Response and Mechanisms of Resistance to Larotrectinib and Selitrectinib in Metastatic Undifferentiated Sarcoma Harboring Oncogenic Fusion of NTRK1

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INTRODUCTION

Oncogenic translocations involving the neurotrophic receptor tyrosine kinase genes (NTRK1, NTRK2, and NTRK3), which encode the 3 tropomyosin receptor kinases (TRKs; TRKA, TRKB, and TRKC), produce fusions linking the NTRK kinase domain to the transcriptional regulatory elements and upstream coding regions of a variety of genes. These fusions lead to aberrant TRK kinase activity, driving oncogenesis.1 TRK fusions can be targeted with TRK inhibitors (TRKis), including larotrectinib2 and entrectinib,3 which are well tolerated and effective in approximately 75% of patients with NTRK-translocated tumors, often producing durable responses.

Acquired resistance to first-generation TRKis arises from secondary mutations within the ATP binding pocket of the kinase domain; these include solvent-front substitutions, gatekeeper mutations, and xDFG-motif substitutions in the activation loop.4,5 Second-generation TRKis such as repotrectinib and selitrectinib,6,7 which are well tolerated and effective in approximately 75% of patients with NTRK-translocated tumors, often producing durable responses.

In a preliminary report, patients with tumors bearing solvent-front substitutions had a response rate of 50% to second-generation TRKis.8 Mechanisms of resistance to second-generation TRKis are not well described.

CASE REPORT

A 47-year-old woman presented to an outside hospital with abdominal pain and bloody diarrhea. A rectal mass was identified and resected (Fig 1A; surgery 1 [S1]). A diagnosis of GI stromal tumor was considered, but immunohistochemistry was negative for c-KIT and DOG-1. At the Dana-Farber/Brigham and Women’s Cancer Center (DF/BWCC), a diagnosis of undifferentiated sarcoma, not otherwise specified, was made. Five months later, the patient developed symptomatic locoregional recurrence with liver and lung metastases, which was resected (S2). A TPM3-NTRK1 fusion was identified using 2 different next-generation sequencing (NGS) panels (Table 1) and retrospectively identified in S1. The patient was enrolled on a phase II trial (ClinicalTrials.gov identifier: NCT02576431) of larotrectinib (100 mg twice a day), with an initial objective partial response (Figs 1B and 1C). After 6 months on study, restaging scans identified an isolated area of progression in the right hepatic lobe, which was resected (S3), followed by resumption of larotrectinib. NGS from S3 identified an NTRK1 G595R solvent-front mutation. Three months later, diffuse disease was noted on restaging scans (Fig 1D). An expanded-access, single-patient protocol was initiated using selitrectinib (100 mg twice a day) with dose escalated at cycle 2 to 150 mg twice a day as a result of low plasma drug levels. A partial response was achieved at 3 months, with dramatic reduction in fluorodeoxyglucose uptake within the tumor (Fig 1E). After 5 months, isolated progression of a perihilar mass was identified and resected (S4). When a second site of progression in the sacrum was identified 1 month later, selitrectinib was increased to 200 mg twice a day with an associated increase in plasma drug levels (Fig 1F). The progressing tumor continued to grow slowly and was resected 3 months later (S5). Selitrectinib was resumed postoperatively, and the patient has remained free of disease progression for > 1 year.

MATERIALS AND METHODS

Informed Consent

Patients provided informed consent for these institutional review board–approved studies and correlative analyses. We obtained all permissions required by law and the DF/BWCC for publication.

Tumor Sequencing and Analysis

Targeted NGS was performed using the DF/BWCC OncoPanel® or FoundationOne Heme (Foundation Medicine, Cambridge, MA) to assess > 400 cancer-associated genes and select translocations. RNA-seq using single-end 75–base pair reads was performed as previously described.10 Fastq files were aligned to
hg19 using STAR,\textsuperscript{11} and expression was quantified using Cufflinks.\textsuperscript{12} Gene set enrichment analysis (GSEA)\textsuperscript{13} was performed using the Hallmark, KEGG, or Reactome databases. Data are publicly available (GSE132439). GUARDANT360 (Guardant Health, Redwood City, CA) circulating tumor DNA (ctDNA) sequencing was also performed.

**Multiplexed Immunofluorescence**

Tissue-based cyclic immunofluorescence (CyCIF) was performed on formalin-fixed paraffin-embedded specimens, as previously described,\textsuperscript{14} using qualified antibodies\textsuperscript{15} listed in Appendix Table A1 and uploaded to cycif.org.

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**FIG 1.** Treatment timeline and assessments. (A) Timeline of diagnosis and therapeutic interventions. Surgeries are numbered sequentially, and boxed lettering indicates the time of computed tomography (CT) and positron emission tomography (PET)-CT imaging. (B–E) Contrast-enhanced CT scans (top panels) and PET-CT images (bottom panels) from patient staging scans as indicated on the timeline. (F) Plasma levels over time of selitrectinib at the indicated dose levels. Data from each dosing point are derived from cycle 1, day 1 pharmacokinetic studies. Dashed lines indicating the 90% inhibitory concentration (IC\textsubscript{90}) of wild-type (WT) and G595R-mutant \textit{TRKA} are shown. BID, twice a day; ctDNA, circulating tumor DNA; POD, progression of disease; S, surgery.
| Surgery No. | Procedure | Pathologic Interpretation | Systemic Treatment | NGS Platform | Results |
|------------|-----------|--------------------------|--------------------|--------------|---------|
| S1         | LAR of primary rectal tumor, total abdominal hysterectomy with bilateral salpingectomy (TAH+BS) | Original diagnosis: GIST with predominantly epithelioid and focal spindle cell features; Revised diagnosis: Malignant epithelioid and spindle cell neoplasm: unclassified sarcoma | None | FoundationOne Heme (Foundation Medicine, Cambridge, MA) | TPM3-NTRK1 fusion, PTEN truncation; TMB = 2.94 |
| S2         | Resection of symptomatic pelvic recurrence | Malignant epithelioid and spindle cell neoplasm: unclassified sarcoma with myxoid features | None | Paradigm (Paradigm Diagnostics, Phoenix, AZ) | TPM3-NTRK1 fusion |
|            |           |                          |                    | OncoPanel (Dana-Farber Cancer Institute and Brigham and Women’s Hospital, Boston, MA) | NTRK1 fusion, PTEN-STAT3 fusion; VUS: EP300, PBRM1, RB1, ZNF217 |
| S3         | Resection of progressive right liver mass | Metastatic epithelioid and spindle cell sarcoma with myxoid features | Larotrectinib 100 mg twice a day | FoundationOne Heme | TPM3-NTRK1 fusion, NTRK1 G595R, PTEN truncation; VUS: NCOR2 fs; TMB = 6 |
|            |           |                          |                    | OncoPanel | TPM3-NTRK1 fusion, NTRK1 G595R, PTEN-STAT3 fusion; VUS: EP300, PBRM1, RB1, ZNF217 |
| S4         | Resection of progressive perihepatic mass | Metastatic epithelioid and spindle cell sarcoma; multiple areas with necrosis and hyalinization; the growing nodule had < 5% treatment effect | Selitrectinib 150 mg twice a day | FoundationOne Heme | TPM3-NTRK1 fusion, NTRK1 G595R, PTEN truncation; VUS: PTCH1 fs; TMB = 4 |
|            |           |                          |                    | OncoPanel | TPM3-NTRK1 fusion, NTRK1 G595R, PTEN-STAT3 fusion; VUS: PTCH1 fs, EP300, PBRM1, RB1, ZNF217; TMB = 4.5 |
| S5         | Resection of progressive presacral mass | Metastatic epithelioid and spindle cell sarcoma; areas of moderate to complete necrosis and hyalinization | Selitrectinib 200 mg twice a day | FoundationOne Heme | TPM3-NTRK1 fusion, NTRK1 G595R, PTEN truncation, KRAS G12V; VUS: PTCH1 fs; TMB = 5 |
|            |           |                          |                    | Guardant (Guardant Health, Redwood City, CA) | NTRK1 fusion, NTRK1 G595R, KRAS G12V; ctDNA undetectable |

Abbreviations: ctDNA, circulating tumor DNA; GIST, GI stromal tumor; LAR, low anterior resection; NGS, next-generation sequencing; TAH+BS, total abdominal hysterectomy with bilateral salpingectomy; TMB, tumor mutational burden in mutations per megabase; VUS, variant of uncertain significance.
Patient-Derived Models

Tumor was implanted subcutaneously into female nude mice (NU/NU; Charles River Laboratories, Wilmington, MA) to produce patient-derived xenografts (PDXs) following protocols approved by the institution’s Institutional Animal Care and Use Committee. Primary cultures were established by generating a tumor suspension and passing adherent cells.

RESULTS

To explore resistance mechanisms to second-generation TRKis, we performed targeted NGS of inhibitor-sensitive and -resistant tumors. Compared with S3, which was sensitive to selitrectinib, S4 harbored a PTCH1 frameshift mutation (Table 1). PTCH1 normally functions as a tumor suppressor,16 and its inactivation promotes Hedgehog signaling. In addition to the PTCH1 mutation, S5 also harbored a KRAS G12V mutation and variants of unknown significance (Table 1). ctDNA sequencing after S5 failed to detect NTRK fusions or known tumor mutations. All sequenced tumors exhibited a likely inactivating PTEN rearrangement.

To characterize potential transcriptional mechanisms underlying selitrectinib resistance, we analyzed S3 and S5 by RNA-seq. A tumor from a separate patient with an ETV6-NTRK3 translocated sarcoma was also analyzed. Compared with the ETV6-NTRK3 tumor, all TPM3-NTRK1 tumors exhibited exclusive expression of NTRK1 exons associated with the oncogenic fusion (Fig 2A). Similar findings were observed in a cell line and PDX generated from S3 and S5 (Appendix Fig A1A). Although all samples expressed TPM3 and ETV6, only the ETV6-NTRK3 tumor expressed detectable NTRK3 transcript (Fig 2B). The S5 tumor treated with selitrectinib expressed lower levels of the TPM3-NTRK1 fusion transcript (Fig 2B). Using GSEA to explore pathways associated with selitrectinib resistance, the S5 tumor exhibited enrichment in KRAS-related signaling as compared with the S3 tumor (Fig 2C), consistent with oncogenic activation of KRAS signaling. An inflammatory response signature was similarly enriched in S5 compared with S3 (Fig 2D), and these gene sets showed similar enrichment in PDXs (Appendix Figs A1B and A1C). Through GSEA comparisons of multiple databases, S5 showed recurrent enrichment of immune- and inflammatory-related signatures as compared with S3 (Fig 2E). To further characterize the inflammatory infiltrate, we performed CIBERSORT analysis17: this showed that M1 macrophages and CD8 T-cell subsets were enriched in the S5 tumor (Fig 2F). Consistent with this analysis, levels for several markers of T cells (CD8A and CD3), T-cell activation (CD48), macrophages (CD68), and several modulators of the immune microenvironment16 were higher in S5 (Fig 2G). Despite the loss-of-function mutation in PTCH1, we found no evidence of activation of the Hedgehog signaling pathway by RNA-seq (Appendix Figs A2A and A2B).

To further characterize the tumor microenvironment and its organization, we performed multiplexed immunofluorescence imaging (CyCIF)14 of S2, S3, and S5 followed by single-cell analysis. All tumors stained positive for TRK, with evidence of inflammatory infiltrates in S3 and S5 (Fig 3A). Compared with S2, S3 and, to a greater extent, S5 had a higher density of CD45+ immune cells including cytotoxic T cells and CD68+ macrophages (Fig 3B). Infiltration by these immune cells was significantly greater in S5, in agreement with transcriptional profiling results. Spatial neighborhood analysis showed that S5 had more CD68+ macrophages and CD8a+ T cells surrounding tumor cells as compared with S3 (Figs 3C to 3E, Appendix Fig A3). Moreover, spatial analysis revealed a higher density of interfacing programmed cell death 1 (PD-1)–positive and programmed death ligand 1 (PD-L1)–positive cells in S3 than S5 (Fig 3F-G).

DISCUSSION

We report here a patient with a TPM3-NTRK1–driven sarcoma that developed resistance to first- and second-generation TRKis. The tumor initially developed resistance to larotrectinib through an NTRK1 solvent-front mutation; resistance was overcome with the second-generation TRKi selitrectinib. Two sites of focal progression were surgically resected while on selitrectinib. No clear secondary oncogenic mutation was identified in S4, and the dose of selitrectinib was increased to 200 mg twice a day. Isolated progression was observed at the high drug dose, the tumor was resected (S5), and the gain-of-function KRAS G12V mutation was identified. Transcriptional profiling was consistent with functional activation of KRAS signaling in this tumor.

Dysregulation of KRAS signaling in an NTRK fusion–driven sarcoma initially treated with effective TRKis is analogous to the well-characterized mechanism of acquired anti–epidermal growth factor receptor (EGFR) antibody resistance in colorectal cancer. In a subset of these tumors, KRAS mutations emerge to drive resistance to EGFR inhibition through reactivation of oncogenic signaling.19,20 We speculate that consequent reactivation of signal transduction pathways by mutant KRAS overcomes effective inhibition of the NTRK fusion oncogene. Elevated levels of TRK protein in S5 relative to S3 despite lower expression of the fusion transcript may further suggest effective TRK inhibition because kinase inhibition can stabilize kinase conformation, decrease protein turnover, and prolong half-life.21,22

Although the etiology of the increased inflammatory infiltrate in the KRAS G12V mutant tumor (S5) is unclear, evidence from other cancer types suggests that KRAS mutations may alter the immune microenvironment.23,24 Several targets of immuno-oncology therapies were expressed at higher levels in S5, suggesting an alternative means of targeting resistant disease (although the number...
**FIG 2.** Expression profiling of selitrectinib-sensitive and -resistant samples. (A) Plot of mapped RNA-seq reads at the NTRK1 locus for an ETV6-NTRK3 tumor and TPM3-NTRK1 tumors from surgery (S) 3 and S5. (B) Heatmap of RNA-seq data demonstrating expression of NTRK genes and fusion partners. (C and D) Hallmark gene sets for KRAS signaling up and inflammatory response comparing S3 and S5. (E) Butterfly plot of all Reactome, KEGG, and Hallmark gene sets (n = 910) comparing S3 and S5 tumors. Immune and inflammatory gene sets are outlined in green. (F) CIBERSORT analysis of S3 and S5 tumors showing relative leukocyte abundance. Cell types with nonzero leukocyte fraction are shown. (G) Heatmap showing relative expression of select immune-related genes. FDR, false discovery rate; NES, normalized enrichment score.
FIG 3. Multiplexed imaging of the immune microenvironment in serial tumor resections. (A) Tissue-based cyclic immunofluorescence images from surgery (S) 2, S3, and S5 samples demonstrating staining for tropomyosin receptor kinase (TRK), CD45, and CD68, with lower panels representing magnified images of the upper panels. (B) Immune cell counts, with digital images from the indicated single or multiplexed antibodies processed as previously described.14 Cell counts were calculated frame by frame and are represented as box plots, with the median indicated in red. (C) Global distribution of cells staining highest for TRK (contour map) and CD68+ cells (heatmap, with red indicating higher cell density) in S3 and S5. (D and E) Histograms representing the number of CD68+ or CD8+ cells neighboring TRK−high cells. Cumulative probabilities in each imaged frame are shown as a box plot (inset). (F) Representative staining of programmed cell death 1 (PD-1) and programmed death ligand 1 (PD-L1) in samples S3 and S5, respectively. (G) Proximity probability of cells staining positive for PD-1 and PD-L1, with histogram representing the number of PD-L1−positive cells neighboring PD-1−positive cells. Cumulative probabilities in each imaged frame are shown as a box plot (inset). A two-sample t-test was used to compare groups, with P values indicated; the frame numbers in each sample are 33 (S2), 127 (S3), and 168 (S5).
of cells expressing PD-1 and PD-L1 protein was discordant with RNA expression, as has been observed elsewhere. Furthermore, data from colorectal carcinoma suggests an association between kinase fusions and response to checkpoint inhibitor therapy, suggesting the rational combination of TRKis and immuno-oncology therapeutics for resistant disease.

As exemplified by this patient, treatment with second-generation TRKis can elicit durable response; the patient remains well > 1 year on selitrectinib after the final resection. Imaging using [18F]fluorodeoxyglucose positron emission tomography may be a useful early marker of effective TRK inhibition and tumor response. Consistent with our findings on reactivation of the mitogen-activated protein kinase (MAPK) pathway as a mechanisms of TRKi resistance, alterations in BREF, KRAS, and MET were recently reported to confer resistance to second-generation TRKis in carcinomas, with clinical benefit derived from targeting the reactivated signal transduction pathways. These results provide insight into mechanisms of pathway reactivation under conditions of continued TRK inhibition and identify changes in the immune microenvironment that may play important roles in the diagnosis and treatment of resistant disease.

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**AUTHORS’ DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST**

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Open Payments is a public database containing information reported by companies about payments made to US-licensed physicians (Open Payments).

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FIG A1. Expression profiling of NTRK translocated models. (A) Plot of mapped RNA-seq reads at the NTRK1 locus for an ETV6-NTRK3 tumor and TPM3-NTRK1 tumors from surgery (S) 3, S3 patient-derived xenograft (PDX), S3 cell line, S5, and S5 PDX. (B and C) Hallmark gene set for KRAS signaling up and inflammatory response comparing S3 and S5 PDX grown in athymic, T-cell–deficient mice. FDR, false discovery rate; NES, normalized enrichment score.
FIG A2. Hedgehog pathway gene expression. (A) Expression of genes in the KEGG Hedgehog signaling pathway gene set. (B) Heatmap of RNA-seq data showing expression of select genes essential to Hedgehog cellular signaling. PDX, patient-derived xenograft; S, surgery.

FIG A3. Colocalization of CD8α+ and TRKhigh cells. Global distribution of cells staining highest for tropomyosin receptor kinase (TRK; contour map) and CD8α+ cells (heatmap, with red indicating higher cell density) in surgery (S) 3 and S5.
| Cycle No. | Antibody Name | Target Protein | Vendor | Catalog No. | Clone | Conjugated Fluorophore |
|----------|---------------|----------------|--------|-------------|-------|------------------------|
| 1        | 488/FITC      | pan-TRK        | Abcam, Cambridge, United Kingdom | ab181560 | EPR17341 | n/a                    |
|          | 555/Cy3       | antipCTD2      | Active Motif, Carlsbad, CA | 61084 | 3E10 | n/a                    |
|          | 647/Cy5       | anti-CD20      | DAKO, Santa Clara, CA | M07555 | L26 | n/a                    |
| 2        | 488/FITC      | CD4-488        | R&D Systems, Minneapolis, MN | FAB8165G | Polyclonal | Alexa Fluor 488 |
|          | 555/Cy3       | CD3D-555       | Abcam | AB208514 | EP4426 | Alexa Fluor 555 |
|          | 647/Cy5       | PD1-647        | Abcam | AB201825 | EPR4877 (2) | Alexa Fluor 647 |
| 3        | 488/FITC      | Ki67-488       | CST, Danvers, MA | 11882 | D3B5 | Alexa Fluor 488 |
|          | 555/Cy3       | FOXP3-570      | eBioscience, Waltham, MA | 41-4777-80 | 236A/E7 | eFluor 570 |
|          | 647/Cy5       | PDL1-647       | CST | 15005 | E1L3N | Alexa Fluor 647 |
| 4        | 488/FITC      | IBA1-488       | Abcam | ab195031 | EPR6136(2) | Alexa Fluor 488 |
|          | 555/Cy3       | CD68-PE        | CST | 79594 | D4B9C | PE |
|          | 647/Cy5       | CD45-647       | BioLegend, San Diego, CA | 304020 | HI30 | Alexa Fluor 647 |
| 5        | 488/FITC      | anti-PTEN      | CST | 9559 | 138G6 | Zenon-488 |
|          | 555/Cy3       | Keratin-570    | eBioscience | 41-9003-80 | AE1/AE3 | eFluor 570 |
|          | 647/Cy5       | CD8a-660       | eBioscience | 50-0008-80 | AMC908 | eFluor 660 |
| 6        | 488/FITC      | p53-488        | CST | 5429 | 7F5 | Alexa Fluor 488 |
|          | 555/Cy3       | pH3-555        | CST | 3475 | D2C8 | Alexa Fluor 555 |
|          | 647/Cy5       | H2ax-647       | CST | 9720 | 20E3 | Alexa Fluor 647 |
| 7        | 488/FITC      | Background     | CST | Background | Kazgenomics | Alexa Fluor 488 |
|          | 555/Cy3       | Background     | CST | Background | BioLegend | Alexa Fluor 555 |
|          | 647/Cy5       | Background     | CST | Background | Abcam | Alexa Fluor 647 |

Abbreviations: Ch, channel; CST, Cell Signaling Technology; n/a, not applicable; PE, phycoerythrin; t-CyCIF, tissue-based cyclic immunofluorescence.