Colonic Adenocarcinomas Harboring NTRK Fusion Genes

A Clinicopathologic and Molecular Genetic Study of 16 Cases and Review of the Literature

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Abstract: This study was undertaken to determine the frequency, and the clinicopathologic and genetic features, of colon cancers driven by neurotrophic receptor tyrosine kinase (*NTRK*) gene fusions. Of the 7008 tumors screened for *NTRK* expression using a pan-Trk antibody, 16 (0.23%) had Trk immunoreactivity. ArcherDx assay detected TPM3-NTRK1 (n = 9), LMNA-NTRK1 (n = 3), TPR-NTRK1 (n = 2) and EML4-NTRK3 (n = 1) fusion transcripts in 15 cases with sufficient RNA quality. Patients were predominantly women (median age: 63 y). The tumors involved the right (n = 12) and left colon unequally and were either stage T3 (n = 12) or T4. Local lymph node and distant metastases were seen at presentation in

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Conflicts of Interest and Source of Funding: The authors have disclosed that they have no significant relationships with, or financial interest in, any commercial companies pertaining to this article.

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6 and 1 patients, respectively. Lymphovascular invasion was present in all cases. Histologically, tumors showed moderate to poor (n = 11) differentiation with a partly or entirely solid pattern (n = 5) and mucinous component (n = 10), including 1 case with sheets of signet ring cells. DNA mismatch repair–deficient phenotype was seen in 13 cases. Tumor-infiltrating CD4/CD8 lymphocytes were prominent in 9 cases. Programmed death-ligand 1 positive tumor-infiltrating immune cells and focal tumor cell positivity were seen in the majority of cases. CDX2 expression and loss of CK20 and MUC2 expression were frequent. CK7 was expressed in 5 cases. No mutations in *BRAF*, *RAS*, and *PIK3CA* were identified. However, other genes of the PI3K-AKT/MTOR pathway were mutated. In several cases, components of Wnt/β-catenin (*APC*, *AMER1*, *CTNNB1*), p53, and TGFβ (*ACVR2A*, *TGFBR2*) pathways were mutated. However, no *SMAD4* mutations were found. Two tumors harbored *FBXW7* tumor suppressor gene mutations. *NTRK* fusion tumors constitute a distinct but rare subgroup of colorectal carcinomas.

Key Words: colorectal carcinoma, immunohistochemistry, TRK expression, NTRK1, 2 and 3, fusion genes, next-generation sequencing

(*Am J Surg Pathol* 2020;44:162–173)

Colonic adenocarcinoma (from here on "colon cancer") is genetically heterogenous and characterized by a range of genomic and epigenomic alterations, most of which are mutations in oncogenes or tumor suppressor genes.[1] Through recent advances in sequencing technology, oncogenic fusion genes, earlier known from sarcomas and lymphomas, have been also identified in epithelial cancers, including colorectal carcinoma (CRC).[2–4] Fusions frequently involve genes encoding receptor tyrosine kinases and result in the expression of chimeric proteins. These proteins typically contain activated kinases, inducing MAPK and AKT downstream and signaling pathways that promote tumorigenesis.[5]

Neurotrophic receptor tyrosine kinase (*NTRK*) genes, *NTRK1* (chromosome 1q21-q22), *NTRK2* (chromosome 9q22), and *NTRK3* (chromosome 15q25), encode a family of transmembrane receptor tyrosine kinase proteins, TrkA, TrkB, and TrkC. Trk proteins, activated by neurotrophins, are expressed in neuronal tissue and play a central role in the development and function of the human nervous system.[6] Molecular alterations (predominantly gene fusions) and aberrant activation of *NTRK* genes have been reported in different types of epithelial, hematopoietic, and mesenchymal malignancies. Typically, the 3′ region of the *NTRK* gene fuses to the 5′ region of a gene partner, forming a chimeric gene/oncogene that expresses constitutively activated tyrosine kinase.[7] Detection of an oncogenic *NTRK* fusion has immediate clinical implications. Recently developed Trk inhibitors have shown significant efficacy in the treatment of advanced and metastatic tumors, including CRCs harboring oncogenic *NTRK* fusion genes.[7–9]

In colon cancer, the first fusion between *TPM3* (tropomyosin 3) and *NTRK1* was identified > 35 years ago.[10] Subsequently, a small number of CRCs driven by *NTRK1* or *NTRK3* fusions have been described. However, only limited clinicopathologic data of these tumors are available.[4,8,9,11–15] A recent study summarized clinicopathologic and molecular genetic features of metastatic CRCs driven by different tyrosine kinase fusions, including *NTRK* fusions.[16] The clinicopathologic profile of primary tumors harboring oncogenic *NTRK* fusions remains to be elucidated. A summary of clinicopathologic data of previously published CRCs harboring *NTRK* fusions is available in Supplemental Data Table S1A (Supplemental Digital Content 1, http://links.lww.com/PAS/A853) and Supplemental Data Table S1B (Supplemental Digital Content 2, http://links.lww.com/PAS/A854).

In this study, a large, unselected cohort of colon cancers was screened using immunohistochemistry and targeted RNA sequencing to find tumors with *NTRK* gene fusions. Sixteen colon cancers harboring *NTRK1* or *NTRK3* fusions were identified, and their clinicopathologic, immunohistochemical, and molecular genetic features were studied in detail.

MATERIALS AND METHODS

This study evaluated 7008 anonymized colon carcinomas from Europe (Czech Republic, Finland, Germany, Italy, and Poland), Japan, and the United States. Staging of tumors was provided by contributors following the American Joint Committee on Cancer (AJCC) TNM classification and staging recommendation (http://cancerstaging.org) or Dukes staging system.[17] Histologic classification of tumors was based on World Health Organization classification of tumors of the digestive system.[18] The density of tumor-infiltrating lymphocytes (TILs) was scored following previously reported methods.[19] A score of at least 2 lymphocytes by high-power fields (HPF) was required for high-level TIL.

All patients underwent a partial colectomy. Clinical information with regard to adjuvant chemotherapy was available in 8 cases; it was administered in 5 patients. None of the patients were known to receive tyrosine kinase inhibitor therapy.

Tumor samples were analyzed using tissue microarrays or multitumor blocks. Tissue microarrays were constructed using core biopsies and MicaArray kit (MicaArray, New York, NY) or Manual Tissue Arrayer MTA-1 (Beecher Instruments Inc./Estigen, Tartu, Estonia). Multitumor blocks were built manually using rectangular tissue samples, as previously reported.[20]

Immunohistochemistry

In all, 7008 tumors, *NTRK1*, *NTRK2* and *NTRK3* expression was evaluated using a pan-Trk antibody clone A7H6R (#92991; Cell Signaling Technology Inc., Danvers, MA) and Leica Bond-Max automated immunohistochemistry, Leica Biosystems Inc. (Buffalo Grove, IL) with 25-minute heat-induced epitope retrieval in ER2 buffer.

All pan-Trk-positive tumors were evaluated for the expression of several antigens including DNA mismatch repair (MMR) proteins (MutL Homolog 1 [MLH1], PMS1 Homolog 2 [PMS2], MutS Homolog 2 [MSH2], and MutS Homolog 6 [MSH6]), caudal-type homeobox 2 (CDX2), catenin beta 1 (CTNNB1), cytokeratin 7 and 20 (CK7, CK20), Ki-67, mucin 2 (MUC2), tumor protein p53 (p53), and programmed death-ligand 1 (PD-L1). In addition, TILs and macrophages were characterized with antibodies against the CD4, CD8, and

CD68. A detailed description of antibodies and immunohistochemical protocols are provided in Supplemental Data Table S2 (Supplemental Digital Content 3, http://links.lww.com/PAS/A855).

DNA and RNA Extraction

DNA and RNA were recovered from formalin-fixed paraffin-embedded colon carcinoma specimens using a Maxwell RSC instrument and DNA or RNA FFPE Kit (Promega, Madison, WI), according to the manufacturer's protocols.

ArcherDx Assay

Target-specific libraries for next-generation sequencing (NGS) were constructed using Archer Universal RNA Reagent Kit v2 (ArcherDx, Boulder, CO). Library sequencing was accomplished using a MiSeqDx instrument (Illumina, San Diego, CA). NGS data were analyzed using the Archer Analysis Pipeline Virtual Machine (https://archerdx.com). In 1 case, the result of ArcherDx assay was confirmed in a secondary laboratory that performed a blinded experiment (the prior finding was withheld until after the experiment was completed).

Ion Torrent NGS

NGS was performed by Macrogen USA (Rockville, MD) using the Ion Torrent (Life Technologies/Thermo Fisher Scientific, Waltham, MA) NGS platform. Depending on the DNA quality, either Ion AmpliSeq Comprehensive Cancer Panel (409 gene targets) or Ion AmpliSeq Cancer Hotspot Panel v2 Kit (50 gene targets) was used. All 50 genes targeted by the Cancer Hotspot Panel were included in the Comprehensive Cancer Panel.

Bioinformatics analysis of NGS data was processed by Torrent Server Suite 4.2 and sequences aligned to human genome reference sequence HG-19 (The Genome Reference Consortium). Variant calling was performed using Variant Caller v4.2, which is compatible with the Integrative Genomics Viewer (Broad Institute, Cambridge, MA), a high-performance visualization tool for interactive exploration of large, integrated data sets. Mutation nomenclature was based on recommendations from Human Genome Mutation Society (www.hgvs.org). The FATHMM (Functional Analysis Through Hidden Markov Models), SIFT (Sorting Intolerant from Tolerant), and PolyPhen (Polymorphism Phenotyping) scores predicting functional consequences of coding variants were either obtained from the COSMIC (Catalogue of Somatic Mutations in Cancer) at https://cancer.sanger.ac.uk or assessed during bioinformatic analysis.

MLH1 Promoter Hypermethylation Analysis

MLH1 promoter hypermethylation was evaluated in 2 tumors with loss of *MLH1* expression detected by immunohistochemistry. Sodium bisulfite conversion of genomic DNA was executed using EZ DNA Methylation-Gold kit (Zymo Research, Burlington, ON, Canada) and provided by Zym Research procedure. The methylation-specific PCR amplification of the *MLH1* promoter and evaluation of PCR amplification products were carried out, as previously reported.[21]

TABLE 1. Type of NTRK Fusion and pan-Trk Expression Pattern Identified in 15 Colon Carcinomas

Fusion Gene	No. Tumors	Membrane pan-Trk Staining	Cytoplasmic pan-Trk Staining	Nuclear pan-Trk Staining
TPM3-NTRK1	9	+++	+++	−
LMNA-NTRK1	3	+ or +/−	+++	Focal or scattered cells
TPR-NTRK1	2	+	++	−
EML4-NTRK3	1	−	+	−

RESULTS

Trk Immunohistochemistry and NTRK Fusion Gene Transcripts

Pan-Trk immunostaining was seen in 16 of 7008 analyzed colon cancers (0.23%). Fifteen of the 16 Trk-positive colon cancers contained RNA sufficient for molecular studies. The ArcherDx assay detected NTRK1 (n = 14) and NTRK3 (n = 1) fusion gene transcripts. Nine of these tumors harbored TPM3-NTRK1 chimeric transcripts with either exon (e) 8 to e10 (n = 7) or e8 to e13 (n = 2) fusion breakpoints. In 3 cases, LMNA-NTRK1 fusions with 3 distinctive fusion breakpoints—e4 to e10, e10 to e11, and e11 to e8—were detected. TPR-NTRK1 chimeric transcripts with identical e21 to e10 fusion breakpoints were identified in 2 tumors. One colon cancer harbored EML4 (EMAP Like 4)-NTRK3 transcripts with an e2 to e14 fusion breakpoint.

Immunohistochemical patterns for pan-Trk of different types of *NTRK* fusion colon cancers are listed in Table 1. Tumors harboring TPM3-NTRK1 fusions displayed strong cytoplasmic and membrane pan-Trk positivity and lacked nuclear staining (Fig. 1A). Colon cancers with LMNA-NTRK1 fusions showed strong cytoplasmic but weak membrane immunoreactivity and focal nuclear staining (Fig. 1B). The latter was not seen in a tumor harboring an LMNA(e4)-NTRK1(e10) fusion.

TPR-NTRK1 fusion tumors displayed weaker cytoplasmic and membrane pan-Trk staining than TPM3-NTRK1 and LMNA-NTRK1 fusion tumors and lacked nuclear immunoreactivity (Fig. 1C). The sole colon cancer with an EML4-NTRK3 fusion revealed weak but distinct cytoplasmic pan-Trk staining and no membrane or nuclear immunoreactivity (Fig. 1D). In this case, the results of the ArcherDx assay were confirmed by a blinded experiment in a secondary laboratory.

Demographic and Clinicopathologic Features of NTRK Fusion Colon Cancers

Clinical characteristics of *NTRK* fusion colon cancers are summarized in Table 2. Most of these cancers (13/16, 81%) were diagnosed in women. Median ages for women and men were 63 and 71 years, respectively. An overall 38.5% (5/13) of female patients were in the fifth and sixth decades (age range: 46 to 56 y). Tumors harboring *NTRK* fusions involved different portions of the

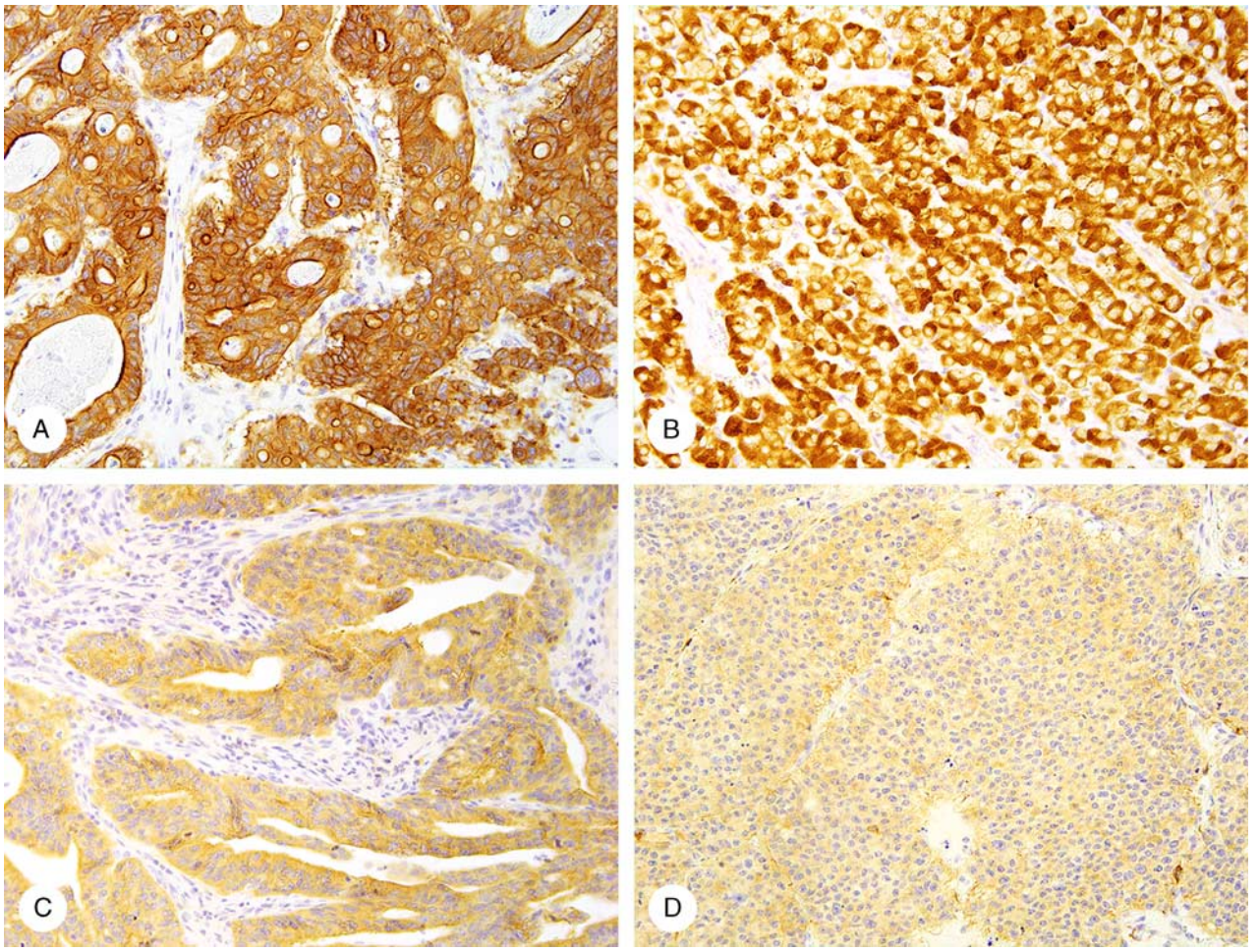

FIGURE 1. Trk-positive immunohistochemistry in colon cancers. TPM3(e8)-NTRK1(e10) fusion tumor (case 8) displayed strong, diffuse cytoplasmic and membrane staining (A); LMNA(e10)-NTRK1(e11) fusion tumor (case 14) with an area of strong nuclear staining and weaker cytoplasmic staining (B); TPR(e21)-NTRK1(e10) fusion tumor (case 5) showed weaker cytoplasmic and membrane staining than TPM3-NTRK1 and LMNA-NTRK1 fusion tumors and lacked nuclear immunoreactivity (C); EML4(e2)-NTRK3(e14) fusion tumor (case 12) displayed weak but distinct cytoplasmic staining and no membrane or nuclear immunoreactivity (D).

large intestine including the cecum (n = 2), ascending colon (n = 1), hepatic flexure (n = 4), transverse colon (n = 2), splenic flexure (n = 3), descending colon (n = 3), and rectosigmoid (n = 1). On the basis of TNM criteria, *NTRK* fusion colon cancers were either T3 (n = 12) or T4 (n = 4). Five patients had local lymph node metastases. In 1 case, both local and distant metastases (liver and lung) were diagnosed at presentation. Follow-up data were available in 12 cases. One patient (case 1) with local and distal metastases died of the disease in 1 month. Two elderly (84 and 86 years old) patients (cases 7 and 9) died of unknown causes after 7 days and 24 months, respectively. Nine patients were alive without disease with follow-up ranging from 11 months to 17 years (median follow-up: 28 mo).

Histologic Features

The majority of *NTRK* colon cancers showed moderate to poor (n = 11) or poor (n = 4) differentiation (Fig. 2A). Eight tumors displayed focal (n = 6) or extensive (n = 3) solid growth areas, whereas 1 revealed solid ribbon-like growth pattern consistent with medullary morphology (Fig. 2B). Focal to extensive mucinous component was seen in 8 tumors, including 1 with sheets of signet ring cells (Fig. 2C). A vague nested pattern was present in 1 case (Fig. 2D). Lymphovascular invasion was present in all tumors. Nine colon cancers had a high level ($\geq$2 TIL/HPF) of TILs. The histologic features of *NTRK* fusion tumors analyzed in this study are summarized in Table 3.

Immunohistochemical Profile

Thirteen of 16 (81%) *NTRK* fusion colon cancers revealed loss of MLH1 and PMS2 expression, indicating MMR protein deficiency. Loss of MSH6 expression was seen in 1 MLH1/PMS2-deficient tumor. All colon cancers expressed MSH2. CK20 expression was variable and presented in 9 of 16 tumors (56%), including 6 with focal immunostaining (Fig. 3A). CK7 was expressed in 5 of 16 tumors (31%); 4 cases showed extensive expression. Although most tumors were

TABLE 2. Clinical Characteristics of 16 Colon Cancers With Positive pan-Trk Immunohistochemistry

Case	Age (y)	Sex	Tumor Site in Colon	Staging System (pTNM, Dukes C)	Follow-Up	NTRK Fusion Gene
1	54	Female	Cecum	pT4aN2bM1b	DOD (1 mo)*	TPM3(e8)-NTRK1(e10)
2	68	Female	Cecum	pT3N0M0	ANED (1 y 5 mo)†	LMNA(e4)-NTRK1(e10)
3	46	Female	Ascending	Dukes C‡	ANED (17 y)§	Unknown‖
4	50	Female	Hepatic flexure	pT3N0M0	ANED (1 y 10 mo)§	TPM3(e8)-NTRK1(e13)
5	53	Female	Hepatic flexure	pT3N1aM0	ANED (2 y 4 mo)§	TPR(e21)-NTRK1(e10)
6	63	Male	Hepatic flexure	pT3N1M0	ANED (7 y)‡	TPM3(e8)-NTRK1(e13)
7	86	Female	Hepatic flexure	pT3N0M0	DOC (7 d)†	LMNA(e11)-NTRK1(e8)
8	77	Female	Transverse colon	pT3N0M0	NA	TPM3(e8)-NTRK1(e10)
9	84	Male	Transverse colon	pT3N0M0	DOC (2 y)†	TPM3(e8)-NTRK1(e10)
10	63	Female	Splenic flexure	pT3N0M0	ANED (3 y 9 mo)§	TPM3(e8)-NTRK1(e10)
11	68	Female	Splenic flexure	pT3N0M0	ANED (11 mo)‡	TPR(e21)-NTRK1(e10)
12	71	Female	Splenic flexure	pT3N0M0	ANED (12 y 4 mo)‡	EML4(e2)-NTRK3(e14)
13	56	Female	Descending	pT4aN2bM0	NA	TPM3(e8)-NTRK1(e10)
14	62	Female	Descending	pT4aN2bM0	ANED (1 y 1 mo)§	LMNA(e10)-NTRK1(e11)
15	71	Male	Descending	pT3N0M0	NA	TPM3(e8)-NTRK1(e10)
16	70	Female	Rectosigmoid junction	pT4aN0M0	NA	TPM3(e8)-NTRK1(e10)

*Adjuvant chemotherapy status unknown.
†No adjuvant chemotherapy.
‡6 cm tumor, metastases in local lymph nodes.
§Adjuvant chemotherapy.
‖RNA failed quality control test.
ANED indicates alive, no evidence of disease; DOC, died of unknown causes; DOD, died of disease; NA, not available.

CDX2-positive, complete or focal loss of expression was seen in 4 cases (Fig. 3B). MUC2 expression was absent (8/16) or focally present (6/16) in 87.5% of tumors (Fig. 3C). The majority (13/16) of colon cancers revealed a high proportion (80% to 100%) of Ki-67-positive tumor cells (Fig. 3D). Confluent nuclear p53 expression was identified in 4 colon cancers, including 3 with MMR proficiency (Figs. 4A, B). One tumor was entirely negative, whereas the remaining 11 displayed a variable number of tumor cells with p53-positive nuclei. Membrane and cytoplasmic β-catenin immunostaining were seen in all cases. However, in 1 MMR-deficient tumor, prominent nuclear β-catenin accumulation was noted (Figs. 4C, D). Detailed tumor immunoprofiles are presented in Table 4.

In 9 cases, CD4 and CD8 immunostaining showed high numbers of tumor-infiltrating T-cell lymphocytes; $CD4^+$ cells were less prominent. Cases with $CD4^+/CD8^+$ TIL immunoreactivity displayed stroma rich in CD68-positive tumor-infiltrating macrophages. Thirteen cases showed a variable PD-L1-positive population of tumor-infiltrating immune cells, while tumor cell positivity was absent to minimal.

DNA Methylation Studies

DNA methylation study was performed on 2 colon cancers (cases 5 and 10) and revealed hypermethylation of MLH1 promoter.

Mutation Profiles of NTRK Fusion Tumors

A total of 409 genes were sequenced in 9 *NTRK* fusion tumors (cases 1, 2, 5, 7, 8, 10, 13, 15, and 16) with well-preserved DNA. Because of insufficient DNA quality, case 11 was evaluated with a panel of 50 gene targets. The genes mutated in these *NTRK* fusion colon cancers are listed in Table 5 and in Supplemental Data Table S3 (Supplemental Digital Content 4, http://links.lww.com/PAS/A856). No mutations in *MLH1*, *PMS2*, *MSH2*, and *MSH6* were identified in 8 MMR-deficient *NTRK* fusion colon cancers with documented loss of MLH1 and PMS2 by immunohistochemistry. However, 1 tumor contained a subclone with a pathogenic *PMS1* mutation (p.Arg93Cys).

None of the 10 *NTRK* fusion colon cancers harbored mutations in the typical CRC drivers *BRAF*, *K-RAS*, *N-RAS*, and *PIK3CA*. However, 8 mutations in different components of the PI3K-AKT/MTOR signaling pathway were identified in 56% (5/9) analyzed tumors. These mutations affected *AKT1* (n = 1), *MTOR* (n = 1), *PTEN* (n = 2), and genes (*PIK3CD* and *PIK3R2*, and *PIK3C2B*) encoding different subunits of class I and II phosphoinositide 3-kinases (PI3Ks).

In 5 tumors, mutations in *APC* (n = 3), *AMER1* (n = 3), and *CTNNB1* (n = 2) components of the Wnt/β-catenin signaling pathway were identified. *TP53* was mutated in 2 colon cancers. Two other tumors contained subclones with mutated *FBXW7*, another cancer-related tumor suppressor gene. Mutations in *ACVR2A* and *TGFBR2*, genes playing a role in the transforming growth factor β (TGFβ) signaling pathway, were identified in 2 tumors. However, there were no *SMAD4* mutations in all the analyzed 10 *NTRK* fusion colon cancers.

DISCUSSION

This study analyzed clinicopathologic and genetic features of 16 colon cancers characterized by Trk expression. Tumors that were immunohistochemically positive with the pan-Trk antibody comprised 0.23% of the screening cohort of 7008 cases. All cases, except 1 with unsatisfactorily preserved RNA, were shown to harbor *NTRK* gene fusions. This suggests a high specificity of pan-Trk immunostaining in colon cancer. Previous studies using different antibodies and automation platforms showed high specificity of TrkA (clone

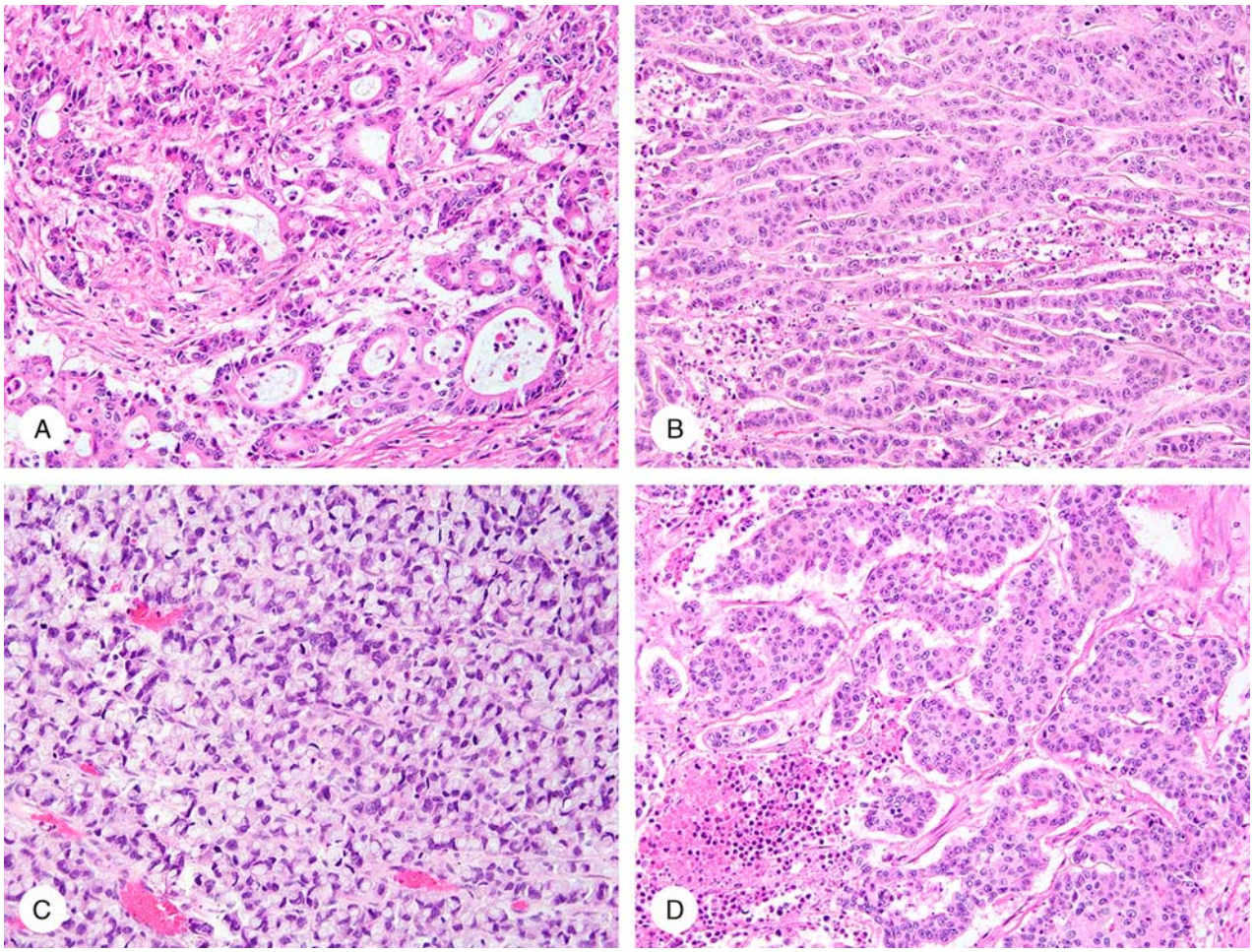

FIGURE 2. Histologic features of Trk-positive colon cancers. TPM3(e8)-NTRK1(e10) fusion tumor (case 13) with moderate to poor differentiation (A); LMNA(e11)-NTRK1(e8) fusion tumor (case 7) with solid areas displaying ribbon-like growth pattern (B); LMNA (e10)-NTRK1(e11) fusion tumor (case 14) with prominent sheets of signet ring cells (C); EML4(e2)-NTRK3(e14) fusion tumor (case 12) showed a vague nested growth pattern (D).

EP1058Y/Ab 76291) or pan-Trk (EPR 17341, C17F1) immunohistochemistry in detecting CRCs harboring *NTRK1* fusions.[8,9,11,12,22–24] However, previously described perinuclear/nuclear membrane staining in tumors harboring LMNA-NTRK1 fusions[22] was not seen in our study. Instead, 2 tumors with the *LMNA* fusion involving e10 and e11 displayed focal nuclear immunoreactivity.

The lack of Trk staining has been occasionally reported in CRCs harboring TPM3-NTRK1 and ETV6-NTRK3 fusions.[22,23] In this study, colon cancer with the EML4-NTRK3 fusion displayed confluent but weak Trk staining. Little is known about Trk immunoreactivity of CRCs harboring *NTRK3* fusions, because all such tumors were identified by the RNA sequencing.[16] However, negative or suboptimal Trk immunoreactivity has been reported in pediatric sarcomas and gliomas harboring *NTRK3* fusions.[22,25] One group of investigators using TrkA antibody (clone OTI5B6) described strong Trk expression in 6% and 15% of colorectal tumors, respectively, in Chinese and Korean populations.[13,26] A high frequency of pan-Trk-positive tumors was also reported in a study using a cocktail of ALK/pan-Trk and ROS1 antibodies to screen CRCs for several different fusions.[27] High frequencies of immunopositivity in those studies most likely included false-positive results, probably due to incomplete specificity of TrkA antibody (clone OTI5B6) or other technical factors. Previous studies applying Trk immunohistochemistry to search for colorectal tumors harboring *NTRK* fusions are summarized in Supplemental Data Table S4A (Supplemental Digital Content 5, http://links.lww.com/PAS/A857).

The sensitivity of Trk immunohistochemistry in search of *NTRK* fusion colon cancers cannot be assessed in this investigation, because negative cases were not genotyped. However, a NGS study of 1272 CRCs estimated the frequency of *NTRK* fusion tumors to be around 0.2%, as found in our study.[23] Results of studies applying molecular genetic screening to search for *NTRK* fusions in CRCs are summarized in Supplemental Data Table S4B (Supplemental Digital Content 6, http://links.lww.com/PAS/A858).[3–5,13,23,28,29]

In CRCs, *NTRK1* has been shown to form fusions with different gene fusion partners including *LMNA*, *PLEKHA6*

TABLE 3. Histopathologic Characteristics of 16 Colon Cancers With Positive pan-Trk Immunohistochemistry

Case	Degree of Glandular Differentiation	Solid Growth Area	Mucinous Component	TILs
1	Moderate to poor	Focal	No	Low*
2	Poor	Extensive	Focal	High†
3	Moderate to poor	No	Focal	Low
4	Moderate to poor	Focal	Extensive‡	Low
5	Moderate to poor	No	Focal	High
6	Moderate	No	No	Low
7	Poor (medullary subtype)	Extensive	No	Low
8	Moderate to poor	Focal	Focal	High
9	Moderate to poor	No	No	High
10	Moderate to poor	No	No	High
11	Moderate to poor	Focal§	Extensive‡	Low
12	None	All	No	High
13	Moderate to poor	Focal	Focal	High
14	Poor	Focal‖	Extensive‡	Low
15	Moderate to poor	No	Yes	High
16	Poor	Extensive	Focal	High

*< 2 TIL/HPF.
† ≥ 2 TIL/HPF.
‡Luminal.
§Minimal.
‖Sheets of signet ring cells.
HPF indicates high-power fields.

(pleckstrin homology domain containing AC), *SCYL3* (SCY1-like pseudokinase 3), *TPM3*, and *TPR*.[8,9,13–16] In this study, TPM3-NTRK1 was the most common (60%) fusion detected in colon cancers. Two other fusion types previously reported in CRCs, LMNA-NTRK1 and TPR-NTRK1, were less common and accounted for 20% and 13% of analyzed cases. No *NTRK1* fusions engaging *PLEKHA6* or *SCYL3* were detected. All these fusions are the result of intrachromosomal rearrangements between chromosome 1q21.3 (*TPM3*), 1q22 (*LMNA*), 1q24.2 (*SCYL3*), 1q31.1 (*TPR*), and 1q32.1 (*PLEKHA6*) and chromosome 1q23.1, *NTRK1* locus (www.genecards.org) Variants of *NTRK1* fusions reported in CRC in this study, and in previous studies, are shown in Table 6.

NTRK1 fusions with *TPM3* and other gene partners have also been reported in papillary thyroid carcinoma, spitzoid melanocytic neoplasms, intrahepatic cholangiocarcinoma, glioblastoma, pediatric high-grade glioma, non–small cell lung cancer, soft tissue and uterine sarcomas, and a low-grade sarcoma called lipofibromatosis-like neural tumor.[30–38]

NTRK3 fusions in CRC seem to be very rare, and only a few cases involving *COX5A*, *EML4*, *ETV6*, and *VPS18* have been reported, with ETV6(e5)-NTRK3(e15) being the most common molecular event.[4,15,16,29] ETV6-NTRK3 fusion resulting from reciprocal t(12;15)(p13;q25) translocation was first described in infantile fibrosarcoma and congenital mesoblastic nephroma.[39] In our study, EML4(e2)-NTRK3 (e14) fusion was identified in 1 tumor with weak Trk staining. Previously, an identical fusion formed by the reciprocal t(2;15)(p21;q25) translocation was reported in a case of colon cancer.[4] In vitro studies documented that EML4-NTRK3 chimeric protein leads to the oncogenic activation of the MAPK/ERK signaling pathway.[4] Moreover, expression of EML4-NTRK3 oncoprotein in NIH 3T3 cells was sufficient for cellular transformation.[40] *NTRK3* fusions involving *EML4*, *ETV6*, and a number of other gene partners were identified in other malignancies originating from different cell lineages. These include secretory breast carcinoma, secretory carcinoma of the salivary gland, papillary thyroid cancer, radiation-associated thyroid cancer, acute myeloid leukemia, melanocytic neoplasms, glioma, infantile fibrosarcoma, and congenital mesoblastic nephroma.[23,29,34,41–46] Variants of *NTRK3* fusions reported in CRC in this and previous studies are shown in Table 7.

The majority of colon cancers harboring *NTRK* fusions revealed some features characteristic of microsatellite-unstable colorectal tumors, such as female predominance, right colon location, presence of mucinous differentiation, and a high level of TILs. In this study, MMR deficiency was identified by immunohistochemistry in 81% (13/16) of analyzed cases. Similar frequency (77%) was previously recorded by a study on metastatic CRCs with *NTRK* fusions.[16] Loss of MLH1 and PMS2 forming MMR protein MuLα complex, lack of mutations affecting *MLH1*, *PMS2*, *MSH2*, and *MSH6* genes, and evidence of *MLH1* promoter methylation suggested that the nature of these alterations was sporadic.

BRAF mutations, typically seen in colon cancer with MMR deficiency, were not detected in the *NTRK* fusion tumors analyzed in this study. In addition, other common CRC drivers—such as *K-RAS*, *N-RAS*, and *PIK3CA*—were not involved. Absence of *BRAF* (V600E) and *RAS* mutations in *NTRK* fusion tumors has previously been reported.[8,9,11,12,16] This highlights a primary oncogenic role of chimeric Trk fusion proteins. Nevertheless, in our cohort, components of PI3K-AKT/MTOR signaling pathway other than *PIK3CA* were affected by mutations implicating this pathway in *NTRK* fusion tumors.

In contrast to the mutual exclusivity of *NTRK1* fusion to oncogenic *BRAF* and *RAS* mutations, this study documented the coexistence of mutations in genes of Wnt/β-catenin and p53 pathways in a majority (7/10) of analyzed cases. β-catenin nuclear accumulation (documented by immunohistochemistry) in a tumor with *APC* mutation and confluent nuclear p53 expression in tumors harboring *TP53* mutations supported functional modification of these pathways. Alterations of Wnt/β-catenin and p53 signaling pathways are more common in nonhypermutated tumors.[47] A recent study reported both *APC* and *TP53* mutations in metastatic *NTRK* fusion CRCs.[16] In 2 tumors, mutations in *FBXW7*, a p53-dependent tumor suppressor gene, were identified. Approximately, 10% of human CRCs harbor *FBXW7* mutations. Mutational inactivation of *FBXW7* contributing to tumor progression is secondary to *TP53* mutation.[48]

In this study, mutations in *ACVR2A* and *TGFBR2*, components of the TGFβ signaling pathway, were identified in 2 MMR-deficient *NTRK* fusion colon cancers. Inactivation of the TGFβ signaling pathway is a common event in CRC. The components of this pathway are mutated in > 85% of hypermutated tumors.[49]

The *NTRK* fusion colon cancers described in this series revealed striking sex predilection to female patients with a 1:4.3 male to female ratio. This simply cannot be explained by

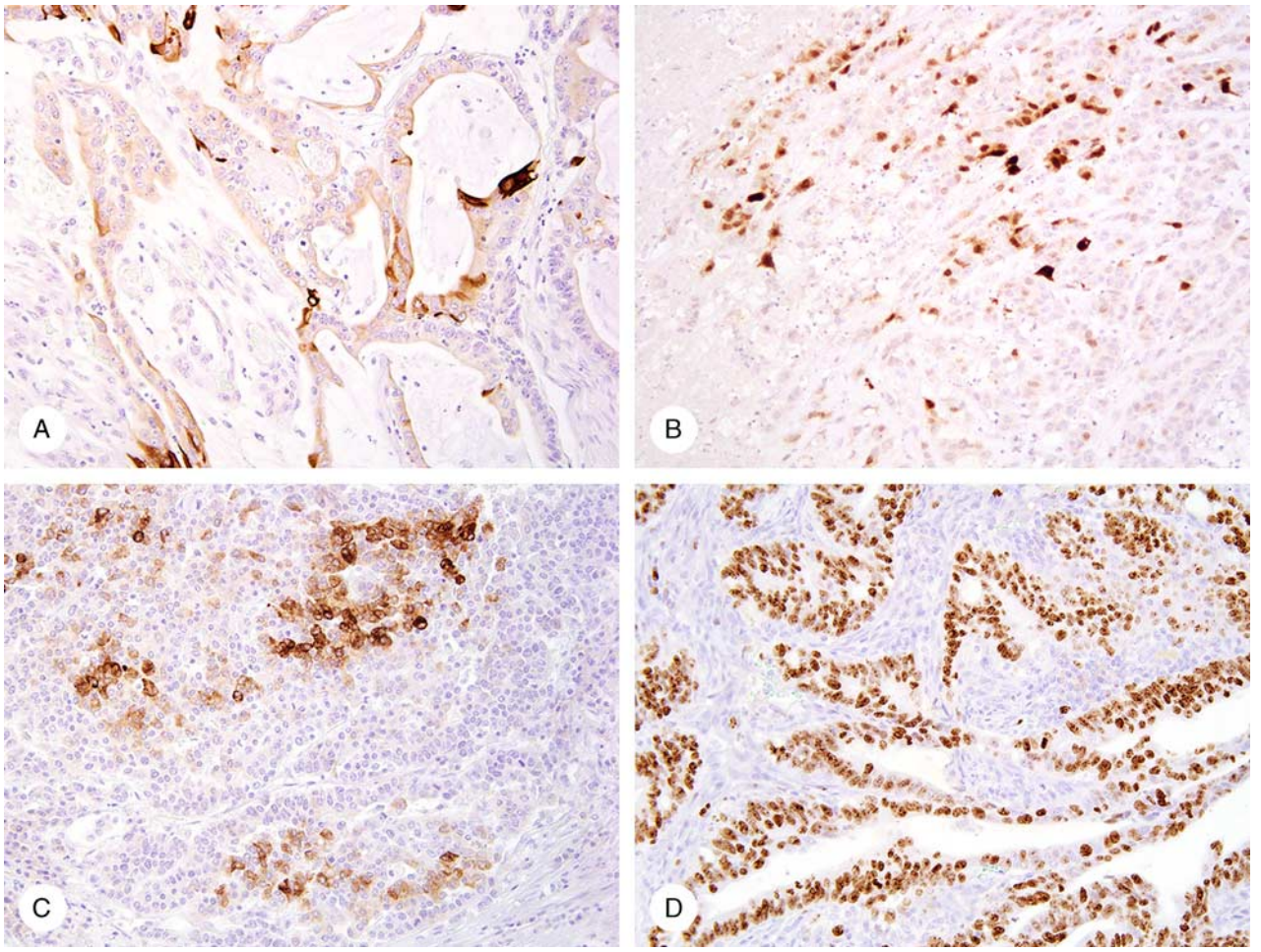

FIGURE 3. Immunohistochemistry of Trk-positive colon cancers. Focal CK20 expression (A) in TPM3(e8)-NTRK1(e10) fusion tumor (case 15); focal CDX2 expression (B) in LMNA(e11)-NTRK1(e8) fusion tumor (case 7); focal MUC2 expression (C) in LMNA(e4)-NTRK1(e10) fusion tumor (case 2); diffuse (near 100%) Ki-67 expression (D) in TPR(e21)-NTRK1(e10) fusion tumor (case 5).

a higher frequency of MMR-deficient CRCs among female patients, as the male to female ratio is around 1:1.5 among MMR-deficient tumors.[50] Furthermore, 30% of female patients were below 60 years old, mimicking cancers with a hereditary predisposition.[51] Yet, no mutations affecting MMR genes (*MLH1*, *PMS2*, *MSH2*, and *MSH6*) were identified.

In this study, a majority of Trk-positive tumors were classified as left-sided on the basis of their occurrence up to the splenic flexure.[52] However, a recent molecular study indicated that classification of CRCs by tumor location better highlights molecular differences.[53] Taking specific location into consideration, a majority (12/16) of Trk-positive tumors were diagnosed in locations designated uncommon, such as hepatic flexure (n = 4), transverse colon (n = 2), splenic flexure (n = 3), and descending colon (n = 3).[51,54] The majority of previously published studies omitted information with regard to the specific location of tumors. Nevertheless, on the basis of current and previous studies, the distribution of *NTRK1*-fusion versus *NTRK3*-fusion tumors seems to be different, with the latter being more equally present in the left and right colon. Three of 7 (43%) *NTRK3* colon cancers compared with only 4 of 15 (27%) *NTRK1*-fusion tumors were diagnosed in the left colon, including the sigmoid colon and rectum (Supplemental Data Table S1A, Supplemental Digital Content 1, http://links.lww.com/PAS/A853 and Supplemental Data Table S1B, Supplemental Digital Content 2, http://links.lww.com/PAS/A854).

Mucinous differentiation was seen in 56% (9/16) of tumors. In contrast, none of the recently reported 8 metastatic CRCs with *NTRK* fusions had mucinous changes.[16] Nevertheless, mucinous differentiation could be seen in 3 of 4 histologic images attached to *NTRK3*-fusion CRCs available at The Cancer Genome Atlas (www.cancer.gov/tcga).

The mechanisms underlying better prognosis of some colon cancers are incompletely understood. Several positive and negative prognostic markers have been implicated. The high level of TILs has been considered a positive prognostic indicator, especially if coupled with deficient MMR status.[55] In this study, 8 of 13 MMR-deficient tumors revealed high levels of CD4 and CD8-positive TILs. These tumors were saturated with CD68-positive macrophages and PD-L1-positive immune cells. However, the tumor cells revealed

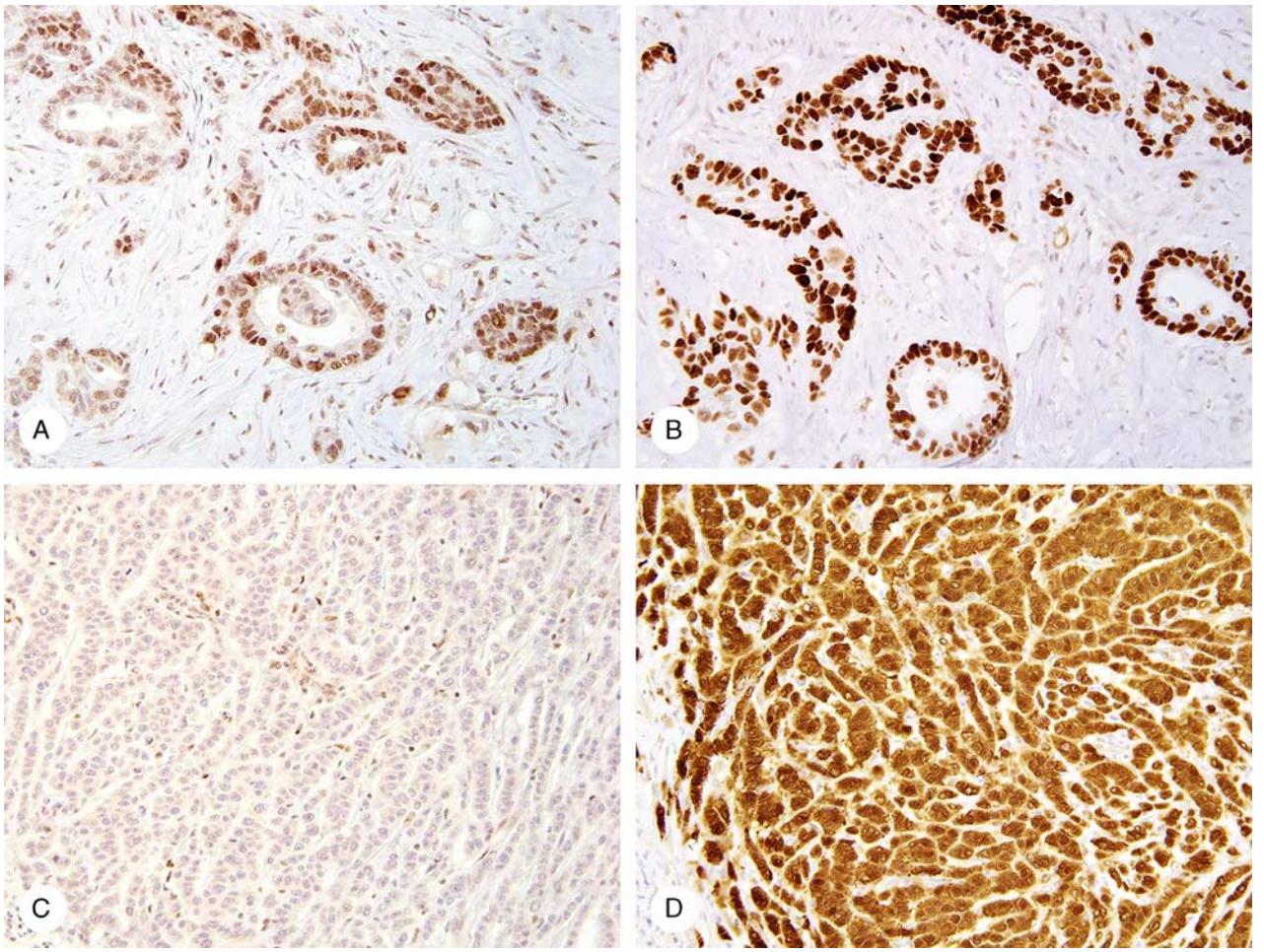

FIGURE 4. Involvement of p53 and β-catenin pathways in Trk-positive colon cancers. TPM3(e8)-NTRK1(e10) fusion tumor (case 1) with retained MLH1 expression (A) harbored a *TP53* mutation (p.Met246Arg) and displayed diffuse p53 immunostaining (B); LMNA(e11)-NTRK1(e8) fusion tumor (case 7) with loss of MLH1 expression (C) harbored an *APC* mutation (p.Cys1502Ter) and displayed nuclear β-catenin accumulation (D).

TABLE 4. Immunophenotype of 16 Colon Cancers With Positive Trk Immunohistochemistry

Case	MLH1	PMS2	MSH2	MSH6	CK7	CK20	CDX2	MUC2
1	Retained	Retained	Retained	Retained	Diffuse	Diffuse	Diffuse	No exp
2	Loss	Loss	Retained	Retained	Diffuse	No exp	No exp	Focal
3	Loss	Loss	Retained	Retained	No exp	No exp	Diffuse	No exp
4	Loss	Loss	Retained	Retained	No exp	Focal	Diffuse	Focal
5	Loss	Loss	Retained	Retained	Diffuse	Focal	Diffuse	No exp
6	Retained	Retained	Retained	Retained	No exp	Diffuse	Diffuse	No exp
7	Loss	Loss	Retained	Retained	No exp	No exp	Focal	No exp
8	Loss	Loss	Retained	Retained	No exp	Focal	Diffuse	No exp
9	Loss	Loss	Retained	Retained	No exp	Focal	Diffuse	Focal
10	Loss	Loss	Retained	Retained	No exp	No exp	No exp	Sc
11	Loss	Loss	Retained	Retained	No exp	Focal	Diffuse	Sc
12	Loss	Loss	Retained	Retained	Diffuse	Sc	Diffuse	Sc
13	Loss	Loss	Retained	Loss	No exp	Sc	Diffuse	No exp
14	Retained	Retained	Retained	Retained	Focal	Diffuse	Diffuse	Diffuse
15	Loss	Loss	Retained	Retained	No exp	No exp	Diffuse	Diffuse
16	Loss	Loss	Retained	Retained	No exp	No exp	Focal	No exp

No exp indicates No expression; Sc, scattered cells.

TABLE 5. Mutated Oncogenes and Tumor Suppressor Genes in *NTRK* Fusion Colon Cancers Reported in This Study

Gene ID	Mutation	Predicted Functional Consequences*	Case
ACVR2A	p.Asp386fs	NA	2
AKT1	p.Ser240Pro	Pathogenic	10
AMER1	p.Arg531Ter	Neutral	8
AMER1	p.Gly140Asp	NA	16
AMER1	p.Val305fs	NA	2
APC	p.Cys1502Ter	Pathogenic	7
APC	p.Pro750Ser	Pathogenic	16
APC	p.Cys1289Tyr	Pathogenic	11
CTNNB1	p.Arg225Cys	Pathogenic	8
CTNNB1	p.Lys354Asn	Pathogenic	2
FBXW7	p.Arg479Gln	Pathogenic	13
FBXW7	p.Trp446Ter	Pathogenic	11
MTOR	p.Ala1792Val	Pathogenic	2
PIK3C2B	p.Arg727Trp	Pathogenic	15
PIK3CD	p.Val370fs	NA	8
PIK3CD	p.Gly245Ser	Pathogenic	15
PIK3R2	p.Val54Met	Pathogenic	5
PTEN	p.His272fs	Pathogenic	1
PTEN	p.Asn323fs	NA	10
TGFBR2	p.Trp529Ter	NA	16
TP53	p.Met246Arg	Pathogenic	1
TP53	p.Pro72Arg	Neutral	11
TP53	p.Cys176Tyr	Pathogenic	15

*On the basis of the FATHMM, SIFT, PolyPhen scores predicting functional consequences of coding variants.
NA indicates not available.

TABLE 7. Types of NTRK3 Fusions Described in 10 CRCs in This (n = 1) and Previously Published Studies*[4,15,16,26] and Available at The Cancer Genome Atlas

	COX5A	ELM4	ETV6	VPS18
NTRK3	e1	e2	e5	e11
e14		2		
e15	1		6	
e18				1
Total		10		

*Excluding 4 cases harboring ETV6-NTRK3 fusions reported without specific data on fusion breaks.[15,16]

variable focal positivity with no tumor being confluently and strongly positive. Expression of PD-L1 and other immune checkpoints in *NTRK* fusion CRCs has not been well studied. One study reported a TPM3-NTRK1 tumor with *PD-L1* amplification and strong diffuse (100%) PD-L1 expression.[23] A durable response to anti-PD-1 treatment has been reported in a case of MMR-deficient colon cancer harboring tyrosine kinase fusion (AML4-ALK) with partial PD-L1 positivity.[16]

In our cohort, colon cancers with *NTRK1* or *NTRK3* fusions commonly showed aberrant immunophenotypes with a frequent loss of CK7, CK20, and MUC2 expression, occasionally accompanied by the loss of CDX2. Such antigenic patterns constituted a highly aggressive subgroup of poorly differentiated CRCs with early recurrences and shorter overall survivals.[56–58] Despite these unfavorable prognostic markers, some patients had longer survival—from 45 months to 17 years. However, conclusions of overall survival are hampered here due to limited follow-up data.

Several previous reports on *NTRK* fusion CRCs presented both disseminated tumors and tumors with no evidence of disease after 4- and 5-year follow-up and no adjuvant chemotherapy.[11] More recent study reported *NTRK* fusion CRCs with synchronous and metachronous lymph node, liver, and peritoneal metastases in 100% (8/8) of analyzed cases and concluded extremely poor prognosis for these tumors.[16] In our cohort, synchronous metastases were documented in 37.5% (6/16) of *NTRK* fusion colon cancers, mostly collected from regional and university hospitals. This might include a bias toward more advanced tumors in studies based on cases from cancer centers. Additional multicenter studies are necessary to better define the biological potential of *NTRK* fusion colon cancers.

To identify patients who could benefit from TRK inhibitor therapy, 2-step screening—using NTRK immunohistochemistry and followed by molecular genetic testing of positive cases—should be considered in all advanced and/or metastatic *BRAF*/*RAS* wild-type CRCs.

In summary, our study presents the clinicopathologic and molecular genetic profile of the rare primary colon cancers harboring *NTRK* gene fusions. Although these tumors displayed some phenotypic and genetic features typically seen in the MMR-deficient colon cancers, the

TABLE 6. Types of NTRK1 Fusions Described in 33 CRCs in This (n = 15) and Previously Published Studies*[8–16,21,26]

	LMNA					PLEKHA6	SCYL3	TPM3		TPR	
NTRK1	e4	e6	e9	e10	e11	e22	e11	e7	e8	e16	e21
e8								2§			
e9									1	1	
e10	1					1		6	10		2
e11		1		2	1†				1‡		
e12			1				1				
e13									2		
Subtotal			6			1	1	22		3	
Total						33					

*Excluding 16 cases harboring 11 TPM3-NTRK1, 3 LMNA-NTRK1, and 2 TPR-NTRK1 fusions reported without specific data on fusion breaks.[15,16]
†Two fusion transcripts, LMNA(e10)-NTRK1(e11) and LMNA(e11)-NTRK1(e11) formed due to alternative splicing.
‡Two fusion transcripts, TPM3(e8)-NTRK1(e11) and TPM3(e8)-NTRK1(e12) in 1 tumor.
§Intraexonic break in 1 case.

separation of *NTRK* fusion tumors from the MMR-deficient tumors into a new molecular subtype seems to be indicated, especially considering targeted treatment inhibiting oncogenic Trk fusion proteins.

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