Cytoplasmic TrkA Expression as a Screen for Detecting NTRK1 Fusions in Colorectal Cancer\textsuperscript{1,2}

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Abstract

NTRK1 gene fusions, the targets of multikinase inhibitors, are promising therapeutic targets for colorectal cancer (CRC). However, screening methods for detecting NTRK1 gene fusions in CRC tissues have not been reported. In this study, we investigated the potential use of immunohistochemistry (IHC) for detecting NTRK1 gene fusions. We performed and compared IHC with fluorescence in situ hybridization (FISH) in 80 CRC patients. TrkA immunostaining was observed to be both membranous and cytoplasmic and was scored semiquantitatively using staining intensity and proportions. The tumors were observed to be NTRK1 gene fusion-positive when \( \geq 20 \) out of 100 nuclei in FISH. A significant correlation between the IHC and FISH results for determination of the NTRK1 gene fusions was observed. We measured the cytoplasmic TrkA expression, which showed an area under the receiver operating characteristic (ROC) curve of 0.926 (range: 0.864-0.987, 95% CI, \( P = .001 \)). By choosing 4.5 (sum of the intensity and proportion scores of cytoplasmic TrkA expression) as the cut-off value for the positive and negative NTRK1 gene fusion groups, the sensitivity and specificity for predicting lymph node metastasis were 100 and 83.8\%, respectively (\( P = .001 \)). Specifically, high cytoplasmic TrkA expression (sum of intensity and proportion scores >4) was associated with the presence of NTRK1 gene fusions (\( P < .0001, r = 0.528 \)). Taken together, our data showed that IHC for TrkA can be used as an efficient screening method for detecting NTRK1 gene fusions in CRC.

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Introduction

Colorectal cancer (CRC) is a major cause of morbidity and mortality worldwide. The incidence of CRC in Korea has increased dramatically over the past few decades; however, the incidence rates of other more common cancers, such as stomach and liver cancers, have decreased [1]. Recently, molecularly targeted drugs, such as cetuximab (Erbitux®; Merck KGaA, Darmstadt, Germany), an epidermal growth factor receptor (EGFR)-targeted monoclonal antibody, and the vascular endothelial growth factor (VEGF)-targeted monoclonal antibody bevacizumab (Avastin®; Genentech Inc., CA, U.S.) [2–4], have been developed to treat metastatic CRC. However, serious limitations are associated with the use of these targeted drugs, and it is thus necessary to develop prognostic biomarkers and novel therapeutic targets based on the molecular mechanisms underlying CRC pathogenesis. Recently, due to the development of next generation sequencing (NGS) technology, several studies have revealed gene fusions as candidate therapeutic targets for treating colon cancer, including fusion generation sequencing (NGS) technology, several studies have revealed CRC pathogenesis. Recently, due to the development of next therapeutic targets based on the molecular mechanisms underlying and it is thus necessary to develop prognostic biomarkers and novel therapeutic targets based on the molecular mechanisms underlying CRC pathogenesis. Thus, we validated IHC TrkA expression as a screening method for detecting NTRK1 fusions in 80 CRC tissue samples.

Materials and Methods

Commercially available split FISH probes were used for detecting the NTRK1 gene fusions in CRC tissues according to the manufacturer’s instructions (FS0024; Abnova, Taipei City, Taiwan). The study included a cohort of 80 patients with T3 or T4 CRC, who underwent resection of the primary tumor at Pusan National University Hospital (PNUH) between January and May 2015. The group consisted of 44 men and 36 women, with a mean age of 67.8 years (range, 31–87 years). Standard FFPE sections were obtained from the Department of Pathology and the National Biobank of Korea, Pusan National University Hospital. All samples from the National Biobank of Korea were procured after obtaining informed consent of the patients and were subjected to institutional review board-approved protocols. Briefly, TrkA expression was confirmed in deparaffinized, protease-treated FFPE tissue sections using an anti-TrkA C-terminal monoclonal antibody (TA806413; OriGene, Rockville, MD, U.S.). Brain ganglia and lymphocytes served as positive and negative controls, respectively. TrkA immunostaining was observed to be both membranous and cytoplasmic (Figure 1) and was semiquantitatively scored (staining intensity: negative (0), weak (1), moderate (2), strong (3); staining percentage: 0%-5% (0), 5%-25% (1), 25%-50% (2), ≥50% (3)). For interpretation of TrkA cytoplasmic and membranous staining, each pathologist (SJL, AK, and DYP) first separately scored and in cases with differing results, samples were reviewed again using a multi-viewer microscope. Simultaneously, deparaffinized and protease-treated FFPE tissue sections were denatured at 75 °C and incubated overnight with 5′-end TexRed-labeled and 3′-end FITC-labeled probes for NTRK1. After washing and DAPI counterstaining (32–804,831; Abbott, Chicago, IL, U.S.), the number and localization of hybridization signals were assessed. Tumors were determined to be NTRK1 gene fusion positive when more than 20 out of 100 nuclei demonstrated break-apart 5′- and 3′-end signals (Figure 2). The relationship between the NTRK1 FISH-positive and TrkA IHC-positive results was assessed using the Spearman’s rank correlation coefficient. The ability of TrkA IHC to predict the detection of NTRK1 gene fusions by FISH was assessed using ROC curve analysis. Clinicopathological features were analyzed for the NTRK1 gene fusions using the Student’s t-test, χ² test, or Fisher’s exact test. The results were considered to be statistically significant at P < .05. Statistical analysis was performed using SPSS version 10.0 for Windows (SPSS Inc., Chicago, IL, USA).

Figure 1. IHC staining patterns of TrkA in CRC tissue samples. (A) Cytoplasmic, (B) membranous, and (C) mixed cytoplasmic and membranous staining patterns are shown.
Results

We detected the expression of NTRK1 gene fusions and TrkA and determined the relationship between FISH and IHC results for NTRK1 gene fusion analysis. We simultaneously performed FISH using split FISH probes and IHC using an anti-TrkA C-terminal monoclonal antibody using FFPE sections obtained from 80 CRC patients. The IHC analysis of TrkA in CRC tissue samples revealed two staining patterns (cytoplasmic and membranous). About 80% of the CRC samples (64/80) showed both cytoplasmic and membranous staining. Additionally, heterogeneous cytoplasmic or membranous staining in the CRC tissue sections was observed. Figure 3 shows heterogeneous cytoplasmic staining of adenocarcinoma tissue, which coincides with FISH-positive NTRK1 gene fusion sites and increased cytoplasmic staining of TrkA.

For the IHC analysis using an anti-TrkA C-terminal monoclonal antibody, we independently evaluated and scored cytoplasmic and membranous TrkA expression. For the intensity scoring of cytoplasmic TrkA expression, negative (0), weak (1), moderate (2), and strong (3) cytoplasmic TrkA expression was noted in 20.0% (16/80), 26.3% (21/80), 38.8% (31/80), and 15.0% (12/80) of samples, respectively. With regard to the proportional scoring of cytoplasmic TrkA expression, 0%-5% (0), 5%-25% (1), 25%-50% (2), and ≥50% (3) cytoplasmic TrkA expression was identified in 21.3% (17/80), 21.3% (17/80), 36.3% (29/80), and 21.3% (17/80) of the samples, respectively. For the intensity scoring of membranous TrkA expression, negative (0), weak (1), moderate (2), and strong (3) membranous TrkA expression was noted in 6.3% (5/80), 40.0% (32/80), 50.0% (40/80), and 3.8% (3/80) of samples, respectively. With regard to the proportional scoring of membranous TrkA expression, 0%-5% (0), 5%-25% (1), 25%-50% (2), and ≥50% (3) membranous TrkA expression was identified in 6.3% (5/80), 11.3% (9/80), 38.8% (31/80), and 43.8% (35/80) of samples, respectively (Figure 3). The NTRK1 gene fusion analysis using FISH indicated NTRK1 gene fusions were present in 7.5% (6/80) of the samples. Comparing the results of the two assays for each sample, we observed heterogeneous TrkA immunostaining was associated with either strong cytoplasmic TrkA expression and frequent NTRK1 rearrangements or weak to moderate cytoplasmic TrkA expression and less frequent NTRK1 rearrangements (Figure 4).

A significant correlation between the FISH and IHC results for NTRK1 gene fusion analysis was seen. We observed cytoplasmic TrkA expression with the area under the receiver operating characteristic (ROC) curve as 0.926 (0.864-0.987, 95% CI, P = .001) (Figure 5). By choosing 4.5 (the sum of the cytoplasmic TrkA expression scores) as the cut-off value for the NTRK1 gene fusion positive and negative groups, the sensitivity and specificity of predictions of lymph node metastasis were 100 and 83.8%, respectively (P = .001). Specifically, high (or strong) cytoplasmic TrkA expression (sum of the intensity and proportion score ≥ 4) was associated with the detection of NTRK1 gene fusions (P < .0001, r = 0.528) (Table 1). In order to assess the characteristics of the NTRK1 gene fusion-positive CRC samples, we analyzed various clinicopathological features in all 80 CRC patients. We observed that the NTRK1 gene fusion-positive tumors were associated with younger patients (P = .051). Other clinicopathological features including sex, tumor location, histological differentiation, perineural invasion, invasion depth, lymphovascular invasion, lymph node metastasis, and microsatellite instability status were not associated with the detection of NTRK1 gene fusions (Table 2).

Discussion

With the recent development of multi-omics data analysis tools and sequencing methods, several studies have revealed novel therapeutic targets such as gene fusions (e.g., the NTRK1 and ROS1 gene fusion in CRC) [5,6]. Among these, the NTRK1 gene fusions, such as TPM3-NTRK1 and LMNA-NTRK1, have been reported in CRC patients [7-10]. NTRK1 gene fusions are therapeutic targets for multitkine inhibitors, such TrkA inhibitors. NTRK1 encodes the TrkA receptor, which is a member of the Trk family of RTKs. Its activation induces the PI3K/AKT, Ras/MAPK, and PLC-gamma signaling pathways [11,12]. The dysregulation of the kinase activity of Trk is associated with carcinogenesis.

We identified NTRK1 gene fusions in 7.5% (6/80) of CRC samples using FISH. This incidence rate is higher than that reported by previous studies [2.7% (2/74, using NGS), 1.7% (1/66, using qRT-PCR), 2% (3/147, using RNA sequencing), and 0.5% (2/408, using IHC and qRT-PCR)] [7-10]. We believe that these discrepancies can be partly attributed to the differences in detection methods or study cohorts. Unlike other studies, we simultaneously performed FISH and IHC, using split FISH probes using an anti-TrkA monoclonal antibody, in FFPE sections obtained from 80 CRC patients. Lee et al. performed IHC for TrkA and confirmed these results using PCR and direct DNA sequencing [7]. Park et al. initially performed RNA-sequencing analysis and then confirmed NTRK1 gene fusions using FISH [10]. Créancier et al. used IHC and qRT-PCR to detect the high expression level of mRNA coding for the intracellular domains of NTRK1 and confirmed these results using PCR and direct DNA sequencing [7]. Park et al. initially performed RNA-sequencing analysis and then confirmed NTRK1 gene fusions using FISH [10]. Créancier et al. used IHC and qRT-PCR to detect NTRK1 gene fusions in CRC samples [8]. However, these methods could potentially yield false-negative and false-positive results. Although testing of fusion genes was based on FISH in many laboratories, FISH and other molecular methods have various limitations (cost, turn-around time, and FFPE specimen quality problems). Conklin et al. reported that IHC was a reliable method to detect ALK rearrangements identified by FISH in non-small cell lung cancer [14].

Based on these data and findings, we performed a correlation analysis between...
Figure 3. Cytoplasmic TrkA immunostaining showing (A) negative, (B) weak, (C) moderate, and (D) strong staining intensities. Membranous TrkA immunostaining showing (E) negative, (F) weak, (G) moderate, and (H) strong staining intensities.
Figure 4. Heterogeneous TrkA immunostaining in the CRC tissues analyzed using FISH. (A & B) Strong cytoplasmic TrkA expression in adenocarcinoma tissues showing frequent NTRK1 rearrangements. (C & D) Weak to moderate cytoplasmic TrkA expression in adenocarcinoma tissues revealing less frequent NTRK1 rearrangements. Split red and green signals (yellow arrows) indicate NTRK1 fusion genes. The scale bar indicates 20 μm.

Figure 5. ROC curve analysis for TrkA immunostaining to predict NTRK1 gene fusions using FISH. Cytoplasmic TrkA expression, yielding an area under the ROC curve of 0.926 (range: 0.864–0.987, 95% CI, $P = .001$) is shown.
the results of IHC for TrkA and FISH and analyzed the ability of this screening method to detect NTRK1 gene fusions in CRC samples, similar to the use of ALK IHC for detecting ALK fusion genes in non-small cell lung cancer.

In this study, we used an anti-TrkA monoclonal antibody that recognizes the C-terminal intracellular domain of TrkA as readout for NTRK1 rearrangements in CRC. However, there have been no previous studies on using the intensity and proportion of cytoplasmic TrkA staining as a predictive marker for NTRK1 gene fusions. Ardini et al. and Créancier et al. reported only strong cytoplasmic TrkA staining [7,8]. Lee et al. and Park et al. also reported only cytoplasmic TrkA staining [9,10]. Furthermore, the positive rate for cytoplasmic TrkA staining differed between the different studies (0.5–5.1%) [7–10]. Our data revealed a higher frequency of strong cytoplasmic TrkA staining rate (15%, 12/80) compared to the previous studies. We believe that these discrepancies may be partly attributed to the differences in the types of primary antibodies or specimens used. Two studies used a rabbit anti-TrkA monoclonal antibody (ab76291; Abcam, Cambridge, U.K.) [7,8]. Lee et al. used an anti-pan-Trk rabbit antibody (C17F1; Cell Signaling Technology). Park et al. and the present study used an anti-TrkA C-terminal monoclonal antibody (TA806413; OriGene, Rockville, MD, U.S.). Additionally, three of the studies [7,8,10] used tissue microarray technology, and one study used tissue blocks for performing TrkA IHC. As shown above, TrkA expression was observed to be heterogeneous. This may have been partly responsible for the high percentage of strong cytoplasmic TrkA expression observed in our study. Furthermore, for practical purposes, a false negative TrkA IHC results will be of concern due to the heterogeneous expression TrkA expression observed in our study.

To overcome this issue, we needed to standardize the TrkA IHC method (primary antibodies, dilution and staining platform of immunohistochemistry) and size of specimen (size of tissue). Hechtman et al. reported that specific staining patterns for Trk expression were related with the subcellular localization of the fusion partners [13]. For example, in case of the NTRKI-LMNA fusion, immunostaining was observed in the nuclear membrane; however, in case of the NTRKI-TPM fusion, mixed cytoplasmic and membranous staining was observed [13]. In the present study, all NTRK1 gene fusion-positive CRC samples displayed mixed cytoplasmic and membranous staining patterns for TrkA. Despite the limitations of our present study (small sample size procured from a single institution), we identified that strong cytoplasmic TrkA expression was associated with the presence of NTRK1 gene fusions. Additionally, IHC for TrkA can be used as an effective screening method for detecting NTRK1 gene fusions in CRC. Further multi-institutional studies encompassing larger cohorts, with other IHC methods and primary antibodies, are needed to confirm our present findings.

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DYP and CHK conceived and designed this study. The experiments were performed by CHK, YC, and YJW. Histopathological slides analysis was carried out by DYP, SJL, AK, YK, and WYP. Clinical data was analyzed by HJJ and GAS. The manuscript was written by YC and edited by DYP and CHK. DYP supervised the research and obtained funding.

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