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# NTRK-fusions in pediatric thyroid tumors: Current state and future perspectives

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ABSTRACT

Pediatric and adult papillary thyroid cancer (PTC) share many similar oncogenic drivers, but differ in the pathological features and outcomes of the disease. The most frequent genetic alterations in adult PTCs are mutually exclusive point mutations in *BRAF* or the *RAS* family. In pediatric PTC, fusion oncogenes involving chromosomal translocations in tyrosine kinase (TK) receptors, most commonly *RET* and *NTRK*, are the most common genetic alterations observed. This review of the literature describes the current state of translational research in pediatric NTRK-driven thyroid cancer and highlights opportunities to improve our understanding and current models of pediatric PTC.



## Introduction

Receptor tyrosine kinases (RTK), such as NTRK, are essential mediators in transducing signals to regulate biological functions including survival, proliferation, and cell differentiation. Their common structures consist of an extracellular ligand binding domain, a transmembrane region, and an intracellular kinase domain. In physiological conditions, their activation upon ligand binding leads to the homodimerization of the receptors and phosphorylation of the downstream signaling cascades. Depending on the phosphorylation site, different pathways such as RAS/MAPK, PI3K/AKT and JAK/STAT can be activated. However, in the case of the chromosomal rearrangements, either a 3'kinase fusion (the C-terminal domain of any RTK gene is combined with the N-terminus of a partner gene) or a 5'kinase fusion (N-terminal domain of the RTK is combined with the C-terminal of a fusion partner gene) generate a chimeric kinase that becomes constitutively active [1]. This gain of intrinsic kinase activity could be the consequence of several different mechanisms: (i) the fusion kinase expression is controlled by the promoter of the partner gene, (ii) the partner gene provides the dimerization domain such as coiled coil, helix-loop-helix, WD repeats or zinc finger domain leading ligand-independent activation in the fusion kinase or (iii) the novel fusion kinase loses its autoinhibitory domain. In addition, the fusion protein could have altered intracellular localization due to conformational changes or it can display new structural binding regions that enhances MAPK and or PI3K/AKT activation [2].

In the physiological state, TRK receptors are activated by neurotrophins. Signaling and interaction is specific to the TRK receptor and neurotrophin (reviewed in [3]). This enables tissue and signaling specificity based on the available receptors and ligands. Activation of TRKA (NTRK1) with nerve growth factor (NGF) leads to the docking of cytoplasmic proteins to activate the RAS/MAPK pathway. Conversely, activation of TRKB (NTRK2) via neurotrophic binding can result in RAS/MAPK pathway activation in addition to PI3K and PLCgamma pathway activation. Neurotrophin 3 binding to TRKC (NTRK3) preferentially activates PI3K/AKT signaling. These pathways are well mapped and understood in neuronal development and maturation, but the specificity of NTRKs with fusion partners and how this preferentially leads to pathway activation in tumors is not well known. (Fig. 1)

Comprehensive genomic studies are continually revealing novel fusion partners in thyroid cancer and other tumor types, as well a tissue tropism for these fusions [4]. For example, NTRK fusions detected in pediatric thyroid tumors predominantly involve NTRK1 and NTRK3, whereas central nervous system tumors primarily have

*Abbreviations:* PTC, Papillary thyroid cancer; NIS, Sodium-iodide symporter; RAI, Radioactive iodine.

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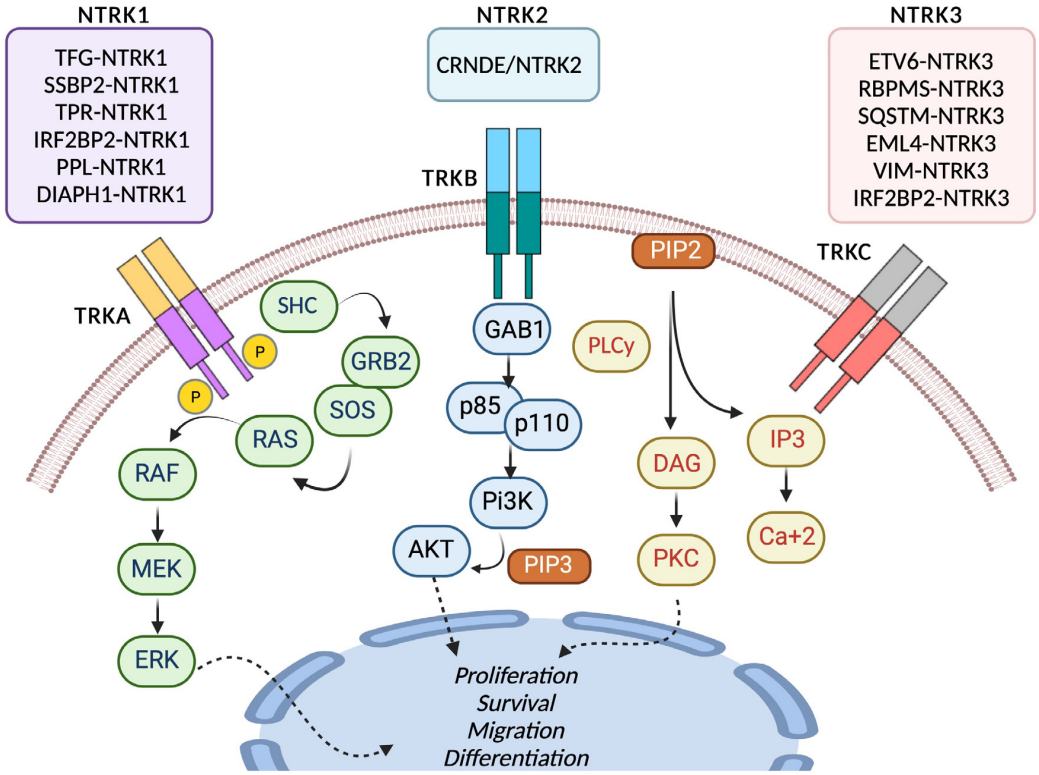


**Fig. 1.** NTRK fusions identified in thyroid cancer. trk fusion receptors lead to constitutive activation of signaling pathways such as Ras, phosphatidylinositol-3-kinase (PI3K) and phospholipase C (PLC)-y pathways. Created with BioRender.com

fusions associated with NTRK2 [4]. Whether this tropism is present throughout pediatric and adult tumors has not yet been delineated. It is also not yet clear whether expression of these fusions in different cells will respond differently to NTRK inhibitors.

Many genetic mutations, primarily activating the MAPK pathway, have been identified in pediatric thyroid cancer [4–7]. The molecular and biochemical responses triggered by NTRK-fusion gene expression confer distinctive clinical presentations for pediatric thyroid cancer patients which include lymphovascular permeation, extrathyroidal extension, regional lymph node invasion and distant metastasis to the lung. Although there is a good prognosis and successful treatment of the disease in many patients, initial diagnostic tests that are more predictive of disease invasion and aggressiveness could help stratify patients that need more aggressive frontline surgery. Due to the highly invasive nature of PTC in pediatric patients, recommendations are usually total thyroidectomy [8] followed by radioactive iodine (RAI) therapy to eliminate residual disease or possible metastases. This can lead to significant morbidities for patients, many of whom may not need such an extensive therapeutic approach. Further, nearly 5% of the patients become resistant to RAI therapy, with very few second line therapeutic options available. Current efforts are focused in reducing the number of surgeries in pediatric patients, to avoid complications and lifetime hormone replacement therapy [9] and could be significantly improved with reliable predictive biomarkers. Lee et al. recently reported that NTRK and RET-fusion oncogenes may confer greater risk for metastatic spread in pediatric PTC, compared to PTCs driven by BRAFV600E [10].

## Development of targeted therapeutics

Genetic profiling of tumor samples at the time of biopsy and diagnosis is being used to discover novel biomarkers, prognosticate regarding outcome, and identify molecular targets which may respond to newer precision therapies. Contrary to chemotherapy drugs which indiscriminately attack proliferative cells, targeted therapeutics offer a more precise effect against cancer cells. These agents block kinase receptors or cytosolic kinases, inhibiting proliferation and blocking cell survival pathways supported by the MAPK and the PI3K pathways. The most common compounds used in thyroid cancer therapy are broad kinase inhibitors (MKIs), such as Sorafenib, Lenvatinib, Cabozatinib, Donafenib, and Vandetanib ([11, 12]). While these MKIs demonstrate efficacy in thyroid cancer treatment, their broad spectrum is often associated with significant side effects. Thus, the latest strategies have shifted towards targeting more specific molecular alterations, such as gene fusions.

Currently, there are two inhibitors approved by the FDA with activity against NTRK fusion kinases in adult and pediatric solid tumors, Entrectinib and Larotrectinib [13–15]. While Larotrectinib has selectivity for TrkA, TrkB and TrkC with high potency ([16, 17]), Entrectinib also inhibits the kinase activity of ROS1 and ALK ([18, 19]). Both of these inhibitors have been tested in tissue-agnostic clinical trials and have shown remarkable pharmacologic efficacy in some patients with NTRK-fusion driven thyroid tumors [20–22]. This expedited, tissue-agnostic trial method is based on the presence of specific gene alterations but independent of the tissue type. Indeed, other malignancies that have shown significant clinical response to these inhibitors include salivary-gland tumors, soft tissue sarcomas, infantile fibrosarcoma, colon cancer, lung cancer, melanoma, gastrointestinal stromal tumors (GIST), cholangiocarcinoma, appendiceal tumors, breast tumors, and pancreatic tumors ([10, 16, 17, 23-33]). Fortunately, these clinical studies have demonstrated significant responses; however, the biochemical mechanisms of action are still not clear, or the long-term effects fully addressed. Very few *in vitro* or preclinical signaling studies have been done to elucidate the impact of these inhibitors on down-

stream signaling cascades, or the durability of inhibition. In a pilot exploratory analysis by Hong et al [19], the efficacy and long-term safety of Larotrectinib treatment were evaluated in 159 patients with NTRK-fusion positive solid tumors. The distribution of the enrolled patients (clinical trials: NCT02122913, NCT02637687 and NCT02576431) by age was 12 adults in phase 1, 97 adolescent and adults in phase 2 and 50 pediatric patients in phase 1/2. Response to Larotrectinib was observed in 121 of 153 patients, including 19 patients with thyroid tumors, with an overall median response duration of 35.2 months. Although the adverse events were primarily of grade 1 and 2 with similar frequency observed across age groups, further mechanistic and biochemical studies will be necessary to fully understand the clinical response of these inhibitors. Further, studies are needed in pediatric patients to determine whether life-long kinase inhibitor therapy will be necessary and to identify potential long-term side effects of prolong targeted therapy.

One common caveat associated with using kinase inhibitors is the acquisition of resistance mechanisms. The emerging mutations found in the TRK kinase domain confer drug resistance by interfering with the drug binding pocket ([34, 35]). Some of the mutations already reported are: TrkA-G595R, -G667S, -G667C, V573M and -F589L, and TrkC-G623R and -G696A [36]. There are ongoing trials in advance phase to develop the second generation of targeted therapy against NTRK fusions. Novel drugs such as Selitrectinib (LOXO-195), Repotrectinib (TPX-0005) [37] and Taletrectinib (DS-6051b) [38] are showing promising results, but more *in vitro* and *in vivo* testing is necessary.

Expression and membrane localization of the sodium-iodide symporter (NIS) are required for cellular uptake and therapeutic function of RAI, and diminished expression and membrane targeting of NIS have been demonstrated in patients with RAI-refractory thyroid cancer [39]. Several kinase inhibitors have shown potential for re-establishing NIS function in RAI-refractory thyroid cancer. Recently, Lee et al. reported that Larotrectinib treatment could restore expression of the NIS gene (*SCL5A5*) [10]. Presumably, the re-expression of NIS is mediated through inhibiting the MAPK pathway as has been previously reported in other studies whereby MAPK-pathway inhibition restores NIS expression and can enhance the uptake of RAI [40–42]. Lee et al. used an *in vitro* model, where non-tumoral adult human primary thyroid follicular epithelial cells (Nthy-ori-3-1) were transfected to over-express a TRP-NTRK1 construct. Treatment with Larotrectinib *in vitro* restored $^{125}$I uptake, increased NIS expression and protein levels, and inhibited cell growth when used alone or in combination with $^{131}$I therapy. Notably, while the *in vitro* effects in this study were consistent with previous reports of thyroid cancer patients treated with Larotrectinib ([17, 19, 43]), the concentrations of Larotrectinib used *in vitro* for these studies were 1000-fold higher compared to those used in other NTRK-fusion driven cells lines [44–46] and higher than the peak concentration achieved in clinical practice [46]. Additional studies are needed to determine whether thyroid cells respond differently than other fusion-driven tumor cells, if NTRK-fusion partner affects response, and whether expression levels of the fusion oncogene impact responsiveness to therapy. These studies could help to refine the clinical use of these inhibitors and allow personalized tailoring of these treatments to specific tumor types and fusion-oncogenes.

## Models of pediatric thyroid cancer

A significant limitation within the thyroid cancer field is the lack of fusion models that recapitulate the disease in both adult and pediatric contexts. Pediatric thyroid cancer is rare, and as a result, the majority of the research and published data are focused on adult thyroid models. The high incidence of fusion oncogene drivers in children combined with the surge of novel NTRK target specific drugs in the last years highlights the need for new models of pediatric thyroid cancer. To date, the only available thyroid cancer cell lines are exclusively from adult patients and none of them express any chromosomal rearrangement involving NTRK. There is one *in vivo* mouse model that recapitulate NTRK1-TPM3 (TRK-T1) fusions in thyroid cancer; however, the fusion gene is expressed in the germline and causes tumor development earlier than expected in sporadic thyroid cancer ([47, 48]). Tissue specific models that can be temporally controlled are needed to more closely recapitulate sporadic tumor development resulting from somatic mutations.

While the development of next-generation sequencing (NGS) has led to a dramatic expansion of the ability to identify driver mutations for individual patient tumors and has generated great enthusiasm about the promise of targeted, mutation-based treatment strategies, this approach may fall short of expectations without validation in appropriate preclinical models. As an example, the Zero Childhood Cancer (ZERO) Program, a precision medicine initiative for pediatric cancer in Australia, conducted a large-scale study that used whole genome, transcriptome, and methylome profiling in a panel of 252 high-risk pediatric tumors to identify targetable alterations. Of these, 93.7% had some detectable alteration and 71.4% were positive for a known therapeutic target. At the time of publication, 38 patients had received treatment with the relevant targeted therapy, and only 31% had shown evidence of objective clinical benefit [49]. Rather than undermining the promise of targeted therapeutics overall, this gap between molecularly targeted therapy and real-world clinical outcomes demonstrates that the complexity of tumor behavior *in vivo* is not fully explained by genomic and transcriptomic analysis [50].

Many oncogenes have been identified as common driver alterations for multiple cancer types. In light of this, while targeted therapy may have shown efficacy in one tumor type, there may be mechanisms for resistance present in other tumor types despite the presence of the same driver alteration. The BRAF$^{V600E}$ mutation has been identified as a therapeutic target in an array of pediatric and adult cancer types, predominantly including papillary thyroid cancer, glioma, melanoma, and colon cancer, among others. Despite the common molecular mechanism, targeted BRAF$^{V600E}$ inhibition has shown differential degrees of clinical success between these different tumor types. While BRAF$^{V600E}$ inhibition showed significant efficacy in BRAF-mutated melanoma, Zhang et al. identified a relative intrinsic resistance to BRAF$^{V600E}$ inhibition in BRAF-mutated gliomas in a preclinical model that was able to be overcome by the addition of a MEK inhibitor [51]. This finding underlines the importance of models specific to each tumor type in preclinical phases in order to optimize the benefit to patients undergoing early clinical trials.

Given the relatively higher predominance of research for adult cancers, models of pediatric cancers with pediatric-specific oncogene profiles are lacking. The few that have been done demonstrate the importance of creating these models to elucidate the molecular mechanisms specific to pediatric tumors that carry oncogenes that are rare in the corresponding adult tumor types. As with thyroid cancer, malignant astrocytoma occurs in both children and adults, and these populations show significant differences in clinical features and frequency of specific oncogene mutations. Schiffman, et al. identified an activating BRAF$^{V600E}$ mutation in 7/31 (23%) of pediatric malignant astrocytoma cases, a mutation that occurs only rarely in adult astrocytomas of the same grade [52]. Five of these seven mutations occurred in conjunction with a homozygous *CDKN2A* deletion leading to loss of expression of *Ink4a-Arf*. They subsequently created a BRAF$^{V600E}$ Cre-inducible heterozygous knock-in mouse model, which expressed mutationally-activated BRAF$^{V600E}$ at physiological levels and led to

tumor formation only when crossed with a *CDKN2A* homozygous knock-out. In this model, CDK4/6 was required for cell cycle progression of tumor cells, and combined inhibition of BRAFV600E and CDK4/6 led to additive anti-tumor effects, indicating a potential clinical benefit for patients co-expressing these mutations [53]. Similarly, unpublished work from the Franco Lab demonstrates that thyroid tumor cells harboring $HRAS^{G12V}$ or $BRAF^{V600E}$ mutations, have different pathogenesis depending on the age of the mouse. These data suggest that age of the patient has significant impact on tumorigenesis, and, therefore, models are needed that recapitulate pediatric versus adult-onset disease.

For pediatric cancers, preclinical models that recapitulate the specific developmental milieu of childhood-onset tumors are also needed in order to investigate differences in clinical outcomes between pediatric and adult tumors of the same histology. Patient-derived xenograft models are valuable but given the rarity of most pediatric tumors, it is difficult to compile a panel that can be used in large-scale studies [50]. Conventional two-dimensional tissue culture techniques have been widely used, but present limitations with regard to the applicability of findings to the 3D complexity of the *in vivo* tumor environment. Recently, spheroid and patient-derived organoid models have shown promise in more faithfully rendering tumor biology and response to therapies *in vitro* ([54, 55]). Potentially the highest yield model systems for pediatric cancers are animal models with regulated expression of gain- or loss-of-function mutations in the target tissue of interest, as has been done in small cell lung cancer among others [56]. With this type of system, the timing of expression of the mutation of interest can be carefully selected to appropriately mimic the developmental timing of tumorigenesis in humans. The potential to create pediatric- and adult-specific cohorts of tumors that share a common driver mutation using this model presents a promising opportunity to characterize age-related differences in tumor behavior. This will be particularly important in NTRK-driven thyroid tumors whereby we observe different prevalence of NTRK-fusions and pathogenesis of NTRK-driven tumors between pediatric and adult patients ([4, 57]).

## Conclusion

Following the identification of NTRK fusions as a predominant driver mutation in pediatric thyroid cancer [4], there has been considerable enthusiasm for the potential use of NTRK targeted inhibitors as an adjunct to standard therapy in cases of advanced pediatric thyroid cancer carrying an NTRK fusion. However, given the complexity of the *in vivo* tumor environment, and the heterogeneous efficacy of targeted inhibitors among different tumor types, preclinical models are needed to characterize the response of pediatric thyroid tumors to these inhibitors in order to best predict their clinical benefit and identify mechanisms of resistance. Several advanced *in vitro* and *in vivo* models of pediatric cancers have been utilized in other cancer types and are needed in pediatric thyroid cancer to improve the field's ability to evaluate new targeted therapies with the goal of improving outcomes for children and adolescents with thyroid cancer.

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## CRediT authorship contribution statement

**Victoria Casado-Medrano:** Conceptualization, Writing – original draft, Investigation. **Alison O'Neill:** Conceptualization, Writing – original draft, Investigation. **Stephen Halada:** Conceptualization, Investigation. **Theodore W. Laetsch:** Conceptualization, Investigation. **Andrew J. Bauer:** Conceptualization, Writing – review & editing, Funding acquisition. **Aime T. Franco:** Conceptualization, Writing – original draft, Investigation, Supervision, Funding acquisition, Project administration.

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