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Pan-TRK immunohistochemistry as a tool in the screening for *NTRK* gene fusions in cancer patients

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ABSTRACT

Therapy with TRK inhibitors is a tumor-agnostic treatment directed against specific molecular changes rather than cancer type. *NTRK* fusions are rare in most prevalent cancers, accounting for less than 0.5% of cases. However, there is a group of rare cancers in which *NTRK* fusion is more prevalent. These include secretory carcinoma of the breast and salivary gland, childhood sarcomas, such as infantile fibrosarcoma, and cellular and mixed congenital mesoblastic nephroblastoma. The most common rearrangement pertains to *NTRK3* and the most common fusion gene is *ETV6*. Identifying patients with *NTRK* gene fusions who would likely benefit from targeted therapy with TRK inhibitors requires practical diagnostic tools and an appropriate management strategy of diagnostic trajectory. The fusions can be detected by molecular biology techniques or pan-TRK immunohistochemistry. The latter detects *NTRK1/2/3* gene fusions independently of the resulting fusion gene but does not determine which of them has been rearranged or what the fusion partner is. The sensitivity and specificity of the method reach 97% and 100%, respectively. Other advantages include the relatively low cost, short duration of examination, and broad accessibility of immunohistochemistry laboratories. These characteristics make this method a useful screening tool for detecting patients with *NTRK* gene fusions.

Keywords: *NTRK* genes, TRK inhibitors, diagnostic methods; immunohistochemistry

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Cancers with *NTRK* gene fusions as a therapeutic target for TRK inhibitors

In recent years, apart from the methods used so far in the treatment of oncological patients, such as surgical treatment or radio- and chemotherapy, an increasing role is played by targeted therapy, including "tumor-agnostic" therapy, directed at specific molecular changes and not cancer type [1, 2]. Tropomyosin receptor kinase (TRK) inhibitors are examples of such therapies [3, 4].

Neurotrophic TRKs are transmembrane tyrosine kinases that are essential for regulating nerve cell growth, proliferation, and differentiation. These include three groups of proteins: TRKA, TRKB, and TRKC, encoded by *NTRK1*, *NTRK2*, and *NTRK3*, respectively [5]. The *NTRK* genes can be rearranged during carcinogenesis. The *NTRK* fusion combines sequences coding for TRK proteins with sequences of other genes, leading to new active protein production [6]. In tumors with *NTRK* gene fusion, constitutive (ligand-independent) activation of intracellular biological pathways leads to a signaling cascade that controls cell cycle progression, proliferation, apoptosis, and/or survival of cancer cells [7, 8].

Tropomyosin receptor kinase inhibitors can be used in patients with confirmed *NTRK* gene rearrangement, regardless of cancer type [3, 5]. Clinical trials with a TRK inhibitor, entrectinib, have shown effectiveness

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Table 1. Methods for detecting *NTRK* gene fusions in tumors

	Sensitivity	Specificity	Detection of all fusions	Detection of fusion partners	Detection of protein expression
IHC	Relatively high*	Relatively high*	Yes	No	Yes
FISH	High	High	One per probe	One per probe	No
RNA NGS	High	High	Yes	Yes	Yes
DNA NGS	Moderate	High	Yes	Yes	No

*Depending on tumor morphology; FISH — fluorescence *in situ* hybridization; IHC — immunohistochemistry; NGS — next-generation sequencing

in treating diverse types of cancer, both locally advanced and generalized [9].

Cancers with *NTRK* gene fusions are rare, regardless of age group, and account for up to 0.3% of all malignancies [10]. *NTRK* gene fusions have been described in over 40 types of solid tumors [11], including pulmonary, colorectal, breast, and thyroid cancers; melanoma; glioblastoma; and several sarcomas [7, 12]. In addition, some rare tumors have a remarkably high incidence of *NTRK* fusions (> 90%). In adults, these tumors include secretory breast and salivary gland cancer, whereas, in children, they include infantile fibrosarcoma, secretory cancer of the salivary gland, and cellular and mixed congenital mesoblastic nephroblastoma [13, 14].

NTRK fusion detection methods

The infrequent occurrence of tumors with *NTRK* gene fusion requires practical diagnostic tools and appropriate diagnostic strategies [6, 7]. These fusions can be detected by immunohistochemistry (IHC), fluorescence *in situ* hybridization (FISH), reverse transcriptase polymerase chain reaction (RT-PCR), and next-generation sequencing (NGS) [7, 14]. These methods have different sensitivities, specificities, strengths, and limitations (Tab. 1).

Using pan-TRK IHC, *NTRK1/2/3* gene fusions are detected independently of the resulting fusion genes. However, it is not possible to determine the fusion partner or the rearranged *NTRK* gene. The sensitivity of the method varies between 75% and 97%, and specificity ranges from 92% to 100%. The advantages of the pan-TRK IHC technique include the relatively low cost of the test, short execution time, and availability of IHC laboratories. Due to the above qualities, this technique can be used to screen patients for *NTRK* fusion [6, 15].

Fluorescence *in situ* hybridization is a widely used diagnostic method that allows for the detection of chromosomal rearrangements. Fusion probes detect a specific type of fusion gene, such as *ETV6-NTRK3*, or break-apart probes that detect breaks such as those in *NTRK3*. However, FISH cannot determine whether the resulting fusion gene encodes a productive in-frame chimeric transcript or not. The recommendations for detecting the *ETV6-NTRK3* fusion gene are the same as the general principles of the FISH method for detecting fusion genes. They include counting the fluorescent signals in at least 50 randomly selected, non-overlapping tumor cell nuclei by at least two experienced specialists. The usefulness of FISH in cancer screening for *NTRK* fusions is limited because of the variety of fusion partners and the ability to evaluate only one gene rearrangement at a time. This method may help detect the *ETV6-NTRK3* fusion gene in tumors where this gene is present in most cases, such as secretory breast and salivary gland cancers [6, 7].

RNA NGS allows the detection of fusion genes that are transcribed. The main limitation of this method is the instability of the RNA material, especially in archival paraffin blocks. Evaluating the quality of RNA is critical for distinguishing possible false-negative results. According to the literature, only approximately 55% of archival samples meet the quality control requirements before sequencing, and the probability of quality control failure increases with the age of the analyzed material [7, 16].

Targeted DNA NGS tests consisting of panels of selected genes are increasingly being used, including those detecting *NTRK1*, *NTRK2*, and *NTRK3* fusions. Although the DNA NGS method successfully detects gene rearrangements, not all *NTRK* fusions can be detected using targeted assays. *NTRK2* and *NTRK3* are particularly problematic, as they have large intronic regions [6]. Moreover, many *NTRK* fusions detected by DNA-based sequencing are of unknown functional significance and require confirmation by other assays such as RNA sequencing or IHC [6, 7].

Performance and interpretation of the pan-TRK IHC test

The IHC test aims to detect tumors with *NTRK* fusions, which will be subjected to further molecular analysis, usually using the DNA NGS technique. Therefore, special attention should be paid to pre-analytical factors

and the assay process to minimize false-negative rates [17]. Proper conduct of the respective phases of the study affects the credibility of the results.

The first step is to select the optimal material for testing. IHC should be performed using histopathological samples that were fixed in 10% buffered formalin. The fixation time depends on the size of the tested sample and is 6–48 hours for small materials and 24–72 hours for larger materials.

Among the available antibodies, the most frequently used and best-characterized clone is EPR17341 [7, 15]. This antibody detects the C-terminal region of TRK proteins A, B, and C, which are conserved in both the wild-type and fusion proteins. Although the expression of the wild-type TRK protein in most solid tumors is minimal and rare, the pan-TRK IHC assay does not distinguish between wild-type and fusion proteins. IHC determination should be performed following the staining protocol provided by the manufacturer [18]. In addition, negative and positive control stains should be performed each time to minimize the incidence of false positive and false negative results. A negative control is performed using rabbit monoclonal antibodies. A positive control is performed using a normal human appendix. The nerves and ganglion cells in the wall show a positive reaction in the pan-TRK IHC test, whereas other structures are not stained. Performing an external positive control allows for verification of the correctness of the IHC staining process, but it does not constitute control of the pre-analytical stage. Therefore, during the assessment of pan-TRK IHC preparations, attention should be paid to whether any neural structures would constitute an internal positive control [6, 14].

The pan-TRK IHC color reaction is highly variable and can be nuclear, perinuclear/nuclear membrane, cytoplasmic, cellular membrane, or a combination of these. In addition, the staining intensity varies from weak to strong. Any of the above types of staining, stronger than that in the background and present in at least 1% of tumor cells, is interpreted as a positive reaction [14, 15, 19]. The percentage of stained cells and the intensity of staining is higher at the periphery of the specimen and lower in the central part. This type of staining is related to pre-analytical factors such as material fixation. Therefore, pan-TRK IHC tests are best performed with a small amount of material, such as a core needle biopsy, rather than with postoperative material [14]. The most common type of staining observed is the cytoplasmic reaction, which is the most common source of false-positive results compared to other types of expression. Moreover, false-positive pan-TRK IHC results are more common in tumors with muscular and nervous differentiation (leiomyosarcoma, glioma, and neuroblastoma) [7, 14]. In addition, there is a link between the type of color reaction and the occurrence of a specific fusion gene. Positive nuclear staining is often associated with *ETV6-NTRK3* and *EML4-NTRK3* fusions, nuclear membrane staining with *LMNA-NTRK1* fusions, and cell membrane staining with *TPM3-NTRK1* and *TRAF--NTRK2* fusions [15].

As mentioned above, the sensitivity of the pan-TRK IHC test has been reported to be between 75% and 97%. Discrepancies in the obtained results may result from different study populations (cancer types and fusion genes present in them) and pre-analytical procedures. The false-negative rate was higher for *NTRK3* gene fusions (21–27%) than for *NTRK1* and *NTRK2* fusions (< 10%) [13].

NTRK gene fusion tumors in the context of pan-TRK IHC results

Common neoplasms with the rare occurrence of *NTRK* gene fusions

This group of cancers includes colorectal, pulmonary, and breast cancers, where *NTRK* gene fusions occur in fewer than 1% of cases [14]. Within the gastrointestinal tract, *NTRK* fusions have also been detected in cancers of the pancreas, biliary tract, liver, appendix, and gallbladder (20). The most commonly described fusion genes include *TPM3-NTRK1*, *LMNA-NTRK1*, *TPR-NTRK1*, and *ETV6-NTRK3* [15, 20]. In wild-type *BRAF*/*RAS* and high-grade microsatellite instability (MSI), an increase in *NTRK* fusions to approximately 5% has been observed [14]. In pan-TRK IHC, these tumors are usually characterized by strong cytoplasmic staining, which may be accompanied by perinuclear staining (*LMNA* fusion partner) or membrane staining (*TPM3* fusion partner) [15].

In non-small cell lung cancer (NSCLC), mainly glandular NSCLC, *NTRK* gene rearrangements have been detected (most commonly *NTRK1*). The prevalence of such detected fusions is less than 1% [8]. In pan-TRK IHC, strong nuclear and cytoplasmic staining is usually observed [14].

In adult thyroid cancers, *NTRK* gene fusions occur in 2–4% of cases, both in well-differentiated, poorly differentiated, and undifferentiated cancers. In the pediatric group, *NTRK* fusions are more common in papillary thyroid carcinoma (8–15%) [21, 22]. The most common fusion gene is *ETV6-NTRK3*. A positive granular cytoplasmic reaction is observed in pan-TRK IHC (Fig. 1A, B). The sensitivity of this method in thyroid cancers is low, and the rate of false-negative results varies between 25% and 50% and is more common in the case of *NTRK3* fusions [13].

Rare tumors with a low prevalence of *NTRK* gene rearrangements include glioblastoma multiforme [15, 23],

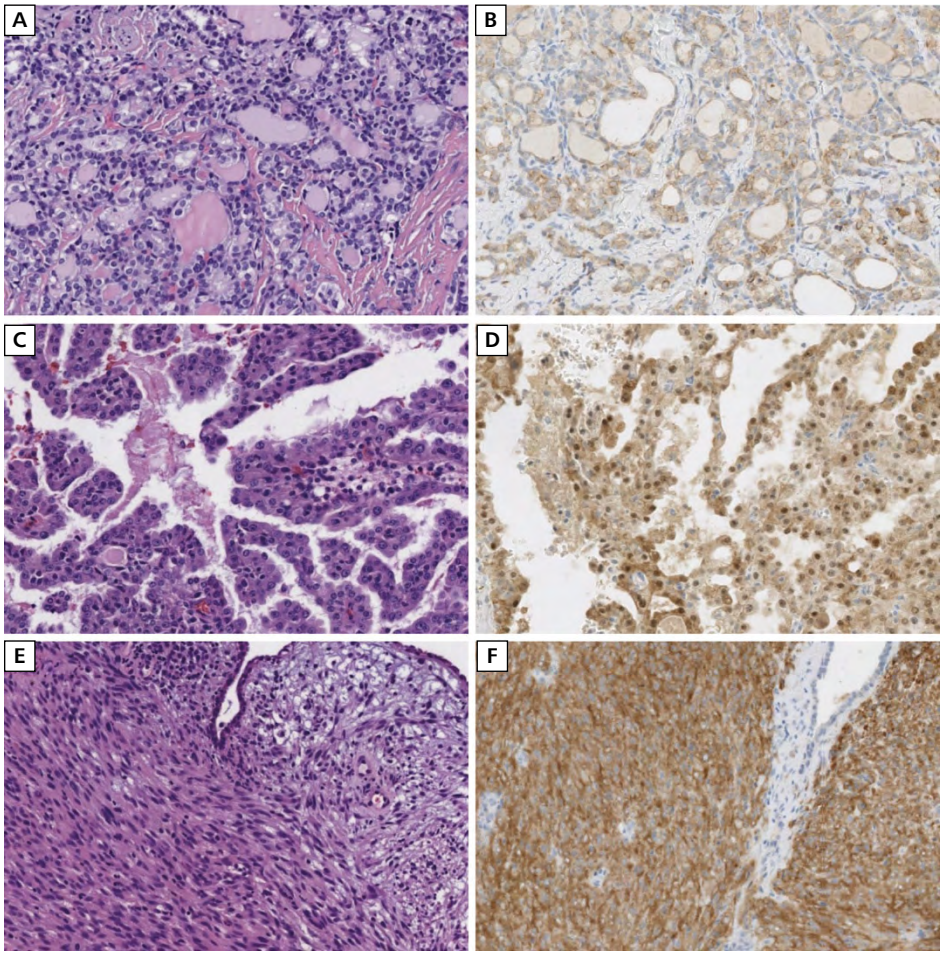

Figure 1. A. Papillary thyroid carcinoma, follicular variant with a confirmed *VIM-NTRK3* fusion gene, HE 200×; **B.** Papillary thyroid carcinoma, follicular variant with a confirmed *VIM-NTRK3* fusion gene; Pan-TRK IHC 200×, with perinuclear and cytoplasmic staining of weak and medium intensity; **C.** Secretory carcinoma of the salivary gland with the detected *ETV6-NTRK3* fusion gene, HE 200×; **D.** Secretory carcinoma of the salivary gland with the detected *ETV6-NTRK3* fusion gene; Pan-TRK IHC 200×, with a strong nuclear and cytoplasmic staining with a weak staining intensity; **E.** Spindle cell sarcoma of the cervix with a confirmed *EML4-NTRK3* fusion gene, HE 200×; **F.** Spindle cell sarcoma of the cervix with a confirmed *EML4-NTRK3* fusion gene; Pan-TRK IHC 200×, a strong cytoplasmic reaction is visible in the tumor cells, no color reaction in the overlying epithelium and the subepithelial layer

a malignant brain tumor with poor prognosis. In the case of this cancer, an effective anti-TRK-targeted therapy would be beneficial. The identification of rare glioblastomas with *NTRK* rearrangements requires reliable diagnostic tests. However, the pan-TRK IHC test is of limited use as a screening method in this group of cancers because of the high rate of false-positive results [23].

Rare tumors with a very high prevalence of *NTRK* gene fusions

This group of tumors includes cancers such as breast secretory carcinoma and salivary gland secretory carcinoma, as well as sarcomas, including infantile fibrosarcoma, cellular and mixed congenital mesoblastic nephroblastoma [14], and the recently described group of low-grade spindle cell sarcomas with *NTRK* gene rearrangements [24, 25].

Secretory carcinoma accounts for fewer than 0.05% of all infiltrating breast cancers and occurs mainly in adult women. In most cases, it is a triple-negative tumor or a tumor with low estrogen and progesterone receptor expression [26]. *ETV6-NTRK3* fusion occurs in over 90% of cases [27, 28]. Pan-TRK IHC is positive in 96% of cases. It is usually characterized by a strong nuclear reaction, and rarely by a nuclear-cytoplasmic reaction of varying intensity. *NTRK* gene rearrangements may also occur in approximately 10% of non-secretory breast cancers, most often *NTRK1* with various fusion

partners [29]. The occurrence of *NTRK* fusions in both secretory and non-secretory breast cancers supports the rationale for performing the pan-TRK IHC test as a screening method to detect patients for treatment with TRK inhibitors [30].

Secretory carcinoma with a morphology similar to that of the breast may develop in the salivary glands, most often in the parotid gland, usually in adults [31, 32]. In nearly 100% of cases, it is characterized by *ETV6* gene rearrangements, with *NTRK3* being the fusion partner in 90% of the cases [31]. On pan-TRK IHC, strong nuclear expression is seen, usually accompanied by low-intensity positive cytoplasmic staining (Fig. 1C, D). The pan-TRK IHC method is characterized by high sensitivity (91%) and specificity (nearly 100%) for the detection of secretory carcinoma of the salivary gland with *ETV6-NTRK3* fusion [33, 34]. In some cases, the nuclear reaction may be weakly intense or occur only focally, making the IHC test challenging. In addition, only cytoplasmic or membrane expression may be present in non-secretory salivary carcinomas [35]. A particular group is adenoid cystic carcinoma, in which a positive pan-TRK IHC test result is found in nearly 40% of cases (strong cytoplasmic staining), which does not correlate with the presence of NTRK gene fusions [36].

Sarcomas with widespread occurrence of *NTRK* gene fusions primarily include childhood cancer. Infantile fibrosarcoma is a fibroblastic tumor that typically affects superficial and deep soft tissues of the limbs, trunk, head, and neck. Analogous tumors in the kidney are termed cellular and mixed congenital mesoblastic nephromas. These cancers usually develop during the first year of life [37]. Approximately 90% of cases are characterized by the *ETV6-NTRK3* gene fusion [38]. Other less common molecular changes include *EML4-NTRK3* fusions or *NTRK1* and *NTRK2* gene rearrangements [16, 38]. Another group of spindle cell sarcomas with *NTRK* gene rearrangement is a newly described group of rare sarcomas with immunohistochemical co-expression of S100 and CD34 in the absence of SOX10 expression. This new category includes tumors previously described as lipofibromatosis-like neural and peripheral nerve sheath tumors. Most of these tumors develop superficially or deep within the extremities or trunk during the first two decades of life [38, 39]. In this group of sarcomas, *NTRK1* fusions with various partners such as *TPR* and *TPM3* are the most common [25]. In the described sarcomas, pan-TRK IHC reaction is positive in most cases (> 90%). In infantile fibrosarcomas, it is a strong nuclear reaction, whereas in neural tumors, similar to lipofibromatosis, it is usually a perinuclear and/or cytoplasmic reaction. In spindle cell sarcomas without *NTRK* fusion, pan-TRK IHC may only be positive in approximately 8% of the cases. The pan-TRK IHC test is characterized by high sensitivity in detecting childhood sarcomas with *NTRK* gene fusions and can be used as a screening method to qualify patients for therapy with TRK inhibitors [24].

A newly described adult sarcoma with an *NTRK* rearrangement is a cervical spindle cell sarcoma. It usually occurs in pre-menopausal women. The co-expression of S100 and CD34 characterizes the tumor cells. Desmin, estrogen receptor (ER), and progesterone receptor (PGR) are not expressed [40]. *NTRK1* and *NTRK3* rearrangements with different fusion partners occur in this group of sarcomas. Fusion genes described so far include but are not limited to *TPM3-NTRK1*, *LMNA-NTRK1*, *TPR-NTRK1*, *SPECC1L-NTRK3*, and *RBPMS-NTRK3* [40–42]. In pan-TRK IHC, TRK expression was observed in tumor cells in all cases (100%). The type of staining (cytoplasmic, perinuclear, or nuclear) may be associated with the formation of the fusion gene (Fig. 1E, F). It should be emphasized that in a low percentage of leiomyosarcomas (approximately 5%), in which there is no *NTRK* gene fusion, a positive pan-TRK IHC test is observed [40].

Tumors expressing pan-TRK IHC without *NTRK* gene fusions

A group of cancers is pan-TRK-positive IHC without *NTRK* gene fusion. Other specific molecular changes may characterize these tumors. Within the head and neck, this group of tumors includes bi-phenotypic sarcomas of the nose and paranasal sinuses (BSNS). The tumor comprises spindle-shaped cells that co-express S100 and SMA but do not express SOX10 [43]. Biphenotypic sarcomas of the nose and paranasal sinuses with non-specific pan-TRK IHC expression have been reported [44]. A characteristic feature of BSNS is rearrangement of the *PAX3* gene with the *MAML3* fusion gene [45]. Because of the microscopic image, S100 expression, and the possibility of a positive pan-TRK IHC result, it is necessary to differentiate this tumor from spindle-cell sarcomas with *NTRK* fusion. Other tumors in this area with frequent positive pan-TRK IHC without *NTRK* rearrangements are olfactory neuroblastoma, childhood small-round-cell tumors, such as Ewing's sarcoma [14, 46], adenoid cystic carcinoma of the salivary gland, and leiomyosarcoma.

Conclusions

Identifying cancer patients with *NTRK* gene fusions who could benefit from targeted therapy using TRK inhibitors requires adequate diagnostic tools. These tumors are diverse and rare. On the one hand, there is a group of rare cancers with widespread occurrence

of *NTRK* gene fusions, and on the other hand, there is a group of common cancers in which such molecular changes occur very rarely.

The pan-TRK method is characterized by high sensitivity and specificity, which may vary depending on the type of cancer. The ability to correctly interpret the results of the pan-TRK IHC test in correlation with the type of cancer is crucial in detecting cancer patients with *NTRK* gene fusions.

The pan-TRK IHC test can be used as a screening method because of its low cost, short execution time, and widespread use of IHC techniques. Pan-TRK IHC-positive tumors should be further investigated by molecular biology techniques to confirm the existence of *NTRK* fusions definitively.

Article Information and Declaration

Author contributions

M.D.: concept and design, analysis and interpretation of data, drafting of the manuscript and critical revision of the manuscript for intellectual content.
I.M.M.: critical revision of the manuscript for intellectual content.

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