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COLORECTAL ADENOCARCINOMAS HARBORING ALK FUSION GENES: A CLINICOPATHOLOGIC AND MOLECULAR GENETIC STUDY OF 12 CASES AND REVIEW OF THE LITERATURE

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Abstract

This study determined the frequency, clinicopathologic, and genetic features of colorectal carcinomas driven by oncogenic fusions of the anaplastic lymphoma kinase gene (*ALK*). Out of 8150 screened tumors, 12 (0.15%) were immunohistochemically ALK-positive with D5F3 antibody. These cancers harbored *CAD-ALK* (n=1), *DIAPH2-ALK* (n=2), *EML4-ALK* (n=2), *LOC101929227-ALK* (n=1), *SLMAP-ALK* (n=1), *SPTBN1-ALK* (n=4) and *STRN-ALK* (n=1) fusions as detected by an RNA-based NGS assay. *ALK* fusion carcinomas were diagnosed mostly in older patients with a 9:3 female predominance (median age, 72 years). All tumors, except a rectal one, occurred in the right colon. Most tumors were stage T3 (n=7) or T4 (n=3). Local lymph node and distant metastases were seen at presentation in 9 and 2 patients. These tumors showed moderate (n=6) or poor (n=3) glandular differentiation, solid medullary growth pattern (n=2), and pure mucinous morphology (n=1). DNA mismatch repair deficient phenotype was identified in 10 cases. Tumor-infiltrating lymphocytes were prominent in 9 carcinomas. In 4 carcinomas, tumor cells showed strong, focal (n=3) or diffuse PD-L1 immunoreactivity. CDX2 expression and loss of CK20 and MUC2 expression were frequent. CK7 was expressed in 5 tumors. Four patients died of disease within 3 years and 7 were alive with follow-up ranging from 1–8 years. No mutations in *BRAF*, *RAS* and genes encoding components of PI3K-AKT/MTOR pathway were identified. However, one tumor had a loss-of-function *PTEN* mutation. Aberration of p53 signaling, *TP53* mutations and/or nuclear accumulation of p53 protein was seen in 9 cases. *ALK* fusion colorectal carcinomas are a distinct and rare subtype of colorectal cancers displaying some features of mismatch repair-deficient tumors.

Keywords

colorectal carcinoma; immunohistochemistry; ALK-expression; ALK fusion genes; next-generation sequencing

INTRODUCTION

Colorectal adenocarcinoma (CRC) is one of the major causes of cancer-related death in the United States and in the world. CRC is a highly heterogeneous disease distinguished by multiple genetic and epigenetic events critical for the tumor initiation and progression.¹ Most CRCs are driven by oncogene or tumor suppressor gene mutations. However, a small subset is characterized by fusions involving receptor tyrosine kinase (RTKs) genes.²⁻⁴ Typically, the 3' region of the RTK gene fuses to the 5' region of a gene partner, forming a chimeric gene/oncogene that expresses a constitutively activated tyrosine kinase capable of inducing downstream signaling pathways that promote tumorigenesis.⁵

Anaplastic lymphoma kinase (ALK), originally identified as the kinase partner in the *NPM1-ALK* fusion in anaplastic large cell lymphoma, is encoded by a gene with high homology to the insulin receptor subfamily of transmembrane tyrosine kinases.⁶ ALK may also be involved in the development and function of the nervous system.⁷ Molecular *ALK* alterations – including amplification and activation of mutations and fusions with multiple 5' partners – have been reported in different types of epithelial, hematopoietic, and mesenchymal malignancies.⁸

Recently, several distinct ALK inhibitors have been approved and show efficacy in the treatment of tumors harboring oncogenic *ALK* fusions.⁹⁻¹⁰ This opened an opportunity for targeted therapy of CRCs driven by oncogenic *ALK* fusions.¹¹

Only a small number of CRCs harboring *ALK* fusions have been reported.^{3,11-23} Previously published *ALK* fusion-positive CRCs are listed in the supplemental data (Table S1). Nevertheless, the clinicopathologic profile of CRCs driven by oncogenic *ALK* fusions is not well established. This study presents a comprehensive clinicopathologic, immunohistochemical, and molecular genetic evaluation of 12 *ALK* fusion-driven tumors identified in a large cohort of CRCs screened with ALK immunohistochemistry.

MATERIALS and METHODS

Eight thousand one hundred fifty de-identified CRCs from Europe (Czech Republic, Finland, Germany, Italy and Poland), Japan, and the United States were studied. Tumor samples were assembled in tissue microarrays (TMAs) or multitumor blocks as previously reported.²⁴

Tumor staging and histologic classification were based on The American Joint Committee on Cancer (AJCC) tumor/node/metastasis (TNM) classification and staging recommendation (<http://cancerstaging.org>) and WHO classification of tumors of the digestive system.²⁵ The density of tumor infiltrating lymphocytes (TILs) was scored as previously described.²⁶

Treatment and follow-up data were available in all cases. Each patient underwent surgery sometimes followed by adjuvant chemotherapy. However, no tyrosine kinase-inhibitor therapy was administered.

Immunohistochemistry

Immunohistochemical ALK expression was evaluated using ALK antibody, clone D5F3 (Cell Signaling Technology, Inc., Danvers, MA), and Leica Bond-Max immunostainer (Leica Biosystems, Inc., Buffalo Grove, IL) with 25-minute heat induced epitope retrieval in ER2 buffer. All ALK-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), Cytokeratin 7 and 20 (CK7, CK20), Mucin 2 (MUC2), Tumor Protein P53 (p53) and Programmed death-ligand 1 (PD-L1, CD274). Details of antibodies and immunohistochemical protocols are provided in the supplemental data (Table S2).

Fluorescence in situ hybridization

Interphase fluorescence in situ hybridization (FISH) was performed on 5 μ -thick formalin-fixed paraffin-embedded (FFPE) tumor sections using Dual Color Break Apart SPEC ALK Probe (ZytoVision, Bremerhaven, Germany) and Histology FISH Acc Kit (Perlan Diagnostics, Gdynia, Poland) following the manufacturer's protocol. Specimens and a probe were denatured for 5 minutes at 73°C and subsequently incubated at 37°C for approximately 12 hours in the hybridization chamber CytoHYB CT500 (CytoTest, Rockville, MD). Following standard washing procedures, sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI). Fluorescence microscope Axio Imager.Z2 (Carl Zeiss Microscopy, Jena, Germany) equipped with DAPI/Green/Red triple-band filter was used for visualization. One hundred cells were evaluated at 100x magnification in each case.

Molecular genetic studies

Detailed protocols of molecular procedures including extraction of nucleic acids, evaluation of *MLH1* promoter hypermethylation status and next-generation sequencing were previously reported.²⁴

Briefly, nucleic acids were recovered from FFPE tumor samples using a Maxwell[®] RSC instrument (Promega, Madison, WI). Target-specific RNA libraries for next-generation sequencing (NGS) were constructed using Archer Universal[®] RNA Reagent Kit v2 (ArcherDx, Boulder, CO) and sequenced using a MiSeqDx instrument (Illumina, San Diego, CA). NGS data was analyzed using the Archer Analysis Pipeline Virtual Machine (<https://archerdx.com>). Genotyping was performed using the Ion Torrent[™] (Life Technologies/ Thermo Fisher Scientific, Waltham, MA) next-generation sequencing platform and either Ion AmpliSeq[™] Comprehensive Cancer Panel (409 gene targets) or Ion AmpliSeq[™] Cancer Hotspot Panel v2 Kit (50 gene targets).

Bioinformatics analysis of NGS-data was processed by Torrent Server Suite 5.12 and aligned to the human genome reference sequence, homo sapiens genome assembly GRCh37

(hg19) from Genome Reference Consortium (www.ncbi.nlm.nih.gov/gre). Variant calling was performed using Variant Caller v5.12, 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.

RESULTS

ALK immunohistochemistry

ALK immunoreactivity showed three patterns of staining: diffuse, focal and luminal. The former appeared as either cytoplasmic or membrane staining. No nuclear staining was seen. All 12 CRCs characterized by diffuse ALK staining harbored *ALK* fusion genes. In contrast, no *ALK* fusions, *ALK* rearrangements and/or *ALK* mutations were identified in tumors showing focal or exclusively luminal ALK positivity.

The intensity of diffuse ALK staining varied. In general, tumors with moderate to strong cytoplasmic ALK immunoreactivity lacked membrane staining, while tumors with strong membrane immunoreactivity displayed weak cytoplasmic staining. Different patterns of diffuse ALK staining in colorectal carcinomas are listed in Table 1 and illustrated in Fig. 1A–D.

The intensity of focal staining varied from strong to weak, while luminal staining was well demarcated in all positive cases. Examples of focal and luminal ALK staining are shown in Fig. 2A–B. There was no significant background staining in all but one case. The latter is illustrated and discussed in the supplemental data (Fig. S1A–D). Clinicopathologic and molecular profiles of selected colon carcinomas with focal and luminal ALK staining are presented in the supplemental data (Table S3).

ALK gene rearrangements and fusion gene transcripts

All tumors with diffuse immunopositivity for ALK showed *ALK* gene rearrangements and chimeric *ALK* fusion transcripts as evaluated by FISH with the break-apart probe and by ArcherDX assay, respectively. *ALK* fusion breaks occurred in exon (e) 20 in all but one case. Seven *ALK* fusion 5' partners were identified: *CAD* (Carbamoyl-Phosphate Synthetase 2, synonymous to Aspartate Transcarbamylase and Dihydroorotase, n = 1), *DIAPH2* (Diaphanous Related Formin 2, n = 2), *EML4* (Echinoderm Microtubule Associated Protein Like 4, n = 2), *SLMAP* (Sarcolemma Associated Protein, n = 1) and *SPTBN1* (Spectrin Beta, Non-Erythrocytic 1, n = 4), *STRN* (Striatin, n=1) and uncharacterized gene locus *LOC101929227* (n = 1). ALK-FISH of a colonic adenocarcinoma harboring a *LOC101929227-ALK* fusion is shown in Fig. 3. All ALK fusion gene transcripts are listed in Table 1.

Association between ALK staining patterns and ALK fusions

The association between the ALK staining pattern and the type of *ALK* fusion is highlighted in Table 1. Distinctive, strong membrane staining was seen in 4 SPTBN1(e7)-ALK(e20) and 1 DIAPH2(e17)-ALK(e20) fusion tumors. Variable membrane staining was seen in a rectal adenocarcinoma with an EML4(e6)-ALK(e20) fusion. Tumors harboring other ALK fusions involving CAD(e35), DIAPH2(e26), EML4(21), SLMAP(e14) and STRN(e3) showed moderate to strong cytoplasmic ALK immunoreactivity. Diffuse but weak cytoplasmic ALK staining was seen in LOC101929227(e2)-ALK(e10) fusion colon cancer. Also, intense perinuclear staining was noticeable in some tumor cells harboring SLMAP(e14)-ALK(e20) fusion. An example of perinuclear staining is shown in the supplemental data (Fig. S2).

Demographic and clinicopathologic features

The demographic and clinicopathologic features of *ALK* fusion CRCs are summarized in Table 2. Seventy-five % (9/12) of *ALK* fusion tumors were diagnosed in female patients with median age 72 years. The median age of the three male patients was 68 years.

All but one *ALK* fusion carcinoma developed in the right-sided colon including cecum (n=3), ascending colon (n=6), hepatic (n=1) and splenic (n=1) flexures. There was 1 rectal tumor. Based on the TNM classification, most (10/12) of the *ALK* fusion colon carcinomas were either T3 (n=7) or T4 (n = 3) with local lymph node (n=9) and distant (n=2) metastases diagnosed at presentation. In two cases, including a rectal tumor, the stage was T2N0M0.

Follow-up data were available in all cases. Four patients with local and distal metastases died of disease in a time span of 1–34 months. Seven patients were alive with (n=1) or without disease with follow-up ranging from 13 to 98 months (median follow-up 32.5 months).

Histologic features

The histologic features of *ALK* fusion CRCs analyzed in this study are summarized in Table 3 and illustrated in Fig. 4A–D. Most tumors showed moderate (n=6) or poor (n=3) glandular differentiation. Mucinous features were seen in three cases including 1 tumor with a pure mucinous morphology. Two carcinomas had solid growth patterns corresponding to medullary subtype. Lymphovascular invasion was present in 67% (8/12) tumors. Eight tumors had a high level (> 2/HPF) of intraepithelial lymphocytes.

Mismatch repair genes status

Mismatch repair (MMR) gene studies are summarized in Table 4. Nine colon carcinomas (75%) were MMR-deficient by immunohistochemistry. Eight tumors revealed loss of MLH1 and PMS2 expression (n=6) or heterogenous loss of MLH1 expression. (supplemental data Fig. S3) In one case, a loss of MSH2 expression was detected. All 12 CRCs expressed MSH6. Methylation studies performed on one of two cases displaying heterogenous loss of MLH1 expression revealed hypermethylation of the *MLH1* promoter. No mutations in *MLH1* and *MSH2* were identified in MMR-deficient *ALK* fusion CRCs. However, 2 MMR-proficient tumors harbored *PMS2* p.Lys647* and *MSH6* p.Asn112Ser mutations, respectively.

P53 pathway alterations

Immunohistochemical and molecular evaluation of p53 pathway is summarized in Table 4. Nuclear p53 immunoreactivity was detected in 11 cases. Six of nine tumors exhibiting focal (n=6) or diffuse (n=3) p53 staining harbored *TP53* mutations. A variable number of scattered p53 positive nuclei were seen in 2 *TP53*-wild type carcinomas, while one cancer was negative for both p53 nuclear accumulation and *TP53* mutation.

CDX2, CK7 and CK20, and MUC2 expression

Immunoprofiles of *ALK* fusion tumors are presented in Table 4. All tumors showed diffuse (n=10) or focal CDX2 expression. CK7 and CK20 were diffusely expressed in 5 and 2 cases, respectively. There was focal CK20 positivity in 5 tumors. Majority of tumors showed total (n=4) or focal loss (n=8) of MUC2 expression.

PD-L1 expression

The results of PD-L1 immunohistochemistry are summarized in Table 4 and illustrated in Fig. S4. Four CRCs showed strong, focal (n=3) or diffuse PD-L1 tumor cell positivity. Abundant PD-L1 positive tumor-infiltrating immune cells were seen in 3 cases. Seven cancers, including 2 with focal PD-L1 positivity of tumor cells, harbored scattered PD-L1 positive tumor-infiltrating immune cells.

Somatic mutations

All somatic mutations identified in this study are listed in the supplemental data Table S4. Tumors (Case 1–6, 8 and 11) with well-preserved DNA were evaluated with comprehensive cancer panel of 409 genes, while genotyping of three cases (Case 7, 9, 10 and 12) with insufficient DNA quality was limited to 50 genes.

None of the 12 *ALK* fusion tumors harbored mutations in the typical CRC drivers *BRAF* (B-raf proto-oncogene, serine/threonine kinase), *K-RAS* (Kirsten rat sarcoma viral oncogene homolog) and *N-RAS* (Neuroblastoma Ras viral oncogene homolog). Two tumors had subclones with additional pathogenic *ALK* mutations, p.Thr1151Met and p.Arg1275Gln.

Genes encoding components of the PI3K-AKT/MTOR (Phosphoinositide 3-kinases-serine/threonine protein kinase/mammalian target of rapamycin) signaling pathway, such as *AKT*, *MTOR*, *PIK3CA* (Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha), and *TSC* (Tuberous sclerosis complex), were not altered. However, a subclone with mutation affecting *PIK3C2B* (Phosphatidylinositol-4-phosphate 3-kinase catalytic subunit type 2 beta) encoding subunits of Class II PI3Ks was identified in 1 case. *PTEN* (Phosphatase and tensin homolog), a regulator of PI3K pathway, carried a loss-of-function mutation (p.Asn63fs) in 1 case.

TP53 was mutated in 50% (6/12) of analyzed CRCs. However, there were no mutations in genes encoding the Wnt/ β -catenin signaling pathway components *APC* (Adenomatous polyposis coli protein) or *CTNNB1* (Catenin beta 1). *FBXW7* (F-box and WD repeat domain containing 7), a tumor suppressor gene, was affected by the loss-of-function mutation (p.Arg367*) in one case.

DISCUSSION

This study analyzed clinicopathologic and genetic features of colorectal carcinomas (CRCs) characterized by immunohistochemical ALK expression. All eleven cases with diffuse cytoplasmic or membrane staining patterns harbored *ALK* fusions (0.15% of the screening cohort of 8150 cases). However, tumors with focal and luminal ALK expression revealed no *ALK* alterations. Thus, in CRC, diffuse cytoplasmic or membranous ALK expression predicts an *ALK* fusion.

Two previous immunohistochemical studies on CRC using anti-ALK 5A4 antibody (Novocastra) and a BenchMark Ultra autostainer (Ventana Medical Systems) and the clone D5F3 with a Leica Bond III autostainer reported a frequency of positive tumors between 0.05% (1 of 1889) and 0.1% (3 of 2980), respectively.^{14, 22} In this study, more sensitive detection of ALK positive tumors may have caused a slightly higher frequency of 0.15%.

Sensitivity of ALK immunohistochemistry in search of *ALK* fusions cannot be assessed in this investigation because ALK-negative CRCs were not genotyped. One hybrid capture-based next generation sequencing study found that 0.19% (6 of 3157) of analyzed tumors harbored *ALK* fusions.²² Another study reported CRC harboring a *C2orf44-ALK* fusion with 89.8-fold increase of *ALK* RNA and negative ALK immunohistochemistry.¹⁹ Although frequency of *ALK* fusion tumors among CRCs identified by immunohistochemical or molecular screening appears to be somewhat similar, it is possible that screening by ALK immunostain is not as accurate as sequencing.

In this study, *ALK* fusion partner genes included *CAD*, *DIAPH2*, *EML4*, *LOC101929227*, *SLMAP*, *SPTBN1* and *STRN*. However, no fusions with *C2orf44* (Chromosome 2 Open Reading Frame 44), *CENPF* (Centromere Protein F), *MAPRE3* (Microtubule Associated Protein RP/EB Family Member 3), *PP4R3B* (Protein Phosphatase 4 Regulatory Subunit 3B), *PPP1R21* (Protein Phosphatase 1 Regulatory Subunit 21), *PRKAR1A* (Protein Kinase CAMP-Dependent Type I Regulatory Subunit Alpha) and *PRKAR1B* (Protein Kinase CAMP-Dependent Type I Regulatory Subunit Beta), previously identified *ALK* partners in CRC, were detected.^{20,22}

EML4-ALK fusion, previously reported in CRC, was less frequent and accounted for 17% (2/12) of analyzed cases. Nevertheless, *EML4* is the most common *ALK* fusion partner in the combined previously published and current fusions in colorectal cancer. The *EML4-ALK* fusion was first discovered in non-small cell lung carcinoma.²⁷ Subsequently, it was found as an alternative molecular aberration in inflammatory myofibroblastic tumors and in a subset of breast, colorectal and non-small cell lung cancers.²⁸⁻³⁰ *EML4* encodes for echinoderm microtubule-associated protein 4, which belongs to a family of proteins that stabilize microtubules.³¹ The involvement of *EML4* protein in microtubule cytoskeleton might be responsible for the cytoplasmic and variable membrane ALK staining seen in *EML4-ALK* fusion tumors.

A *SPTBN1*(e7)-*ALK*(e20) gene was the most common (33%) fusion in our cohort (4/12 cases). This fusion was previously reported in CRC only once.²³ However, an *SPTBN1*(e6)-*ALK*(e20) fusion was identified in a lung adenocarcinoma.³² In chronic myeloid leukemia

and atypical myeloproliferative disorder, fusions with *SPTBN1* are partnered with *FLT3* and *PDGFRB*.^{33,34} *SPTBN1*, a member of the spectrin superfamily, encodes a 247-kDa cytoskeletal protein, which can bind to membrane phospholipids.^{35,36} This could explain a strong cell membrane ALK staining observed in *SPTBN1-ALK* fusion tumors.

One tumor harbored STRN(e3)-ALK(e20) fusion. Previously *STRN* has been shown to be an *ALK* fusion partner in carcinomas of colon, thyroid and kidney.^{15,21,22,37,38} *STRN* is a component of the striatin-interacting phosphatase and kinase (STRIPAK) complex. STRIPAK complexes are highly conserved in eukaryotic organisms and play a role in a variety of physiological cellular processes such as cell cycle control, signaling, migration, apoptosis and Golgi apparatus assembly.³⁹ STRIPAK complex malfunction is associated with different pathological conditions including cancer.^{40,41} In this study, SLMAP, a member of tail-anchored proteins and another component of STRIPAK complex, has been shown to form with ALK chimeric protein.

SLMAP(e14)-ALK(e20) fusion protein has not been previously reported in CRC. However, two similar fusions SLMAP(e12)-ALK(e20) and SLMAP(e13)-ALK(e20) were identified in a lung adenocarcinoma.⁴² A subset of *SLMAP-ALK* fusion tumor cells revealed an intense perinuclear ALK staining spots probably indicating anchoring of the chimeric protein to Golgi apparatus.

The CAD(e35)-ALK(e20) fusion was identified in 1 case. An identical fusion was previously reported in 2 cases, ascending colon and rectal adenocarcinoma.^{11,16} *CAD* consists of 44 exons and encodes a multi-enzymatic protein associated with the enzymatic activity of the enzymes in the pathway of pyrimidine biosynthesis.⁴³ A biallelic *CAD* loss-of-function has been associated with uridine-responsive epileptic encephalopathy.⁴⁴ A patient with disseminated colon carcinoma harboring *CAD-ALK* fusion responded to ALK-inhibitor treatment.¹¹

Fusions of ALK tyrosine kinase domain with new partners, *DIAPH2* and *LOC101929227*, were identified in this study. A *DIAPH2-ALK* fusion occurred in two variants containing either 17 or 26 *DIAPH2* exons. A protein encoded by *DIAPH2* was indicated in the regulation of endosome dynamics and actin filament organization (<https://www.uniprot.org>). ALK immunohistochemistry revealed strong membrane expression in the tumor harboring *DIAPH2*(e17) variant and lack of membrane but strong cytoplasmic expression in the second case. Disruption of *DIAPH2* due to a balanced translocation t(X;12)(q21;p13) has been linked to a family with a premature ovarian failure.⁴⁵

LOC101929227, an uncharacterized RNA gene affiliated with the lncRNA class (www.genecards.org), partnered *ALK* in an unusual fusion involving the first two exons of *LOC101929227* and twenty *ALK* exons (10–29) encoding part of the extracellular domain with second MAM (Meprin, A-5 protein, and receptor protein-tyrosine phosphatase Mu) domain, transmembrane (TM) and intracellular domains. In CRC, noncanonical *ALK* truncation has been identified once in the PPP4R3B(e11)-ALK(e2) fusion containing MAM and TM ALK domains (sequence available at The Cancer Genome Atlas under TCGA-F5–6864 code). The function of ALK extracellular domains, including MAM domains, is not

well understood.⁴⁶ This case was the only one that exhibited diffuse, weak cytoplasmic staining, but the fusion was confirmed by FISH.

Most *ALK* fusions reported in this study resulted from intrachromosomal rearrangements engaging *ALK* on chromosome 2p23.1 and fusion partner genes *EML4* (ch2p21), *CAD* (ch2p23.3), *SPTBN1* (ch2p16.2) and *STRN* (2p22.2) located in its vicinity. However, in three cases, *DIAPH2* (Xq21.33)-*ALK*, *LOC101929227* (11q23.3)-*ALK* and *SLMAP* (3p14.3)-*ALK* fusion genes were formed due to interchromosomal rearrangements. A few colorectal carcinomas harboring fusion genes, *CENPF* (1q41)-*ALK*, *PRKAR1A* (17q24.2)-*ALK* and *PRKAR1B* (7p22.3)-*ALK*, resulting from interchromosomal translocation have been described.^{13,22} Variants of *ALK* fusions reported in colorectal carcinomas in this study and previous studies are shown in Table 5.

ALK fusion tumors were diagnosed predominantly in women in their sixties or seventies (median age 72 years). In comparison, a median age of 50 years was previously reported for 11 patients with metastatic *ALK* fusion CRCs.²⁰ Because cases included in the current study were mostly from regional and university hospitals, this discrepancy might be related to the bias toward more advanced tumors in younger patients (in 4th or 5th decade) in cancer centers.

In this study, 11 *ALK* fusion tumors were classified as right sided based on whether they occurred before or after the splenic flexure, as this classification may better reflect tumor biology at different sites.^{47,48} Most of previously reported *ALK* fusion CRCs occurred in the right colon or rectum and only few were diagnosed in descending colon location. (Supplemental data Table S1).

Mucinous differentiation was seen in 25% (3/12) of tumors. Similarly, 31% (4/13) of previously reported *ALK* fusion tumors (Table S1) displayed mucinous component. This remains in the range of the previously published frequency of combined mucinous tumors and tumors with mucinous component in colorectal carcinomas.⁴⁹ However mucinous histology was seen only in 1 of 8 metastatic colorectal carcinomas with *ALK* fusions.²⁰ *ALK* fusion colorectal carcinomas showed aberrant immunophenotypes with a frequent loss of CK7 and CK20 and MUC2 expression, occasionally accompanied by the partial loss of CDX2. Such antigenic patterns constituted a highly aggressive subgroup of poorly differentiated CRCs with early recurrences and shorter overall survivals.^{50–52}

In colorectal cancer, a high level of tumor infiltrating lymphocytes has been considered a positive prognostic marker, particularly if paired with MMR-deficient status.⁵³ In our cohort, 80% (8/10) of MMR-deficient tumors revealed high levels of tumor-infiltrating lymphocytes. Also, 4 MMR-deficient tumors showed diffuse or focal strong PD-L1 staining of tumor cells. A robust influx of PD-L1 positive tumor infiltrating immune cells were seen in 3 cases including one PD-L1 negative cancer. An association between PD-L1 expression and MMR-deficiency has been previously documented in CRCs.⁵⁴ One study reported a durable response to anti PD-1 treatment in a case of MMR-deficient, *EML4-ALK* fusion colon cancer with partial PD-L1 positivity.²⁰ Thus, screening of CRCs driven by *ALK* fusions for PD-L1 expression could be beneficial for the selection of checkpoint inhibitor therapy.

In this study, CRCs harboring *ALK* fusions revealed features characteristic of microsatellite-unstable colorectal tumors, such as female predominance (1:3 male to female ratio) and right colon location (>90%). Mismatch repair (MMR)-deficiency was identified by immunohistochemistry in 83% (10/12) of *ALK* fusion tumors analyzed in this study. A similar frequency of 73% (8/11) was previously recorded by a study on metastatic CRCs with *ALK* fusions.²⁰ However, a review of previously reported *ALK* fusion CRCs with known MMR status revealed that 56% (9/16) were mismatch repair proficient tumors.^{3,13–17,19,21,22} This questions the proposed rationale for *ALK* fusion screening for only MMR-deficient CRCs.²¹

BRAF mutations, typically seen in colon cancer with MMR-deficiency, were not detected in the *ALK* fusion tumors analyzed in this study. Additionally, other common colorectal carcinoma drivers such as *K-RAS* and *N-RAS* were not involved. This highlights a primary oncogenic role of chimeric *ALK* fusion proteins. No *BRAF*p.V600E mutation has been identified in previously reported *ALK* fusion CRCs.^{14–16,20–22} However, 2 other *BRAF* mutations (one with biological significance) have been reported among 11 cases studied using next-generation sequencing.^{20,22} Likewise, no oncogenic *RAS* mutations was identified in *ALK* fusion CRCs except *KRAS* p.G12A coexisting with *EML4-ALK* fusion in highly advanced, disseminated rectal tumor and *KRAS* p.G13D acquired during disease progression by *STRN-ALK* fusion cecal cancer.^{12,22} Thus, RAS involvement in that case appears secondary to an oncogenic *ALK* fusion.

In our cohort, genes encoding components of PI3K-AKT/MTOR signaling pathway, such as *AKT*, *MTOR*, *PIK3CA*, and *TSC*, were not affected by mutations. Previous studies reported similar results.^{20,22}

In most cases, *TP53* mutations and nuclear accumulation of p53 protein (documented by immunohistochemistry) indicated pathologic modification of this pathway. This is in line with previously published studies reporting frequent *TP53* missense mutations in *ALK* fusion colorectal carcinomas.^{20,22} One of *TP53* mutants harbored a *FBXW7*p.Arg367* mutation. Mutational inactivation of *FBXW7*, a p53-dependent tumor suppressor gene, has been reported in approximately 10% of colorectal carcinomas and linked to tumor progression.⁵⁵ Several *ALK* fusion CRCs have been reported to carry *FBXW7* missense and truncating mutations.^{20,22}

Molecular alterations of Wnt/ β -catenin signaling pathway, common in non-hypermutated tumors, were not identified in this study. Also, previously published *ALK* fusion tumors infrequently carried Wnt/ β -catenin signaling pathway mutations.^{20,21} However, relatively frequent mutations in the gene encoding RNF43, a protein considered to be negative regulator of the Wnt signaling, were reported.⁵⁶ *RNF43* was not included in the gene panel evaluated in this study.

Also, loss-of-function mutations affecting tumor suppressor genes *ARID1A* (AT-rich interactive domain-containing protein 1A) and *ARID1B* (AT-rich interactive domain-containing protein 1B), components of the human SWI/SNF chromatin remodeling complex,

have been reported in *ALK* fusion tumors.²⁰ No *ARID1A* mutants were identified in this study, while *ARID1B* was not tested.

A recent study on *ALK* fusion colorectal carcinomas concluded extremely poor prognosis for these tumors.²⁰ In our cohort, 33% of patients died of the disease during a period of 2 and a half years. However, there were longer survivals (78 and 98 months alive without disease) including two patients with advanced (T3/T4) tumors and local metastases. Also, one recent study reported a patient with *ALK* fusion pT3N1M0 tumor as disease-free for 10 years following surgery and standard chemotherapy.¹⁷ Definitive conclusions of overall patient survival in *ALK* fusion CRC are currently limited by a small number of cases and relatively short follow-ups.

In summary, this study described clinicopathologic and molecular genetic features of rare colorectal cancers with oncogenic *ALK* fusions. Although these tumors had characteristics of MMR-deficient colon cancers, separation of *ALK* fusion tumors into a new molecular subtype is indicated due to the presence of additional therapeutic options.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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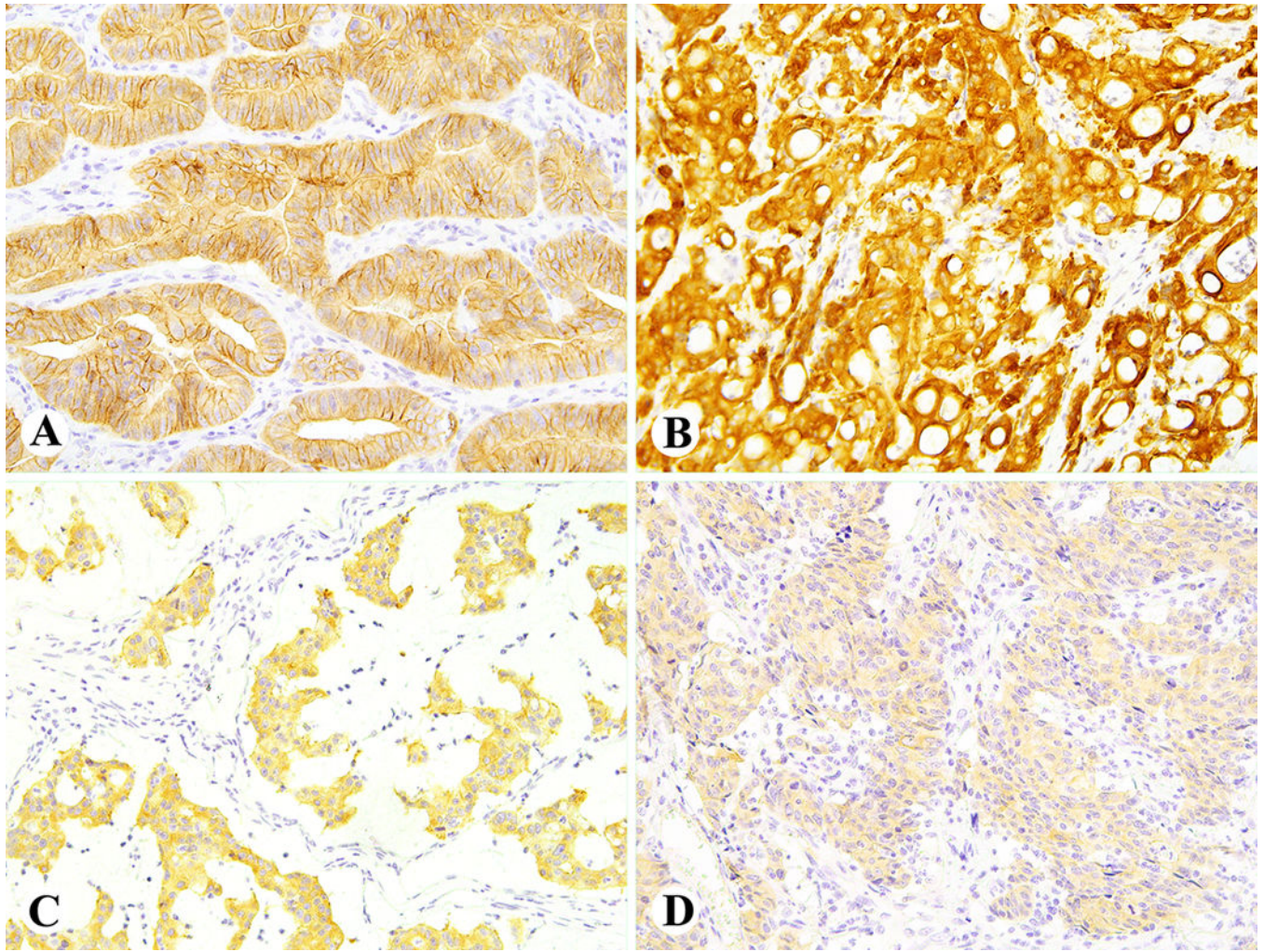


Figure 1. Example of diffuse ALK immunoreactivity in colorectal carcinomas. Strong membrane staining for ALK in Case 1 with a *SPTBN1-ALK* fusion (A); strong cytoplasmic ALK staining in Case 6 with a *DIAPH2-ALK* fusion (B); moderate cytoplasmic ALK staining in Case 8 with an *EML4-ALK* fusion (C); weak cytoplasmic ALK staining in Case 9 with a *LOC101929227-ALK* fusion (D).

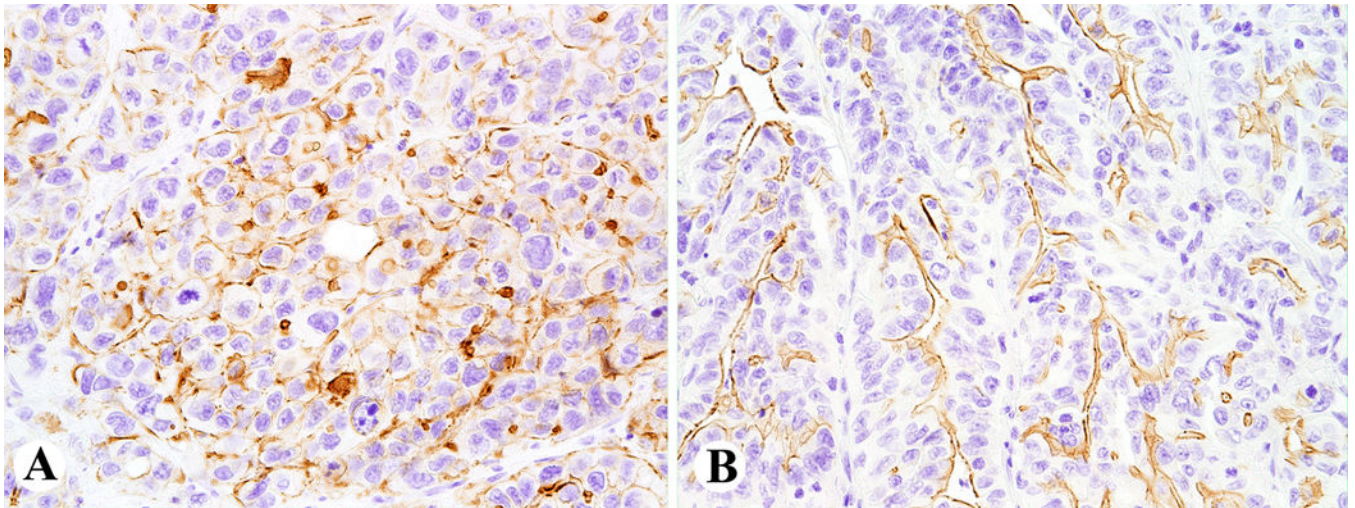


Figure 2.
Example of focal (A) and luminal (B) ALK immunoreactivity in ALK fusion negative colon carcinomas.

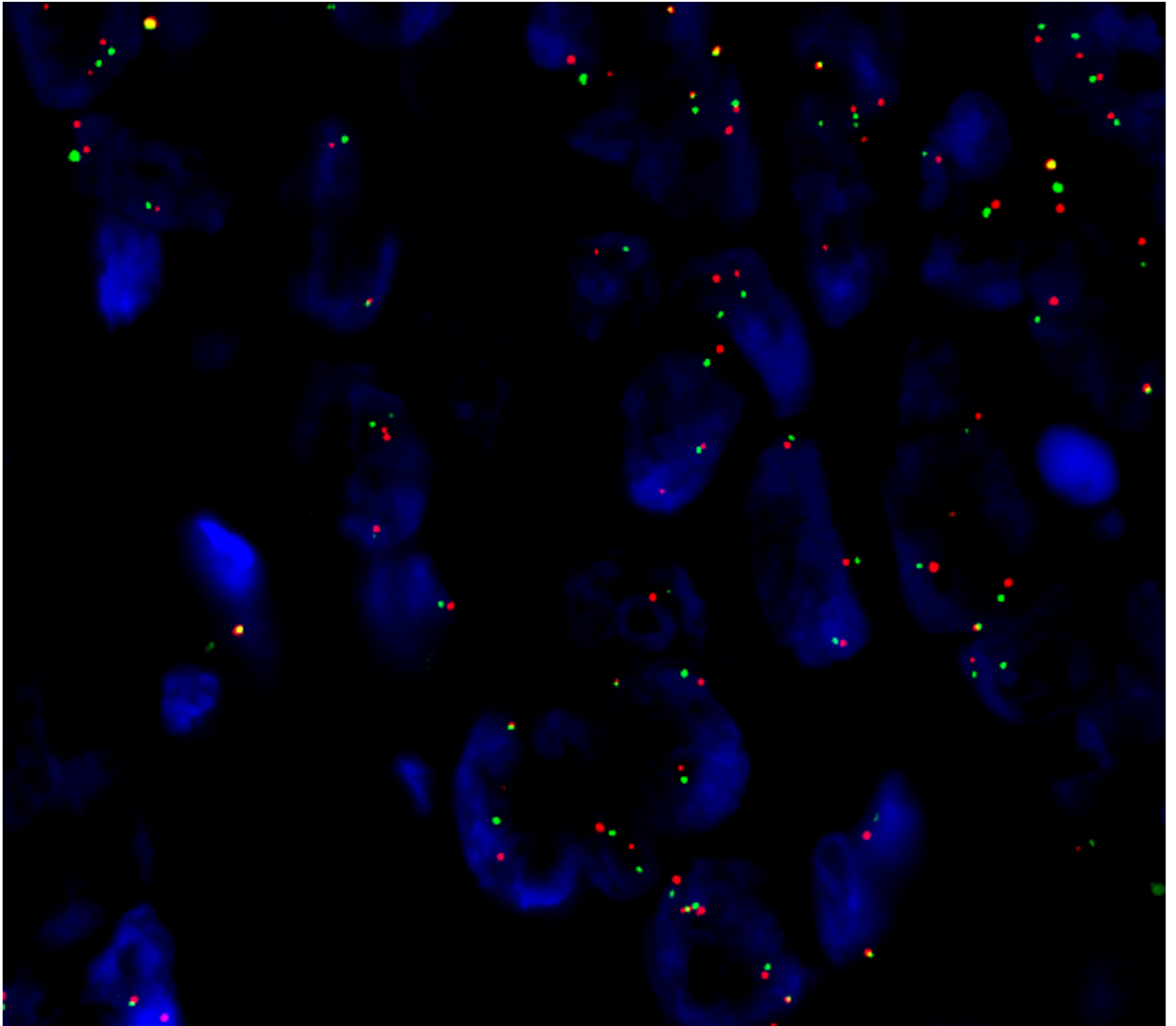


Figure 3. A dual-color FISH analysis performed on interphase nuclei using a break-apart ALK gene probe in CRC (Case 9) harboring a *LOC101929227-ALK* fusion. A split of orange and green signal indicates an *ALK* rearrangement.

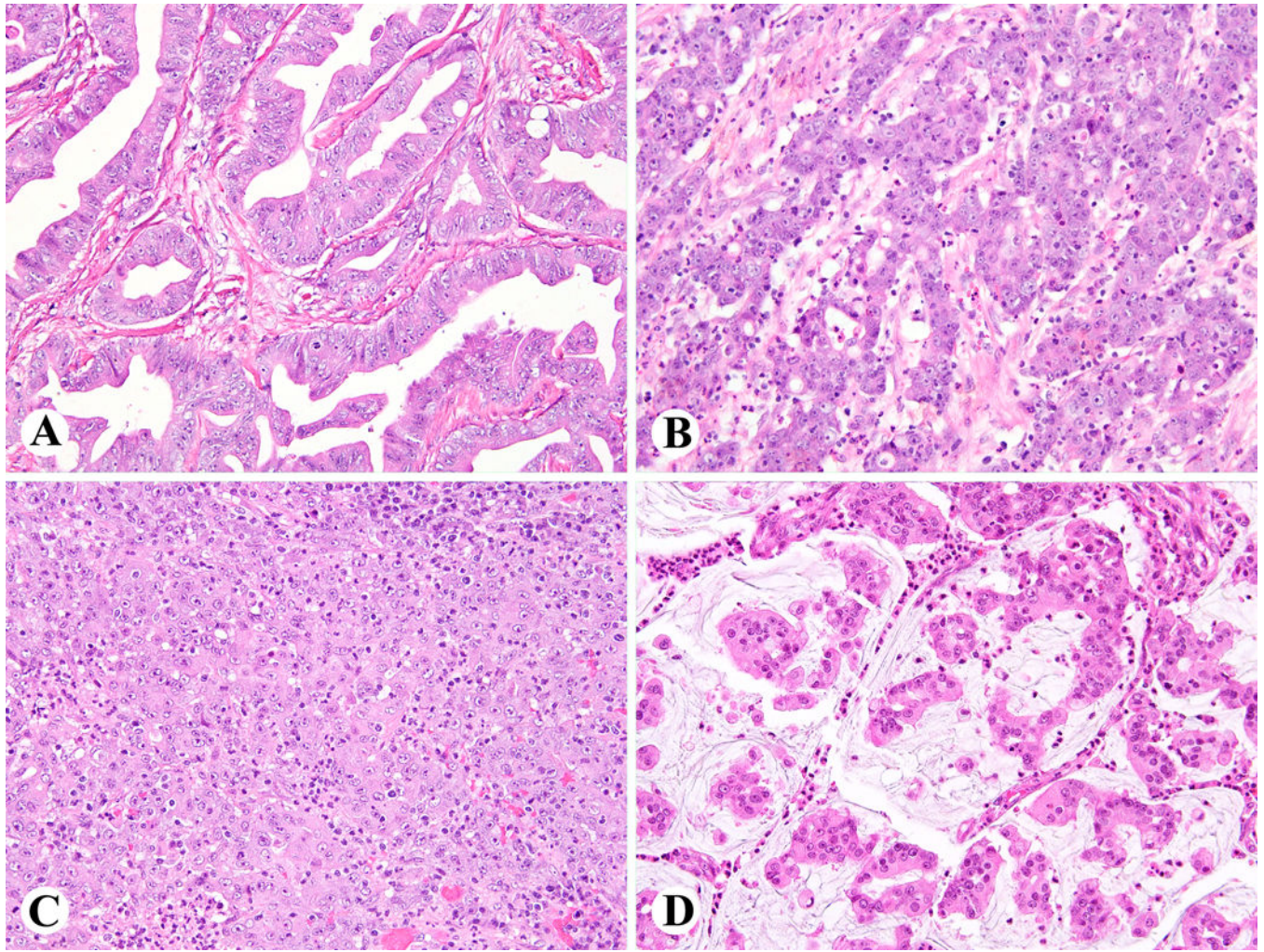


Figure 4. Histologic features of colorectal carcinomas with diffuse ALK immunoreactivity. Case 1, moderately differentiated CRC harboring *SPTBN1-ALK* fusion (A); Case 2, poorly differentiated CRC harboring *SPTBN1-ALK* fusion (B); Case 12, medullary CRC harboring *EML4-ALK* fusion (C); Case 8, mucinous tumor harboring *EML4-ALK* fusion (D)

Table 1. ALK immunoreactivity in 12 colorectal carcinomas harboring *ALK* fusion genes.

| ALK fusion gene transcript | No. of cases | ALK immunoreactivity | |
|----------------------------|--------------|----------------------|------------|
| | | Cytoplasmic | Membrane |
| CAD(e35)-ALK(e20) | 1 | Moderate to strong | Absent |
| DIAPH2(e17)-ALK(e20) | 1 | Weak | Strong |
| DIAPH2(e26)-ALK(e20) | 1 | Strong | Absent |
| EML4(e21)-ALK(e20) | 1 | Moderate to strong | Absent |
| EML4(e6)-ALK(e20) | 1 | Weak to moderate | Variable |
| LOC101929227(e2)-ALK(e10) | 1 | Weak | Absent |
| SLMAP(e14)-ALK(e20) | 1 | Moderate to strong* | Absent |
| SPTBN1(e7)-ALK(e20) | 4 | Weak | Strong |
| STRN(e3)-ALK(e20) | 1 | Moderate | Variable** |

Symbols:

* = some cells with a dot-like perinuclear staining,

** = stronger on luminal side.

Table 2.

Clinical characteristics of 12 colorectal carcinomas harboring *ALK* fusion genes.

| Case | Type of <i>ALK</i> fusion | Age | Sex | Tumor site | pTNM | Follow-up (in months) |
|------|---------------------------|-----|-----|-----------------|------------|-----------------------|
| 1 | SPTBN1(e7)-ALK(e20) | 53 | M | Cecum | pT3N1aM0 | ‡ANED (30m) |
| 2 | SPTBN1(e7)-ALK(e20) | 66 | F | Cecum | pT3N0M0 | ‡ANED (20m) |
| 3 | CAD(e35)-ALK(e20) | 87 | F | Cecum | pT4bN1bM1a | ‡DOD (1m) |
| 4 | SPTBN1(e7)-ALK(e20) | 64 | F | Ascending colon | pT3N2M0 | ‡ANED (98m) |
| 5 | SLMAP(e14)-ALK(e20) | 69 | M | Ascending colon | pT3aN1cM1c | ‡DOD (8m) |
| 6 | DIAPH2(e26)-ALK(e20) | 72 | F | Ascending colon | pT3N1bM0 | ‡DOD (34m) |
| 7 | SPTBN1(e7)-ALK(e20) | 75 | F | Ascending colon | pT4aN2bM0 | ‡ANED (78m) |
| 8 | EML4(e21)-ALK(e20) | 76 | M | Ascending colon | pT3N1M0 | ‡AWD (54m) |
| 9 | LOC101929227(e2)-ALK(e10) | 77 | F | Ascending colon | pT3pN1aM0 | ‡DOD (12m) |
| 10 | DIAPH2(e17)-ALK(e20) | 72 | F | Hepatic flexure | pT4aN2aM0 | §ANED (13m) |
| 11 | STRN(e3)-ALK(e20) | 65 | F | Splenic flexure | pT2N0M0 | ‡ANED (15m) |
| 12 | EML4(e6)-ALK(e20) | 76 | F | Rectum | pT2N0M0 | §ANED (35m) |

Abbreviations and symbols: e = exon, ANED = alive, no avoidance of disease, DOD = died of disease, NA = not available,

‡ = adjuvant chemotherapy,

§ = no adjuvant chemotherapy,

‡ = adjuvant chemotherapy status unknown.

Table 3. Histopathological characteristics of 12 colorectal carcinoma harboring *ALK* fusion genes.

| Case | Type of <i>ALK</i> fusion | Degree of glandular differentiation | Mucinous component | Lymphovascular invasion | Tumor infiltrating lymphocytes |
|------|---------------------------|-------------------------------------|--------------------|-------------------------|--------------------------------|
| 1 | SPTBN1(e7)-ALK(e20) | Moderate | No | Yes | Low |
| 2 | SPTBN1(e7)-ALK(e20) | Poor | No | Yes | High |
| 3 | CAD(e35)-ALK(e20) | Moderate | No | Yes | High |
| 4 | SPTBN1(e7)-ALK(e20) | Moderate | Yes | No | High |
| 5 | SLMAP(e14)-ALK(e20) | Moderate | No | Yes | High |
| 6 | DIAPH2(e26)-ALK(e20) | Poor | No | Yes | High |
| 7 | SPTBN1(e7)-ALK(e20) | Poor | Yes | Yes | High |
| 8 | EML4(e21)-ALK(e20) | Mucinous | Yes | No | Low |
| 9 | LOC101929227(e2)-ALK(e10) | Solid (medullary) | No | Yes | High |
| 10 | DIAPH2(e17)-ALK(e20) | Moderate | No | Yes | Low |
| 11 | STRN(e3)-ALK(e20) | Moderate | No | No | High |
| 12 | EML4(e6)-ALK(e20) | Solid (medullary) | No | No | High |

Table 4.

Mismatch repair status, p53 alterations, CDX2, CK7, CK20, and MUC2 immunophenotypes, and PD-L1 expression in 12 colorectal carcinomas with *ALK* fusions.

| Case | ALK fusion partner gene | Mismatch repair status | | | | p53 pathway alterations | Immunopositivity | | | | PD-L1 expression | |
|------|-------------------------|------------------------|----------|----------|----------|--------------------------------|----------------------|----------|----------|----------|------------------|---------------------------------|
| | | MLH1 | PMS2 | MSH2 | MSH6 | | CDX2 | CK7 | CK20 | MUC2 | Tumor cells | Tumor infiltrating immune cells |
| 1 | SPTBN1 | Retained | Retained | Loss | Retained | Focal (p.Arg273Cys) | Diffuse | Diffuse | Negative | Focal | Negative | Focal |
| 2 | SPTBN1 | Loss | Retained | Retained | Retained | Focal (p.Gly199 ^F) | Diffuse | Diffuse | Negative | Negative | Focal | Diffuse |
| 3 | CAD | Retained | Retained | Retained | Retained | Diffuse (p.Met237Ile) | Diffuse | Diffuse | Focal | Focal | Negative | Focal |
| 4 | SPTBN1 | Loss | Loss | Retained | Retained | Focal (WT) | Diffuse | Diffuse | Diffuse | Diffuse | Negative | Focal |
| 5 | SLMAP | HLoss | Retained | Retained | Retained | Diffuse (p.Arg248Trp) | Diffuse | Diffuse | Negative | Negative | Negative | Negative |
| 6 | DIAPH2 | Loss | Loss | Retained | Retained | Focal (p.Tyri26His) | Diffuse | Diffuse | Negative | Negative | Focal | Focal |
| 7 | SPTBN1 | Loss | Loss | Retained | Retained | IPC (WT) | Diffuse | Diffuse | Negative | Negative | Focal | Focal |
| 8 | EML4 | Retained | Retained | Retained | Retained | Focal (WT) | Diffuse | Diffuse | Negative | Diffuse | Negative | Focal |
| 9 | LOC101929227 | Loss | Loss | Retained | Retained | Negative (WT) | Focal | Diffuse | Focal | Diffuse | Focal | Focal |
| 10 | DIAPH2 | HLoss | Retained | Retained | Retained | Diffuse (p.Arg282Trp) | Diffuse | Diffuse | Diffuse | Diffuse | Negative | Negative |
| 11 | STRN | Loss | Loss | Retained | Retained | IPC (WT) | Diffuse [‡] | Diffuse | Focal | Focal | Negative | Diffuse |
| 12 | EML4 | Loss | Loss | Retained | Retained | Focal (WT) | Focal [*] | Negative | Negative | Negative | Diffuse | Diffuse |

Abbreviations and symbols: HLoss = heterogeneous loss, WT = wild type, IPC = isolated positive cells

[‡] = methylated promoter,

^{*} = weak staining, - = negative,

Table 5.

Types of ALK fusions described in 38 colorectal carcinomas reported in this (n=12) and previously published studies.^{3,11–23}

| Fusion gene partner | ALK | | | | | |
|---------------------|------|---|----|----|----|----|
| | Exon | 2 | 10 | 19 | 20 | NA |
| C2orf44 | 4 | | | | 1 | |
| CAD | 35 | | | | 3 | |
| | NA | | | | | 1 |
| CENPF | 11 | | | | 1 | |
| DIAPH2 | 17 | | | | 1 | |
| | 26 | | | | 1 | |
| EML4 | 2 | | | 1 | 1 | |
| | 6 | | | | 2 | |
| | 13 | | | | 1 | |
| | 20 | | | | 2 | |
| | 21 | | | | 3 | |
| | NA | | | | | 4 |
| LOC101929227 | 2 | | 1 | | | |
| MAPRE3 | 7 | | | | 1 | |
| PPP4R3B | 11 | 1 | | | | |
| PPP1R21 | 17 | | | | 1 | |
| PRKAR1A | 2 | | | | 1 | |
| PRKAR1B | 4 | | | | 1 | |
| SLMAP | 14 | | | | 1 | |
| SPTBN1 | 7 | | | | 5 | |
| STRN | 3 | | | | 4 | |
| Subtotal | | 1 | 1 | 1 | 30 | 5 |
| Total | 38 | | | | | |