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Original Article

A Two-Step Diagnostic Approach for *NTRK* Gene Fusion Detection in Biliary Tract and Pancreatic Adenocarcinomas

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Abstract

Background: It is of interest to determine the incidence and molecular characteristics of *NTRK* gene fusions in patients with bilio-pancreatic cancers, because of possible treatment with TRK inhibitors for advanced tumors. The aim of the present study was to apply the guidelines for NTRK testing algorithm to a series of patients with bilio-pancreatic cancers.

Methods: Immunohistochemistry screening was applied on formalin-fixed paraffin-embedded archival blocks from surgical resections, biopsies, or cytological samples of biliary tract and pancreatic adenocarcinomas. The presence of at least a weak staining in rare tumor cells led to testing by 2 RNA-based NGS panels.

Results: For biliary tract tumors, 153 samples have been selected. A total of 140 samples were suitable to perform IHC, and 17 samples were IHC positive. RNA NGS testing of the 17 IHC-positive samples revealed a single *NTRK*3 gene fusion (*ETV6*(4)-*NTRK3*(14)) that was detected by both NGS panels. In this perihilar cholangiocarcinoma, IHC performed on a biopsy showed a weak focal cytoplasmic and nuclear staining. No other *NTRK* fusion was detected on the 16 other samples with both panels. Overall in the patients screened by IHC and confirmed by NGS, the percentage of *NTRK* fusions was 0.7%. For pancreatic cancers, 319 samples have been selected and 297 were suitable to perform IHC. Nineteen samples were IHC positive. No fusion was detected by NGS.

Conclusion: *NTRK* gene fusions are rare in bilio-pancreatic cancers but testing is of high interest due to possible treatment with specific TRK inhibitors.

Key words: *NTRK*; *NTRK* gene fusions; immunohistochemistry; NGS; RNA-based NGS panels; biliary tract and pancreatic adenocarcinomas.

Implications for Practice

The present study used the diagnostic approach proposed by different guidelines, that is, screening by immunohistochemistry and targeted RNA next-generation sequencing for immunohistochemical positive cases, to study the incidence of *NTRK* gene fusions in bilio-pancreatic cancers. This approach allowed the detection of one case with an *NTRK* gene fusion on a series of 437 bilio-pancreatic cancers, including cytologies, biopsies, and resections. Even if *NTRK* gene fusions are rare in bilio-pancreatic cancers, it is of high interest to detect them because of possible treatment for advanced tumors with specific TRK inhibitors that are FDA and EMA approved.

Introduction

The tropomyosin receptor kinase (TRK) receptor family comprises 3 transmembrane proteins referred to as TRK A, B, and C (TRKA, TRKB, and TRKC) receptors that are encoded by the *NTRK*1, *NTRK*2, and *NTRK*3 genes, respectively.[1,2] These tyrosine kinase receptors are expressed in human neuronal tissue and play an essential role in the physiology of development and function of the nervous system.[2] After embryogenesis, the expression of TRK proteins is limited to the nervous system, testis, and smooth muscle.[2]

Fusions involving one of the 3 *NTRK* genes have been identified in cancers of children and adults. Fusion of the C-terminal tyrosine kinase of a *NTRK* gene with an N-terminal fusion partner leads to transcription of chimeric TRK proteins with constitutively activated (ligand-independent phosphorylation) or overexpressed kinase function conferring oncogenic potential. There are multiple

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possible fusion partners and inconsistent breakpoints. *NTRK* gene fusions are frequent in some rare cancers, such as secretory carcinomas of the breast and of the salivary glands (ie, mammary analog secretory carcinoma of the salivary glands), congenital fibrosarcoma and cellular mesoblastic nephroma.[2-4] On the other hand, *NTRK* gene fusions have been described at low frequency (<5%) in more common solid tumors such as papillary thyroid cancer, glioma, non-small cell lung cancer, gastrointestinal stromal tumor (GIST), or colorectal cancer. In these cancers, *NTRK* gene fusion is described as mutually exclusive from other oncogenic driver alterations.[2,5,6]

NTRK gene fusions have only been reported in few series of bilio-pancreatic cancers. In the study reported by Ross et al. in 2014,[7] 1 patient out of 28 intra-hepatic cholangiocarcinomas tested had an *NTRK1* fusion (incidence 3.6%). More recently after the beginning of our trial, Allen et al.[8] reported 3 *NTRK* gene fusions on 400 patients with pancreatic adenocarcinoma (incidence of 0.8%), and the study of Solomon et al. reported 5 *NTRK* gene fusions on 1492 pancreatic adenocarcinomas (0.34%) and 2 *NTRK* gene fusions on 787 cholangiocarcinomas (0.25%).[9] Although the incidence of *NTRK* fusions in bilio-pancreatic adenocarcinomas is low, it is of high clinical interest for a possible treatment with TRK inhibitors.

NTRK gene fusions are possible therapeutic targets in cancer treatment,[10,11] using highly selective tyrosine kinase inhibitors (TKI) of the 3 TRK proteins (ie, larotrectinib or entrectinib). These inhibitors disclose anti-tumor activity regardless of tumor type and *NTRK* fusion type. *NTRK*-fusion-positive cancers can be treated by larotrectinib, as recently reported in the NEJM.[12] In this trial, anti-tumor activity was reported regardless the tumor type and the fusion type. Moreover, objective response rate was high, duration of response was long, and associated with a good safety profile. It is of interest therefore to determine incidence and molecular characteristics of *NTRK* gene fusions in patients with bilio-pancreatic cancers regarding possible treatment with specific TKI.

Different techniques are available to detect *NTRK* gene fusions such as immunohistochemistry (IHC), in situ hybridization, or next generation sequencing (NGS).

Given the high clinical impact of *NTRK* gene fusion detection but the low frequency of these alterations, algorithms of testing have been proposed taking into account cost, turnaround time, or expertise.[5] Different authors proposed a diagnostic algorithm for *NTRK* gene fusion identification in solid tumors.[2,5,13] The approach of these guidelines is to propose a screening by IHC for tumors with a low prevalence of *NTRK* gene fusion and for which molecular testing is not routinely performed. Indeed, different studies have demonstrated that IHC using a pan-TRK antibody is an effective approach for *NTRK* gene fusion screening. Immunohistochemistry showed overall sensitivity ranging from 75% to 96.7% and specificity ranging from 81.1% to 100%, with higher sensitivity for NTRK1 and NTRK2 fusions and lower sensitivity for NTRK3 fusions.[9,14-17] IHC-positive cases have to be tested by RNA NGS for *NTRK* gene fusion confirmation.

In this single-center retrospective study, the primary endpoint is to apply, on archival pathology material, the guidelines for NTRK testing algorithm (ie, screening by IHC and targeted RNA-based NGS for IHC positive cases) to a series of patients with biliary tract tumors (BTC—including intra-hepatic (IH), extra-hepatic (EH), perihilar (PH) cholangiocarcinomas, and gallbladder tumors (G)) and pancreatic adenocarcinomas to evaluate the percentage of *NTRK* gene fusions. Moreover, Kirchner et al. underlined in their in silico analysis that panels used for targeted RNA sequencing have different efficiency to detect gene fusion.[18] This is why in the present study, we evaluated IHC-positive cases with 2 different NGS panels, ie, the Oncomine Focus Assay (OFA) and the Archer FusionPlex Expanded Lung panel (AFPEL).

Material and Methods

Samples

This retrospective study has been approved by the local ethics committee of CUB Hôpital Erasme (Brussels, Belgium) on December 6, 2018.

After review of the pathology database (Biobanque du Laboratoire d'Anatomie Pathologique, reference B2009/002) and the clinical GI oncology database (using SNOMED codes) of the CUB Hôpital Erasme (Université Libre de Bruxelles, Brussels, Belgium), patients with histologically proven biliary tract tumor (including IH, EH, PH, and G tumors) or pancreatic adenocarcinoma, diagnosed between January 2010 and October 2019, were selected to create the specific database dedicated to the trial. Based on this database, formalin-fixed paraffin-embedded archival blocks from surgical resections, biopsies, or cytological samples (including fine needle aspiration, brushing, or ascites) were evaluated for the feasibility of the pathologic tests (IHC and NGS) (Table 1). In case of insufficient residual material, the sample was ineligible for the study.

IHC

IHC for TRKA, TRKB, and TRKC expression was performed with a pan-TRK monoclonal antibody (mAb) (clone EPR17341; ref. ab181560 (Abcam, Cambridge, UK)). The antibody is reactive to a homologous region of TRKA, TRKB, and TRKC near the C-terminus. Appendix was used as positive control. All assays were performed on a Dako Omnis automated stainer platform (Agilent Technologies Inc.). Prior to staining, paraffin-embedded tissue sections were subjected to deparaffinization and hydration followed by heat-induced epitope retrieval in Dako Target Retrieval Solution pH9 (Agilent Technologies, Inc., ref. GV804) 30 min at 97°C. Pan-TRK antibody was diluted 1/200 and incubated for 20 min at 32°C. Detection was performed with Dako Envision Flex detection system (Agilent Technologies, Inc., ref. GV800) according to the manufacturer's protocol. The sections were

Table 1. Pathology samples.

Location	Number
Biliary tract, *n* = 140	
Cytology	14
Biopsy	41
Surgical resection	85
Pancreas, *n* = 297	
Cytology	93
Biopsy	18
Surgical resection	186

counterstained with hematoxylin (Agilent Technologies, Inc., ref. GC808).

Staining intensity (negative, weak, moderate, or strong), pattern (diffuse, focal, or rare positive cells), and localization (cytoplasmic or nuclear) were evaluated.

NGS

RNA Extraction and Quantification

Nucleic acids were extracted from FFPE tumor samples, after macrodissection of the tumor area, using the Maxwell RSC Instrument (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions and as already detailed.[19,20] The H&E stained slide from the same block, previously reviewed by a pathologist who circled the tumor area and evaluated the tumor percentage, was used as a guide for the macrodissection. The percentage of tumor cells in the samples range from 10% to 50%. The RNA yield was quantified using a Qubit 2.0 Fluorometer (ThermoFisher Scientific, Waltham, MA, USA).

NGS Oncomine Focus Assay

For library construction, 10 ng of RNA were retro-transcribed using the SuperScript VILO (ThermoFisher Scientific, Waltham, MA, USA) in accordance with the manufacturer's instructions. The AmpliSeq Oncomine Focus RNA Assay (ThermoFisher Scientific, Waltham, MA, USA) was used to manually prepare the libraries. The RNA panel can identify known rearrangements in 23 genes: *ALK, RET, ROS1, NTRK1, NTRK2, NTRK3, FGFR1, FGFR2, FGFR3, MET, BRAF, RAF1, ERG, ETV1, ETV4, ETV5, ABL1, AKT3, AXL, EGFR, ERBB2, PDGFRA,* and *PPARG*. The panel consists of 5X primer pairs that target 271 fusion genes and 5 human expression controls (*TBP*, *MYC*, *HMBS*, *ITGB7,* and *LRP1*). Amplification condition was 98°C for 2 min for initial denaturation, followed by 30 cycles at 98°C for 15 s and 60°C for 4 min. Next, the amplicons were digested, barcoded, and purified using AMPure XP Beads (Beckman Coulter, Brea, CA, USA). The libraries were amplified by PCR according to the manufacturer's instructions and size selection was performed using AMPure XP Beads (Beckman Coulter, Brea, CA, USA). Libraries were quantified using the Qubit fluorometer and the Qubit dsDNA HS assay kit (ThermoFisher Scientific, Waltham, MA, USA). The Ion 510 & Ion 520 & Ion 530 Kit—Chef and the Ion Chef (ThermoFisher Scientific, Waltham, MA, USA) were used for template preparation and chip loading. Sequencing was performed using the S5 Gene Studio instrument (ThermoFisher Scientific, Waltham, MA, USA).

NGS data analysis was carried out using Ion Torrent Suite Browser version 5.12 and Ion Reporter version 5.12. The Torrent Suite Browser was used to perform initial quality control including chip loading density, median read length, and number of mapped reads. Fusion detection and sequence analysis were performed on the Ion Reporter using the Oncomine Focus—520—w2.5—Fusions—Single Sample workflow. Final reports included sequence read counts for all targets, number of total mapped fusion panel reads, expression of the 5 control genes, and 3′–5′ imbalance data. A sample was considered contributive when a minimum number of 20 000 total mapped reads were detected and a minimum of 3 out of the 5 control genes were expressed. A minimum of 20 reads must be allocated to a fusion target to be considered positive. This type of NGS analysis for fusion detection is already used in routine in the Department of Pathology and is accredited to ISO15189.

NGS Archer FusionPlex Expanded Lung

The Archer FusionPlex Expanded Lung panel (ArcherDX, Invitae, San Francisco, CA) is designed to identify mutations and known and unknown fusions in 17 genes: *ALK, BRAF, EGFR, ERBB2, FGFR1, FGFR2, FGFR3, KRAS, MET, NRG1, NTRK1, NTRK2, NTRK3, NUTM1, PIK3CA, RET,* and *ROS1*. For library construction, 250 ng of RNA was used in accordance with the manufacturer's instructions. Purified libraries were quantified using the KAPA Universal Library Quantification kit (Roche, Basel, Switzerland) and pooled to equimolar concentrations. The Ion 510 & Ion 520 & Ion 530 Kit—Chef and the Ion Chef (ThermoFisher Scientific, Waltham, MA, USA) were used for template preparation and chip loading. Sequencing was performed using the S5 Gene Studio instrument (ThermoFisher Scientific, Waltham, MA, USA). Results were analyzed using the Archer Suite Analysis software version 6.2.7 (ArcherDx, Invitae, San Francisco, CA). A sample was considered contributory when the following quality control criteria were obtained: Presequencing Cp value <28.5, a minimum of 500 000 reads (total fragments) and a minimum of 95% of reads (unique fragments) on target. A fusion was considered as present when a minimum of 3 single start sites were obtained, a minimum of 10 reads supported the fusion and the fusion was in frame.

Results

Biliary Tract Cancers

For BTC, 153 archival tumors samples have been selected including 140 samples suitable to perform IHC (Table 1). Of these 140 samples, 17 samples were IHC positive including 11 IH, 2 PH, 1 EH, and 3 G tumor samples. Intensity of staining was weak in 16 samples and moderate in one. Staining location was cytoplasmic in 14/17 samples, nuclear in 2/17 samples, and nuclear + cytoplasmic in one sample. Pattern of staining was rare positive cells (2/17), focal (4/17), and diffuse (11/17).

The presence of at least weak staining in rare tumor cells led to testing the sample by NGS.

OFA NGS testing of the 17 IHC-positive samples revealed a single *NTRK*3 gene fusion (*ETV6*(4)-*NTRK3*(14)) (Fig. 1). In this biopsy of a poorly differentiated PH tumor (71 years female patient), pan-TRK IHC had a weak focal cytoplasmic and nuclear staining (Fig.2). Immunohistochemistry for MLH1, MSH2, MSH6, and PMS2 did not show loss of nuclear expression of the MMR proteins.

Archer testing confirmed the single *NTRK*3 gene fusion (*ETV6(4)-NTRK3(14)*) for the same patient and did not reveal other *NTRK* fusion in the other 16 IHC-positive samples. However, an *FGFR2* gene fusion, not targeted by the OFA panel was detected using the AFPEL panel in an intra-hepatic cholangiocarcinoma. Moreover, an *FGFR3(17)-TACC3(8)* gene fusion was detected by both panels in a G adenocarcinoma.

Overall in the patients screened by IHC and confirmed by NGS in BTC, the percentage of *NTRK* fusions was 0.7%.

Pancreatic Adenocarcinomas

For pancreatic adenocarcinomas, 319 archival tumor samples have been selected including 297 samples suitable to

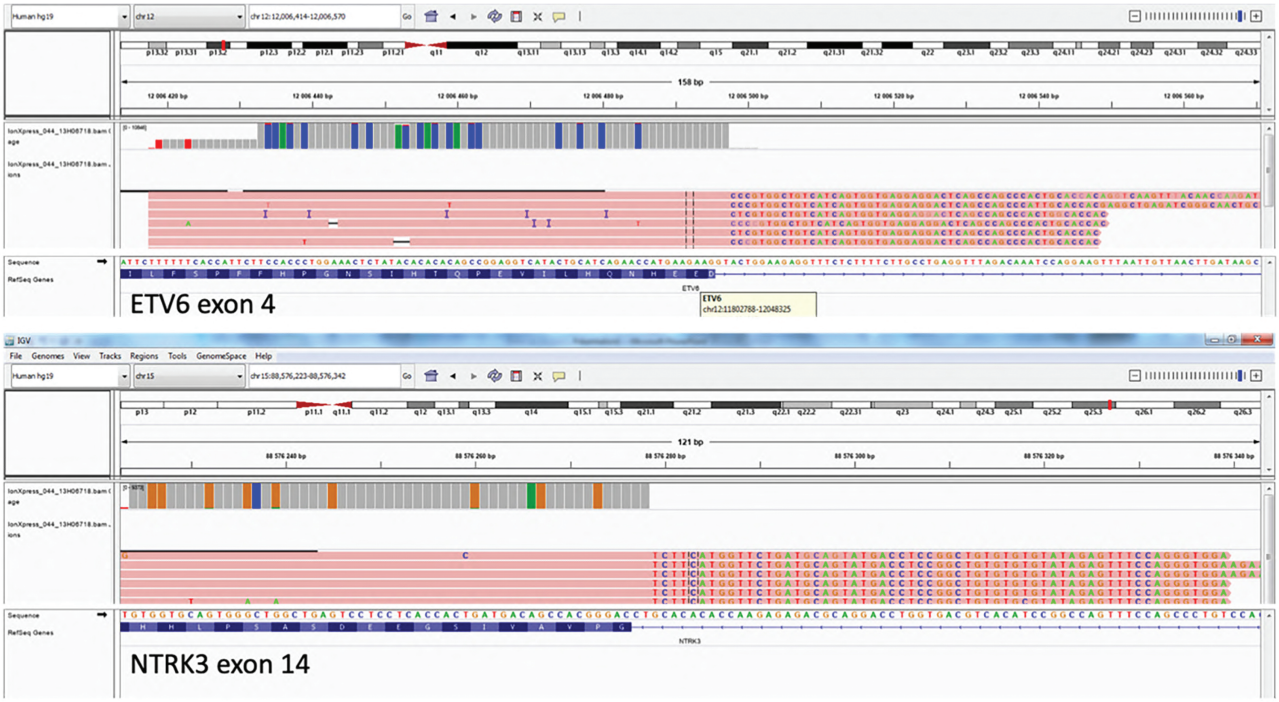

Figure 1. Integrated genomics viewer (IGV) shows a structural variant involving *ETV6* Exon 4 (top panel) and *NTRK3* exon 14 (bottom panel).

perform IHC. Of these 297 samples, 19 were IHC-positive. Intensity of staining was weak in 18 samples and moderate in one. Staining location was cytoplasmic in 18/19 patients and nuclear in one patient. Pattern of staining was focal in 2 cases and diffuse in 17 cases.

No *NTRK* gene fusion or other gene fusion was detected by either Oncomine NGS or Archer testing (one case was fusion negative with the OFA panel and non-informative with the AFPEL panel).

Discussion

NTRK gene fusions are rare in solid tumors and observed in around 1% of the cases, except for tumors such as infantile fibrosarcoma and secretory breast tumors. *NTRK* gene fusions have been reported in few series of bilio-pancreatic cancers.[7-9] In this larger retrospective study, we have confirmed this rare incidence, <1%, in bilio-pancreatic adenocarcinomas in a retrospective trial using a two-step diagnosis method, as proposed by different guidelines.[2,5,13] This two-step diagnosis method begins with immunohistochemistry screening followed by NGS analysis for IHC-positive cases. Taking into account the rare incidence but the high therapeutic impact of *NTRK* gene fusions, it is of the utmost importance to have an efficient and cost-effective algorithm for testing. IHC is a cheap and an easily performed test with a short turnaround time. Restriction of RNA-NGS testing to IHC-positive samples only spares both time and money. Moreover, RNA-based NGS panels are not accessible in every hospital. In the present study, we observed 36 IHC-positive cases, with only one *NTRK* gene fusion detected. The threshold to consider molecular testing was low since weak cytoplasmic staining in rare tumor cells led to NGS testing. By acquiring more experience in using this IHC, the threshold of positivity leading to molecular testing could be refined.

Kirchner et al. underlined in their in silico analysis that panels used for targeted RNA sequencing have different efficiency to detect gene fusion.[18] In their study, Kirchner et al., showed that more variants are covered with the Archer panels in comparison to the OFA. To evaluate discrepancies, we tested IHC-positive samples with 2 panels (AFPEL and OFA). In our trial, Archer confirmed the only *NTRK3* fusion already detected by the Oncomine assay. No other *NTRK* gene fusion was detected on the 35 other IHC-positive cases tested. The advantage of OFA is that the quantity of RNA input is lower (10 ng vs. 250 ng for Archer), this should be taken into account as for some patients, the only available material is cytology or small biopsies.

The advantage to use NGS panels over other techniques such as RT-PCR and ISH is the simultaneous evaluation of other gene fusions. In the present study, NGS panels allowed us to detect an *FGFR3* gene fusion in a gallbladder adenocarcinoma (detected with both panels) and an *FGFR2* fusion in an intra-hepatic cholangiocarcinoma using the Archer Panel (the fusion was not targeted by the OFA panel).

Notwithstanding the low incidence of *NTRK* gene fusion in bilio-pancreatic cancers, screening by IHC seems important to perform in advanced tumors because treatments by specific TRK inhibitors, larotrectinib and entrectinib, are now available. Both are FDA and EMA approved. Moreover, anti-tumor activity was reported regardless of tumor type and of the fusion type, the objective response rate was high, duration of response was long, and associated with a good safety profile. In the study of Drilon, 18 advanced previously treated GI tumors (on a total of 55 patients included in the trial), including 3 patients with cholangiocarcinoma and 2 patients with pancreatic adenocarcinoma, have been included and treated with larotrectinib.[12] The updated results of the GI cohort have been presented at ESMO-GI 2021.[21] After a median follow-up of 20.3 months, median PFS of

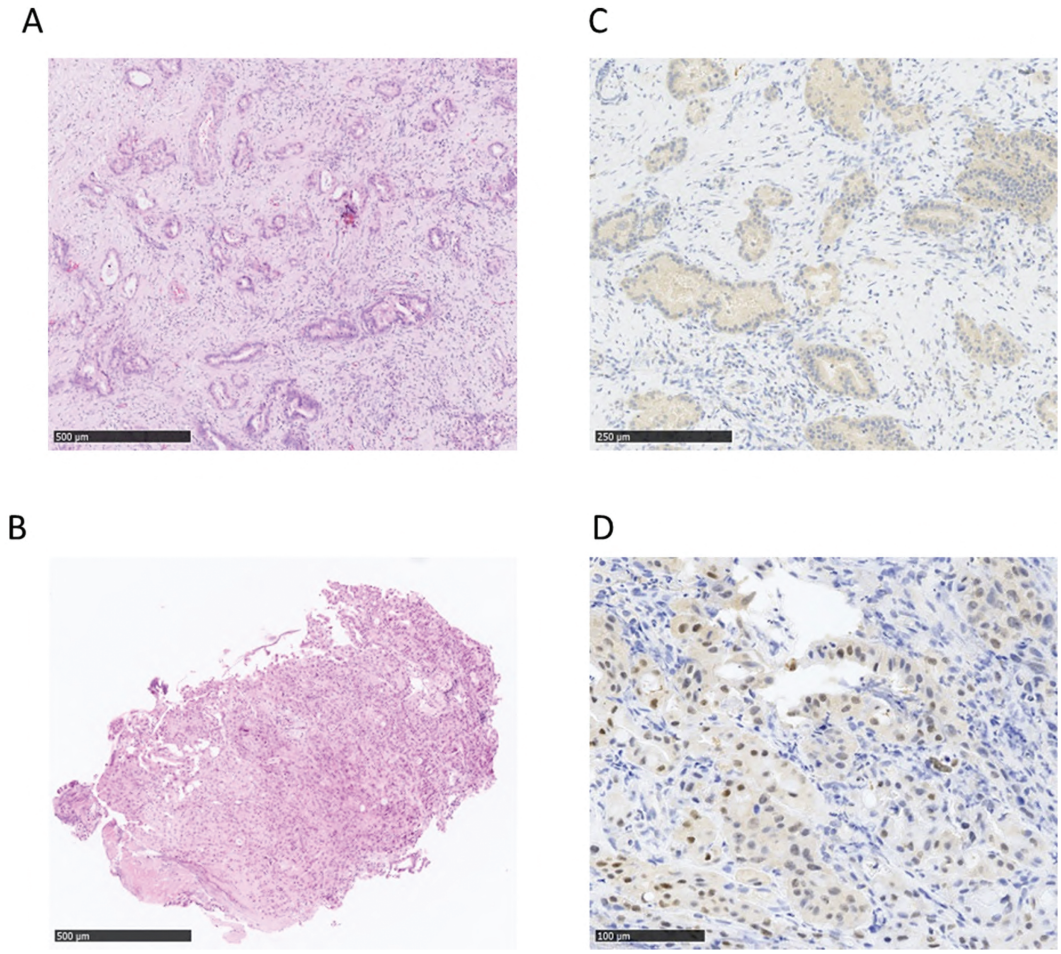

Figure 2. H&E (**A**, **B**—magnification ×5) and Pan-TRK staining patterns (**C**, **D**). C: Diffuse and weak cytoplasmic staining (magnification ×10). No *NTRK* fusion was detected by NGS. D: Focal and weak nuclear and cytoplasmic staining (magnification ×20). Fusion *ETV6(4)-NTRK3(14)* was detected by NGS.

the GI cohort (95% CI) was 5.4 months (2.2–11.6) which is good for heavily pre-treated patients (72% of the patients had received more than 2 lines). Two patients (one cholangiocarcinoma patient and one pancreatic cancer patient) experienced a partial response. The favorable safety profile of larotrectinib was also confirmed in the GI cohort. Four bilio-pancreatic cancer patients were also included and treated with entrectinib in 3 phase 1–2 trials (ALKA-372-001, STARTRK-1, and STARTRK-2) reported in a same publication by Doebele.[22]

In clinical practice, screening of *NTRK* fusions in bilio-pancreatic tumors can be done easily and is cheap when using IHC on archival material as a first screening test. Only positive IHC samples have then to be confirmed by an RNA-based NGS. Specific TKI treatment can be offered to positive advanced tumor patients.

Conclusions

NTRK gene fusions are rare in bilio-pancreatic cancers, incidence is less than 1% as in other solid tumors. Our results support the use of RNA-based NGS to confirm positive IHC results during diagnostic screening.

Even with the low incidence of *NTRK* gene fusion in this GI cancers population, testing is of high interest due to the possible treatment of advanced tumors with specific TRK inhibitors with good efficacy and safety profiles.

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Conflict of Interest

Anne Demols received fees for expert testimony, advisory boards, IDMC, or presentations from Bayer Healthcare, Servier, Incyte, Merck, Ipsen, Basilea, Vifor, Roche, and Teva, and received research grants/funding from Bayer Healthcare. Nicky D'Haene received fees for expert testimony, advisory board, or presentations from Pfizer, AstraZeneca, Biocartis, Merck, Lilly, Roche, Bayer, BMS, and Boerhinger. The other authors indicated no financial relationships.

Author Contributions

Conception/design: A.D., I.S., N.D. Provision of study material or patients: A.D., J.C., V.L. Collection and/or assembly of data: A.D., M.D., N.D.N., C.V.C., S.D.C., N.D. Data analysis and interpretation: A.D., L.R., L.P.C., M.C., N.D.N., A.R., C.V.C., S.D.C., C.M., N.D. Manuscript writing: A.D., C.V.C., S.D.C., N.D. Final approval of manuscript: All authors.

Data Availability

The data underlying this article will be shared on reasonable request to the corresponding author.

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