin-fixed, paraffin-embedded (FFPE) tumor specimens and reverse transcribed into complementary DNA (cDNA). The cDNA is amplified with a panel of primers targeting fusion and native control transcripts. The resulting libraries are sequenced on Ion Torrent instruments and the sequence reads are then enumerated using a custom pipeline. Identified fusion transcripts are confirmed in the Integrative Genomics Viewer (IGV) to check that sequence reads span both fusion partners. (C) Fused genes are detected by PCR amplicons that span a known fusion breakpoint. (D) Novel fusions may also be detected based on overexpression of the kinase domain of selected targets. Figure 4a reproduced with permission from Fehr A, Loning T, Stenman G. Am J Surg Pathol 2011;35(10):1600–2. URL: https://journals.lww.com/ajsp/Citation/2011/10000/Mammary_Analogue_Secretory_Carcinoma_of_the.20.aspx. Figures 4**b**–**d** reproduced with permission from Beadling C, et al. J Mol Diagnostics. 2016;18(2):165–175

fusions involving *NTRK3*, which may reduce the overall sensitivity of IHC as the *ETV6-NTRK3* fusions has been reported as the most common TRK fusion in pan-cancer studies [33, 81]. An example of the detection of a protein resulting from the *LMNA-NTRK1* fusion using IHC can be found in Fig. 5 [28]. A pan-TRK monoclonal antibody has been recently approved for in vitro diagnostic use (Ventana Medical Systems), which should provide a more reproducible reagent for the detection of TRK expression. One caveat when using IHC to detect TRK is that the antibody does not discriminate between expression of the wildtype and fusion protein. Therefore, strong staining may indicate either expression of the wildtype protein or the presence of a TRK fusion protein. Interpretation of IHC results can also be subjective due to the heterogeneity of normal tissue expression and thus requires strict controls. However, IHC remains a cost- and sample-effective method with quick turnaround times. Moreover, IHC is commonly used and widely available in most pathology laboratories and may be an effective initial screening step for TRK fusions prior to confirmation with a secondary method such as NGS.

NGS and Other Multiplexed Assays

The most comprehensive and inclusive method of identifying fusions is through NGS assays such as whole genome, targeted panel, and RNA sequencing (Fig. 4b–d). As the power of NGS

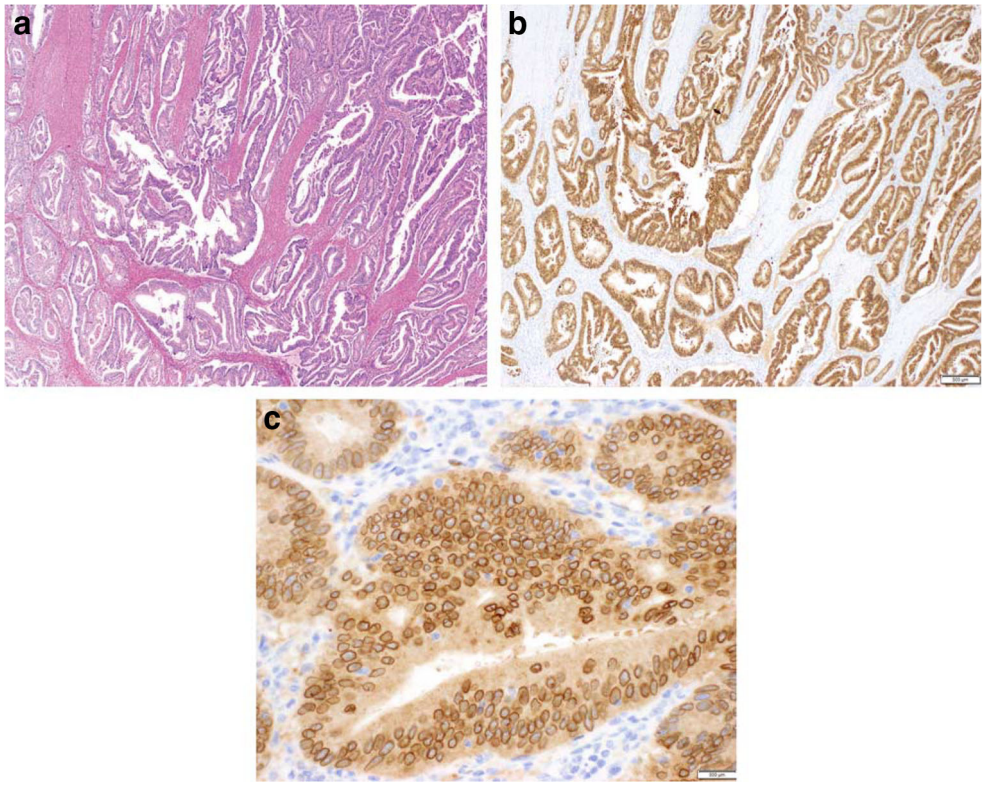

Fig. 5 Example pan-tropomyosin receptor kinase (TRK) immunohistochemistry (IHC) staining pattern in a patient with colorectal carcinoma with an ***LMNA-NTRK1*** fusion [28]. A moderately differentiated colorectal carcinoma with conventional histology (hematoxylin and eosin) and an *LMNA* exon 12-*NTRK1* exon 12 fusion (A) displays diffuse cytoplasmic and nuclear membrane staining for pan-TRK IHC (pan-TRK IHC clone EPR17341, Abcam, Cambridge, MA) (B, C). Reproduced with permission from Hechtman JF, Benayed R, Hyman DM, Drilon A, Zehir A, Frosina D, et al. Am J Surg Pathol 2017;41(11):1547–51. URL: https://journals.lww.com/ajsp/Abstract/2017/11000/Pan_Trk_Immunohistochemistry_Is_an_Efficient_and.13.aspx

technology has increased, the cost of analyzing each sample has also decreased. However, with all NGS-based assays, the need for analytic and bioinformatic support may be prohibitive for many laboratories. Although not primarily used for the detection of fusions, whole genome studies have led to the discovery of novel, recurrent, and rare fusions [82]. The usefulness of these data is limited because, on the genomic level, fusions are often found as passengers of general genomic instability, a hallmark of cancer. However, whole genome, in conjunction with RNA sequencing, has been integral for validating and determining the biological relevance and potential downstream effects of genomic fusions [82, 83]. Whole-genome sequencing has clear benefits and uses in terms of fusion discovery and basic biology research, although they are currently not suitable as a universal method in a clinical setting due to the intensive bioinformatics required to interpret the data generated.

The most common method of detecting fusion events utilizing NGS is through analyzing a specific panel of genes. The genes to be sequenced are isolated by either an amplicon-based or hybrid capture methodology. Amplicon-based methods enrich for target genes by PCR amplification of a distinct set of genes which requires less input DNA but can only detect fusion partners that are also included in the panel. In contrast, hybrid capture enrichment targets specific genomic regions through hybridization with a substrate (streptavidin/biotin), and thus can be used to identify an unknown fusion linked to the target sequence. Detection can still be challenging with low complexity sequencing, as the breakpoint may be located within a large intron, with adequate coverage costly and potentially unfeasible. This is particularly problematic with *NTRK2* and *NTRK3* fusion testing by DNA sequencing, with the entire intron sequence needing to be included in testing panels. Lastly, RNA-based panel sequencing provides the most utility by enriching for specific expressed transcripts without the complication of large introns. These panels, such as the Illumina TruSight 170 panel (TST170) and the Archer FusionPlex assay, are designed to target and enrich for hundreds of fusions involving specific genes using hybrid capture and anchored multiplex PCR technologies, respectively [46]. The advantage of this technology is that knowledge of only one of the partners is required, allowing for the potential discovery of novel fusion partners. The use of messenger RNA (mRNA) also provides confidence that the fusion is expressed. However, this method is still limited in that one of the partner genes must be present on the panel.

Although the turnaround time for NGS technology can be long (6–21 days), it provides an extremely comprehensive,

specific, and sensitive technique with extensive multiplex capabilities. Many of the larger clinical laboratories have moved towards integrating NGS testing into routine clinical workups such as the Memorial Sloan Kettering-Integrated Mutation Profiling of Actionable Cancer Targets (MSK-IMPACT), which has been used since 2014 and was approved by the Food and Drug Administration (FDA) in 2017. The MSK-IMPACT panel is hybrid-capture-based and includes 341 key cancer genes of which 14 are recurrently rearranged genes [69]. However, for many clinical laboratories, the bioinformatics demands, costs, and availability of NGS facilities/personnel can be prohibitive.

Lastly, although not considered NGS, the Nanostring nCounter Vantage 3D is an extensively multiplexed high-throughput hybridization assay that uses target-specific probes to detect fusion transcripts. Although the Nanostring platform requires more input RNA (~100–300 ng) compared to NGS-based panels (~10–100 ng), this technique does not introduce PCR amplification biases or sequencing errors since the assay uses native, unamplified RNA. Instead, the Nanostring platform detects fusions by using probes that are designed to bind directly to the fusion junction. Therefore, fusions that occur at non-canonical breakpoints or are not present in the panel would not be detected. At this point in time, the existing fusion platform only tests for two specific *NTRK1* fusions, although additional assays can be created to detect the full complement of known *NTRK* gene fusions.

Summary of Testing Methods Used to Identify Patients with TRK Fusion Cancer

Due to the large variability in *NTRK* gene fusion partners and the limitations of many of the available testing methods, testing for *NTRK* gene fusions often makes use of two independent testing methods in order to provide a reliable diagnosis [81]. The method used by nearly all studies has been RNA-based NGS due to the comprehensive and extensively multiplexed nature of this technology. Several studies including the phase I trials for entrectinib have used RNA-based anchor multiplexed NGS to identify gene fusion events in patients followed by FISH confirmation with break-apart probes [84] or IHC [85]. Other studies including the phase II basket STARTRK-2 trial have used a two-step IHC-NGS technique [86]. In this method, IHC screening is performed using a cocktail of pan-tyrosine kinase antibodies that detect expression of TRKA, TRKB, TRKC, ROS1, and ALK followed by NGS using RNA-based anchored multiplex PCR to determine the exact fusion, similarly to the previously mentioned studies. This two-step diagnostic test using IHC as an initial screen followed by NGS appears to be a quick and cost-effective method for screening out *NTRK* fusion-negative cases. A caveat is that strong IHC staining may also indicate overexpression of wildtype TRK proteins, requiring validation by NGS [86]. Indeed, strong wildtype TRK protein detection is a major caveat of using antibodies alone, which do not discern between wildtype proteins and TRK fusion proteins. Issues with the scoring methods used by pathologists may also lead to these discrepancies with IHC alone. However, if the goal is to solely detect *NTRK* fusions leading to enhanced TRK expression, the availability of a reliable pan-TRK antibody would be a very useful first step for increasing the detection rate.

NTRK Fusion Testing in Clinical Practice: Challenges and Future Perspectives

Data from the larotrectinib and entrectinib phase I and II clinical trials have shown durable benefit and well-tolerated toxicity, which warrants the introduction of routine testing for *NTRK* gene fusions. However, challenges persist in incorporating these tests into routine laboratory diagnostics. Notably, oncogenic *NTRK* gene fusions have been identified in ~1% of solid tumors [81]. Therefore, routine testing may be limited to tumors where canonical driver mutations are not identified. However, in cancers that are typically driven by oncogenic fusions, including sarcomas, use of targeted sequencing platforms such as Childseq [87], or more recently the extensively multiplexed Nanostring platform, has already been shown to be more cost-effective and comparably reliable in identifying the driver fusion compared to more traditional methods such as IHC, FISH, and RT-PCR [88]. Recent NGS panels such as the Illumina Trusight fusion panel [89] and MSK-IMPACT [90] have also been found to be as effective in detecting gene fusion events involving *ROS1, ALK,* and *RET* fusions. These studies provide support towards using NGS molecular assays as routine diagnostic tests in preference to more traditional methods as the costs become lower and the testing panels become more inclusive.

Traditional methods often suffer from limitations. IHC relies on the availability and efficacy of antibodies, which can vary greatly from lot-to-lot and does not conclusively identify the presence of a fusion protein. However, this variability might be mitigated by the availability of the pan-TRK IHC assay from Ventana. FISH relies on human interpretation of the fluorescent signals and cannot be multiplexed, and RT-PCR requires knowledge of the fusion junction. The greatest barriers to routine use of NGS assays are the need for stringent validation of results over the traditional methods and the bioinformatic pipeline/expertise required for analyzing the data. Many companies and third parties have begun to address the bioinformatic issue by

creating user-friendly software to accompany their assays, allowing for rapid and straightforward analysis of the data generated. Although globally available, country and regional variations in access to NGS testing and the high costs of testing may pose challenges for ensuring broad patient access to these tests.

Conclusions

NTRK gene fusions have garnered much clinical attention recent years due to the efficacy of small molecule inhibitors such as the recently FDA-approved use of larotrectinib (Vitrakvi®) and entrectinib in clinical trials. These drugs have also shown penetrance through the blood-brain barrier which will provide much-needed therapeutic options to patients with CNS malignancies, a field which has struggled to find durable and effective treatment options. *NTRK* gene fusions have been identified using several different approaches including FISH, RT-PCR, and NGS. IHC provides a useful screening technique to identify tumors with potential *NTRK* gene fusions that warrant further confirmation with NGS or other robust techniques, but there is a need to overcome the lack of sensitivity to detect fusions involving *NTRK3*. *NTRK* gene fusions have been identified in a broad range of cancers and appear to be tumor agnostic driver events. Although they may only be present in a small proportion of tumors, identifying these patients will be crucial for providing precision therapeutic options going forward. Therefore, robust testing methods are essential to identify the patients that harbor TRK fusion cancer in order to provide them with the benefit of precision medicine.

Acknowledgements This work was supported in part by funds from CIHR Foundation grant FDN-143280 (to PHS), BrainCare BC (to SY), and the BC Cancer Foundation (to PHS). Medical writing support, including assisting authors with the development of the outline and initial draft, incorporation of comments and preparation of tables and figures, was provided by Cindy Cheung, MBBS, and Alison Scott, PhD; editorial support, including fact-checking, referencing, figure preparation, formatting, proofreading and submission was provided by Annabel Ola, MSc; all of Scion (London, UK), supported by Bayer Healthcare according to Good Publication Practice guidelines (http://annals.org/aim/article/2424869/good-publication-practice-communicating-company-sponsored-medical-research-gpp3).

Compliance with Ethical Standards

Conflict of Interest Dr. Wong declares no conflicts of interest. Dr. Yip declares consultation fees from Bayer and Pfizer for his participation in advisory boards and travel expense reimbursement from Roche/Foundation Medicine. Dr. Sorensen declares that he is an advisor for Bayer Pharmaceuticals but holds no financial interest in the company.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

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