What hides behind the MASC: Clinical response and acquired resistance to entrectinib after ETV6-NTRK3 identification in a mammary analogue secretory carcinoma (MASC)


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ABSTRACT

Background: Mammary analogue secretory carcinoma (MASC) is a recently described pathologic entity. We report the case of a patient with an initial diagnosis of salivary acinic cell carcinoma later reclassified as MASC after next-generation sequencing revealed an ETV6-NTRK3 fusion.

Patients and methods: This alteration was targeted with the pan-Trk inhibitor entrectinib (Ignyta), which possesses potent in vitro activity against cell lines containing various NTRK1/2/3 fusions.

Results: A dramatic and durable response was achieved with entrectinib in this patient, followed by acquired resistance that correlated with the appearance of a novel NTRK3 G623R mutation. Structural modeling predicts that this alteration sterically interferes with drug binding, correlating to decreased sensitivity to drug inhibition observed in cell-based assays.

Conclusions: This first report of clinical activity with TrkC inhibition and the development of acquired resistance in an NTRK3-rearranged cancer highlights the utility of comprehensive molecular profiling and targeted therapy for rare malignancies (NCT02097810).

Key words: ETV6-NTRK3, TrkC, mammary analogue secretory carcinoma, entrectinib
Key Message: "Here we describe the dramatic response of a patient with an ETV6-NTRK3-driven mammary analogue secretory carcinoma to treatment with a pan-Trk inhibitor, and the development of acquired resistance linked to a novel NTRK3 mutation that interferes with drug binding. This case highlights how molecular profiling can identify therapies for rare diseases and dissect mechanisms of drug resistance."

Introduction

Mammary analogue secretory carcinoma (MASC) of the salivary gland is a recently identified salivary cancer subtype that commonly originates in the parotid gland (1). Prior to its designation as a separate entity, MASCs were predominantly grouped with other low-grade salivary cancer histologies (most commonly acinic cell carcinoma). In 2010, identification of the \textit{ETV6-NTRK3} translocation t(12;15)(p13;q25) confirmed these tumors to be a molecularly distinct disease; the histologic resemblance to secretory carcinoma of the breast (which also harbors this fusion gene) inspired the designation "mammary analogue secretory carcinoma" (2,3). Of note, while \textit{ETV6-NTRK3} represents the most common fusion in MASCs, some cases have been found to harbor rearrangements involving \textit{ETV6} and a non-\textit{NTRK3} partner (4).

Recurrent gene rearrangements such as \textit{ETV6-NTRK3} are a critical mechanism of oncogenic activation for the neurotrophic tyrosine receptor kinase genes, \textit{NTRK1}, \textit{NTRK2}, and \textit{NTRK3}, in human malignancies (5). These genes encode a family of tropomyosin receptor kinase proteins (TrkA, TrkB, and TrkC, respectively) that are involved in nervous system development. Apart from salivary gland tumors, these rearrangements have been identified in lung, thyroid, and colon cancers, as well as sarcomas, spitzoid neoplasms, and primary brain tumors (6).
Fusion of the intact tyrosine kinase domain of \textit{NTRK1}, \textit{NTRK2}, or \textit{NTRK3} with a variety of upstream partners results in dysregulated activation of several biochemical signaling pathways that promote oncogenic initiation and growth \cite{7}.

Here we provide the first report of a dramatic clinical response to TrkC inhibition in an \textit{NTRK3}-rearranged malignancy and the development of acquired resistance linked to a novel genomic mechanism.

\textbf{Materials and methods}

\textbf{Genomic profiling.} Broad, hybrid-capture next-generation sequencing was performed using the Integrated Mutational Profiling of Actionable Cancer Targets (MSK-IMPACT) assay \cite{8} and sequenced on a HiSeq 2500 (Illumina, San Diego, CA). 410 cancer-related genes were interrogated, capturing base substitutions, small indels, copy number alterations, and select rearrangements. To detect somatic structural aberrations via MSK-IMPACT, a framework was developed that first aligns raw reads to the reference human genome (hg19) using the Burrows-Wheeler Alignment tool. Duplicates are then filtered using the Picard-tools java package (samtools) and searched for candidate structural rearrangements using DELLY. All candidate somatic structural aberrations were filtered, annotated using in-house tools, and manually reviewed using the Integrative Genomics Viewer.

The FACETS algorithm was used to estimate tumor purity, ploidy, and allele-specific copy number \cite{9}. Cancer cell fraction was estimated from mutant allele frequency and corrected for tumor purity and copy number state of the region the mutation resides in. RNA sequencing was performed using the Archer™ FusionPlex™ assay (ArcherDx, Boulder, CO);
libraries were sequenced on an Illumina MiSeqDx. NTRK3 break-apart was assessed by a fluorescence in situ hybridization (FISH). For TrkC immunohistochemistry (IHC), four-micrometer tissue sections on positively charged slides were deparaffinized and rehydrated, and antigens were retrieved for 30 min in a Tris EDTA (pH 9) at 98 °C. Both a TrkC-specific antibody [TrkC Antibody (798): sc-117, Santa Cruz Biotechnology] (Santa Cruz Biotechnology, Dallas, TX) and a primary antibody mixture that consisted of a cocktail of anti pan-Trk (C17F1 Rabbit mAb, 1:25 dilution, Cell Signaling), anti-ROS1 (D4D6 Rabbit mAb, 1:500 dilution, Cell Signaling), and anti-ALK (C17F1 Rabbit mAb, 1:500 dilution, Cell Signaling) were used to amplify signal development.

**Targeted therapy administration.** Crizotinib was obtained from a commercial supply. Computed tomography (CT) imaging was performed at baseline and at various time points on therapy (3, 10, and 18 weeks) to monitor response. The TrkA, TrkB, TrkC, ROS1, and ALK tyrosine kinase inhibitor entrectinib (RXDX-101) was administered on a phase I clinical trial (NCT02097810). The primary endpoint of the dose escalation phase of this trial was to determine the maximum tolerated and recommended phase II doses of entrectinib. A conventional 3+3 dose escalation design was used. The patient was enrolled in the 400 mg/m² cohort of the dose escalation phase and a fixed daily dose of entrectinib was calculated. Response was assessed via the Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1 at protocol-defined intervals (10). Treatment was administered until disease progression or unacceptable toxicity.

**Functional and structural studies.** cDNAs encoding various NTRK1-3 gene rearrangements (TPM3-NTRK1, LMNA-NTRK1, ETV6-NTRK1, VCL-NTRK2, AFAP1-NTRK2, ETV6-
NTRK2, ETV6-NTRK3, and ETV6-NTRK3 G623R) were inserted into the lentiviral vector pVL-EF1a-MCS-IRES-Puro (BioSettia, San Diego, CA) and introduced into the murine IL-3 dependent pro-B cell Ba/F3. Proliferation assays were conducted with various concentrations of vehicle, entrectinib, TSR-011, and LOXO-101. LOXO-101 (11) and TSR-011 (12) were synthesized by Bioduro (Beijing, China) based on publicly-disclosed structures. Plates were incubated at 37°C in 5% CO₂ for 72 hours, after which cell viability was assessed by measuring ATP content using Cell Titer-Glo® Luminescent Cell Viability assay (Promega). IC50s were determined by 4-parameter curve fit with variable slope.

To assess the impact of entrectinib upon signaling pathways, Ba/F3-ETV6-NTRK3 cells were treated with 300 nM entrectinib, a concentration that is approximately 50% of the clinically achievable minimal concentration (Cmin). Cell lysates were prepared 4 hours post treatment and probed for phosphorylated/total Trk, PLCγ1, PI3K (p85), MAPK and Stat3. All primary antibodies were purchased from Cell Signaling Technology except for anti-β-actin (Millipore). Structural assessments of entrectinib binding were obtained using Glide docking implemented in Maestro (13). Receptor coordinates were obtained from PDB (code: 4YMJ).

Results

ETV6-NTRK3 identification and tumor reclassification. A 34-year-old female presented in January 2006 with a growing left parotid mass. A left superficial parotidectomy was performed, revealing a stage III (pT3N0M0) parotid cancer initially classified as acinic cell carcinoma (AciCC) (Figure 1A). Surgical margins were microscopically involved with tumor and
post-operative intensity-modulated radiation therapy was administered to the left parotid and ipsilateral lower neck.

In August 2011, the patient was diagnosed with biopsy-confirmed metastatic disease involving the lung, pleura, mediastinum, and chest wall. She was initially asymptomatic and managed with active surveillance. She eventually developed pleuritic right-sided chest pain caused by enlarging right-sided pleural metastases which were treated with two palliative surgical resections and three different lines of cytotoxic chemotherapy (vinorelbine, carboplatin and paclitaxel, and doxorubicin). A more detailed history is outlined in Table S1.

Pathologic review of a right lower lobe paraesophageal mass resected in February 2013 revealed a carcinoma morphologically similar to the patient’s initial salivary tumor (Figure S1A). Broad, hybrid capture-based next-generation sequencing of the mass identified an ETV6-NTRK3 t(12;15)(p13.2;q25.3) rearrangement (Figure 1B). Additional testing by fluorescence in situ hybridization (FISH) using an NTRK3 break-apart probe was positive (Figure 1B). Immunohistochemistry performed with a TrkC-specific antibody (Figure 1B) and a Trk antibody cocktail that also detects TrkC (Figure S1B) revealed strong staining, confirming TrkC expression. RNA sequencing confirmed the presence of ETV6-NTRK3 (Figure S1C). The patient’s malignancy was thus reclassified as a MASC.

**Crizotinib therapy.** Based on in vitro data indicating that the multi-targeted kinase inhibitor crizotinib may modestly inhibit TrkC kinase in the context of the ETV6-NTRK3 rearrangement (8), the patient was treated with crizotinib. With the initiation of therapy and the performance of intercostal neurolysis, she quickly experienced resolution of her pleuritic pain. Repeat computed tomography (CT) imaging at 3 and 10 weeks revealed stable disease (2%
and 19% reduction in disease burden, respectively). A CT scan at 18 weeks unfortunately revealed disease progression accompanied by recurrent, tumor-related, chest wall pain. Additionally, the patient developed acute, painful swelling of the hands, legs, and feet bilaterally, determined to be consistent with a paraneoplastic hypertrophic osteoarthropathy in the setting of progressive malignancy. Non-steroidal anti-inflammatory therapy was initiated with minimal improvement in symptoms.

Response and acquired resistance to entrectinib. We hypothesized that TrkC activity remained the central oncogenic driver of the patient’s disease despite progression on crizotinib, and that inhibition with a potent Trk inhibitor would produce tumor regression. She was thus enrolled onto a phase I clinical trial of the pan-Trk inhibitor, entrectinib (NCT02097810). The patient noted a substantial decrease in hypertrophic osteoarthropathy-related limb edema within the first 4 weeks of treatment. These symptoms resolved by week 8 of therapy. CT imaging at 9 weeks (Figure 2) revealed a dramatic partial response with decreased pleural-based metastases. Response was confirmed at 13 weeks, and further disease shrinkage noted at 21 weeks (89% reduction in tumor burden).

After 7 months of therapy, imaging revealed asymptomatic disease progression in a solitary tumor site in the right lower lobe of lung. Though this met RECIST criteria for progressive disease, given stable response at all other metastatic sites and the absence of tumor-related symptoms, she continued entrectinib at an increased dose (Table S1). Serial imaging at 8 and 9 months showed more modest growth of the same mass and continued stable response at other sites. Given ongoing clinical benefit and acceptable tolerability, she remained on entrectinib. Assessment 10 months into treatment unfortunately revealed further
disease progression in not only the right lower lobe tumor, but also several other adjacent pulmonary nodules, leading to discontinuation of entrectinib.

**NTRK3 G623R identification.** Next-generation sequencing was performed on three tumor biopsies: M1 (paraesophageal right lower lobe mass acquired prior to crizotinib), M2a (separate pleural-based right lower lobe mass acquired prior to entrectinib), and M2b (pleural-based right lower lobe mass immediately adjacent to M2a that progressively enlarged on entrectinib therapy) (Figure 3A). While *ETV6-NTRK3* was detected in all three tumors (Figure S2), a novel *NTRK3* exon 16 G623R (c.1867G>A) mutation was identified in the M2b tumor, correlating to the development of entrectinib resistance (Figure 3B). The cancer cell fraction estimate for *NTRK3* G623R was 90%, indicating its presence at a clonal level. Concurrent mutations in *RB1* and *MYC* identified in M2a were maintained in M2b (Figure S3).

A review of histology did not identify morphologic changes associated with the development of acquired resistance to therapy. While the patient’s primary tumor resected in 2006 showed a lower-grade histology with a mitotic count of 2/10 high-power fields and no tumor necrosis, pathologic features of all the metastatic tumors (M1, M2a, and M2b) were similar and more aggressive, each with a mitotic count of 6/10 high-power fields and notable tumor necrosis.

**Functional and structural evaluation.** To evaluate the hypothesis that the *NTRK3* G623R mutation mediates resistance to entrectinib, we investigated Trk inhibitor susceptibility in Ba/F3 cell lines overexpressing *NTRK* constructs. Entrectinib demonstrated potent anti-proliferative activity in cell lines overexpressing various *NTRK* family rearrangements (*TPM3-NTRK1, LMNA-NTRK1, ETV6-NTRK1, VCL-NTRK2, AFAP1-NTRK2, ETV6-NTRK2, ETV6-NTRK3*) with
IC$_{50}$ values ranging between 1-5 nM (Figure 3C). This effect was Trk fusion-specific as
entrectinib had no effect on parental Ba/F3 cells (IC$_{50}$ >1000 nM) or Ba/F3 cells transfected with
empty lentiviral vector.

For the ETV6-NTRK3 fusion, entrectinib was more potent than other Trk inhibitors: TSR-
011 (Tesaro), LOXO-101 (LOXO), and crizotinib (IC$_{50}$ values of 2 nM for entrectinib, 14 nM for
LOXO-101, 59 nM for TSR-011, and 88 nM for crizotinib, Figures S4A and S4B). Western blots
confirmed Trk targeting with entrectinib as phosphorylation of both TrkC and PLCγ1 was
substantially reduced with drug exposure. Other signaling proteins downstream of TrkC also
had reduced phosphorylation with entrectinib, including PI3K (p85), MAPK and Stat3 (Figure
3D).

Introduction of the NTRK3 G623R mutation to the ETV6-NTRK3 construct (Ba/F3-ETV6-
NTRK3 G623R) conferred reduced sensitivity to entrectinib, increasing the IC$_{50}$ value in the
proliferation assays more than 250-fold (2 nM to 507 nM) relative to the Ba/F3-ETV6-NTRK3
cells (Figure 3E). The NTRK3 G623R mutation conferred even greater loss of sensitivity to the
other tested Trk inhibitors, TSR-011 (Tesaro) and LOXO-101 (LOXO), eliciting IC$_{50}$ proliferation
values of >1000 nM (Figure S4C).

Investigation into the structural impact of the G623R point mutation revealed a
potential mechanism of relative resistance to entrectinib and other Trk inhibitors. The glycine at
position 623 lies in the kinase domain of TrkC, a codon that homology alignment suggested is
highly conserved in other kinases, including ALK (position 1202), ROS1 (position 2032), and
NTRK1 (position 595, Figure 3F). Structural analysis (Maestro) revealed extensive hydrogen
bonding and hydrophobic interactions between entrectinib and the ATP binding pocket of wild
type TrkC where the G623 residue is located. The \textit{NTRK3} G623R mutation creates steric hindrance that functionally reduces the binding of entrectinib with mutant TrkC (Figure 3G).

\textbf{Discussion}

In this article, we describe the first clinical response to TrkC inhibition in a patient with an \textit{NTRK3}-rearranged malignancy, underscoring the role of Trk fusion proteins as targetable drivers of oncogenesis. This observation has widespread implications across a number of adult and pediatric hematologic/non-hematologic cancers where \textit{NTRK3} rearrangements have been identified (7). We demonstrate that entrectinib (RXDX-101), a multikinase inhibitor with activity against TrkA, TrkB, TrkC, ROS1, and ALK, has potent \textit{in vitro} activity against a variety of \textit{NTRK} rearrangements, including \textit{TPM3-NTRK1}, \textit{LMNA-NTRK1}, \textit{ETV6-NTRK1}, \textit{VCL-NTRK2}, \textit{AFAP1-NTRK2}, \textit{ETV6-NTRK2}, and \textit{ETV6-NTRK3}.

While crizotinib has modest \textit{in vitro} activity against Trk (14), it is significantly less potent against \textit{ETV6-NTRK3} in comparison to entrectinib, correlating to the modest benefit achieved clinically in this patient. This observation suggests that the degree of Trk inhibition may be a critical determinant of response, emphasizing the need for potent Trk inhibitors such as entrectinib to achieve meaningful clinical outcomes in patients with Trk-driven tumors. Drug development strategies for these agents will need to take into account that \textit{NTRK} rearrangements are high-incidence events in rare tumor types, as well as low-incidence events in more common tumors.

This experience also represents the first reported case of resistance to TrkC inhibition mediated by the appearance of an \textit{NTRK3} G623R mutation. Structural and cellular studies
suggest that this alteration reduces entrectinib (and other tested Trk inhibitors) inhibition of TrkC by sterically disrupting drug binding to the kinase domain. Interestingly, solvent front mutations resulting in amino acid substitution at paralogous positions of ALK and ROS1 (ALK G1202R ([15,16] and ROS1 G2032R ([17])), have previously been identified as mechanisms of acquired resistance to ALK and ROS1 inhibition by crizotinib in ALK- and ROS1-rearranged tumors, respectively. More importantly, the TrkC G623 and TrkA G595 residues are paralogous, confirming the work performed by Russo and colleagues who identified NTRK1 G595R as a mechanism of acquired resistance to entrectinib in a patient with LMNA-NTRK1-rearranged colorectal cancer ([18]). We recommend that molecular profiling be performed at the onset of acquired resistance to Trk inhibition in NTRK-rearranged tumors to identify NTRK3 G623R, NTRK1 G595R, and the analogous mutation NTRK2 G639R. Ongoing drug discovery efforts should focus on the development of targeted therapies with activity against these mutations.

Finally, this report highlights the tremendous potential of comprehensive molecular profiling to impact oncologic diagnostics and therapeutics for cancer patients (19), particularly those with rare malignancies for whom effective or standard therapies are lacking (11,20). In this case, profiling prompted pathologic reclassification of the patient’s diagnosis from AciCC to MASC and identified a class of novel drugs that would not have otherwise been considered for her. In a series of non-parotid AciCC cases, 79% of tumors harbored ETV6-NTRK3 rearrangements, resulting in a change in diagnosis to MASC (21). While salient histologic features may suggest a diagnosis of MASC (22), we recommend that all suspected MASC neoplasms, including zymogen-poor AciCC and other morphologically-similar low-grade salivary tumors, undergo molecular profiling for ETV6-NTRK. For those with recurrent or metastatic
disease, such an approach could potentially translate to clinically-meaningful therapeutic options.

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Disclosures

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References


Figure 1. *ETV6-NTRK3* identification results in tumor reclassification. In Panel A, pathology from a superficial parotidectomy revealed an infiltrative carcinoma with predominantly tubular and microcystic/macrocystic growth patterns and intraluminal secretions. Tumor cells exhibited granular eosinophilic cytoplasm with occasional vacuolation and had relatively bland cytology with mild atypia. A diagnosis of acinic cell carcinoma (AcicCC) was made. In Panel B, the *ETV6-NTRK3* fusion detected via broad, hybrid-capture next-generation sequencing is depicted. Reciprocal translocation between chromosome 12 and chromosome 15 resulted in fusion of *ETV6* exons 1-5 to *NTRK3* exons 15-20 containing the receptor tyrosine kinase (RTK) domain. On the lower left, a positive break-apart fluorescence *in situ* hybridization (FISH) *NTRK3* assay is shown with split signals (arrows). On the lower right, immunohistochemistry for TrkC revealed strong staining signifying TrkC overexpression. The patient’s diagnosis was reclassified as mammary analogue secretory carcinoma (MASC).

Figure 2. A durable partial response is achieved with entrectinib therapy in an *ETV6-NTRK3*-rearranged mammary analogue secretory carcinoma. Computed tomography (CT) imaging of the patient after progression on crizotinib and prior to entrectinib therapy is shown on the left. Repeat CT imaging at 9 weeks revealed a dramatic partial response to therapy (RECIST v1.1) with an interval decrease and resolution of pleural-based metastases in the right hemithorax (arrows). This response was confirmed at 13 weeks and further shrinkage was noted at 21
weeks. A best radiologic response of 89% reduction in tumor burden from baseline was achieved.

Figure 3. The development of clinical entrectinib resistance is mediated by the appearance of a novel \textit{NTRK3} G623R mutation. In Panel A, areas of tumor acquisition via serial biopsies are depicted: prior to crizotinib (M1, paraesophageal right lower lobe mass), after progression on crizotinib and prior to entrectinib (M2a, pleural-based right lower lobe mass), and after progression on entrectinib (M2b, pleural-based right lower lobe mass immediately adjacent to M2a). In Panel B, broad, hybrid capture-based next-generation sequencing confirmed the appearance of an \textit{NTRK3} G623R mutation after progression on entrectinib (M2b) that was not present in pre-entrectinib tumor samples (M1 and M2a). Panel C depicts the antiproliferative activity of entrectinib in engineered Ba/F3 cells expressing a variety of Trk fusion proteins with IC50s ranging from 1.4-4.5 nM. Entrectinib was found to inhibit phospho-TrkC and phospho-PLCY1, with less inhibition of PI3K, MAPK, and Stat3 as depicted in Panel D. In Panel E, introduction of the \textit{NTRK3} G623R mutation into the \textit{ETV6-NTRK3} construct (Ba/F3-ETV6-NTRK3 G623R) conferred reduced sensitivity to entrectinib, increasing the IC50 value in the proliferation assays by more than 250 fold relative to the Ba/F3-ETV6-NTRK3 cells. Homology alignment in Panel F suggests that the native glycine at position 623 of TrkC is highly conserved among TrkC paralogs. A comparison to glycine residues at position 1202 of ALK, position 2032 of ROS1, and position 595 of TrkA, in addition to other paralogs, is shown. Panel G depicts the binding of entrectinib to both wild type TrkC and \textit{NTRK3} G623-mutant TrkC. Extensive hydrogen bonding and hydrophobic interactions between wild type TrkC and entrectinib occur in the ATP
binding pocket where the G623 residue is located (left). The substitution of arginine for glycine at position 623 results in steric hindrance that decreases the binding of entrectinib to mutant TrkC (right).
A  Pathologic Analysis of Resected Tumor

Initial Classification: “Acinic Cell Carcinoma” (AciCC)

B  Molecular Profiling

Next-Generation Sequencing Identifies *ETV6-NTRK3*

- Chromosome 12
- Chromosome 15

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FISH

IHC for TrkC

Reclassification: “Mammary Analogue Secretory Carcinoma” (MASC)