**Background**

**OBJECTIVE:** The STARTTRK-2 trial is a potentially registration-enabling Phase 2 global basket trial of the tyrosine kinase inhibitor entrectinib in patients with solid tumors harboring NTRK1, NTRK2, NTRK3, ROS1, or ALK gene rearrangements (fusions). Phase 1 studies of entrectinib reported a 79% ORR across multiple histology types in patients with gene fusions who were naïve to inhibitors of these targets, received an efficacious dose, and had extracranial disease. Patients harboring these gene fusions are rare (<3%); however, sarcoma patients may have a relatively higher prevalence, particularly in the NTRK2 genes (that encode the Trk proteins), where preliminary clinical evidence of benefit has been demonstrated with Trk inhibitors. Unfortunately, diagnostic testing to identify sarcoma patient populations with such low molecular prevalence poses efficiency and cost challenges, as molecular testing for these gene fusions is not yet part of standard clinical practice. We report on the prevalence of NTRK, ROS1, and ALK fusions in sarcoma based on Ignyta’s internal and 3rd party testing. We also discuss the development of an assay for sarcoma samples and report on immunohistochemistry (IHC) screening rates in a clinical sarcoma cohort.

**METHODS:** The prevalence of NTRK, ROS1, and ALK gene fusions were reviewed for sarcoma from Ignyta’s aggregate diagnostic experience to date (internal testing in Ignyta’s CLIA/CAP lab and testing by third parties). In order to effectively identify sarcoma patients eligible for STARTTRK-2, we developed a 2-step diagnostic test to identify NTRK1, NTRK2, NTRK3, ROS1, and ALK gene fusions in FFPE clinical specimens. This test is comprised of IHC screening using an antibody cocktail followed by an RNA-based anchored multiplex PCR next generation sequencing (NGS) assay.

**Targeting NTRK, ROS1 & ALK**

Entrectinib inhibits TrkA, TrkB, TrkC proteins (encoded by the genes NTRK1, NTRK2, and NTRK3 respectively) as well as ROS1 and ALK.

<table>
<thead>
<tr>
<th>Target</th>
<th>TrkA</th>
<th>TrkB</th>
<th>TrkC</th>
<th>ROS1</th>
<th>ALK</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC50 (nM)</td>
<td>1.7</td>
<td>0.1</td>
<td>0.1</td>
<td>0.2</td>
<td>1.6</td>
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- **Most potent pan-Trk inhibitor in clinical development with activity against most of the known Trk-resistant mutants.**
- **30x more potent against ROS1 than crizotinib; High potency against ALK.**
- **Designed to cross blood brain barrier (BBB) and to address CNS metastases, a common complication of advanced solid tumors.**
- **Entrectinib-mediated inhibition of oncogenic fusion proteins results in rapid tumor response in preclinical models and in selected patient populations.**

**Phase 1 Data**

- **The most frequent (> 10% incidence) treatment-related adverse events were fatigue (44%), dysgeusia (41%), paresthesias (28%), nausea (24%), and myalgia (22%).**
- **The vast majority of treatment-related adverse events were Grade 1 or 2 in severity.**
- **The most frequent (> 2% incidence) Grade 3 treatment-related adverse events were fatigue (4%) and anemia (3%).**
- **Adverse events were reversible with dose modification.**
- **There was no evidence of cumulative toxicity, hepatic or renal toxicity, or QTc prolongation.**
- **No responding patient has discontinued due to safety/interolerability.**

**Case:** 48F with NTRK-rearranged low-grade recurrent endometrial stromal sarcoma

**Detecting NTRK, ROS1 & ALK**

NTRK, ROS1, and ALK fusions are characterized by overexpression of protein. A cocktail IHC assay containing three antibodies (pan-Trk, ROS1, ALK) was developed as a screening approach to eliminate negative samples from further NGS steps.

**How to find that small population of patients which respond to specific therapy?**

- Multiplex molecular profiles provide a large amount of information, FISH is established for gene rearrangements. Both are time consuming and expensive
  - Solution: IHC "phenotyping".
  - Rapid screening multiple markers (any positive vs negative).
  - High sensitivity, but with poor specificity (no false positive).

**Screening with a Cocktail IHC**

**Step 1:** Cocktail IHC screen

1. IC50 control to establish IC50 range for TrkA, TrkB, TrkC
2. IHC cocktail to identify NTRK rearrangements
3. NTRK1, NTRK2, NTRK3, ROS1, ALK
4. ROS1
5. ALK
6. DD53
7. D4D6
8. D25

**Step 2:** NGS RNA-based Sequencing

- **Out of 144 sarcoma samples and several thousand samples from other histologies, no instances were detected where the IHC was screened negative and gene fusions were observed by NGS.**
- **During the course of the study, an antibody with a lower level of non-specific binding was identified, and the new antibody had a composite screening rate of 25% (n=620) compared to the prior screening rate of 52% (n = 82).**
- **Table 1 and Table 2 show the blinded rate using both antibodies by sarcoma location and histology respectively.**

**Table 1. Representative IHC positive screening rate by sarcoma location (n = 144)**

**Table 2. Representative IHC positive screening rate by sarcoma histology or subtype (n = 144)**

**Table 3. Prevalence of NTRK1, ROS1, and ALK gene rearrangements from internal testing and partner collaborations.**

**Conclusion**

It is of critical importance to develop effective diagnostic testing algorithms that include identifying appropriate patients for targeted therapy. Sarcoma patients have substantial prevalence of NTRK, ROS1, and ALK fusions, and a testing approach should balance cost of testing methods considerations in sarcoma centers and the need to efficiently identify patients. Relatively inexpensive IHC screening is a potentially useful tool for improving the cost-effectiveness of expensive NGS testing.

NTRK, ROS1, and ALK fusions have a substantial prevalence in soft tissue sarcoma in important histological subtypes. Depending on the antibody, this 2-step assay is an efficient method for detection of gene rearrangements in both high-volume clinical testing and studies of archival formalin-fixed paraffin-embedded specimens. However, because IHC signal comes from both increased expression of full-length protein, as well as constitutively active fusion proteins, NGS is required to correctly identify gene rearrangements. As newer diagnostic antibodies are developed for these targets, the screening approach will continue to improve in efficiency.

**References**


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