Guidelines for the detection of NTRK fusions. A report from the Belgian Molecular Pathology Working Group

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INTRODUCTION

The Neurotrophic Tyrosine Receptor Kinase (NTRK) genes encode a family of receptor tyrosine kinases that serve important roles in cell survival, proliferation and cellular differentiation in healthy human tissues.[1]

Three NTRK genes are known: NTRK1, NTRK2 and NTRK3. In 1986, a Nature paper appeared where NTRK1 was initially identified in a transfection assay designed to screen for transforming sequences in DNA isolated from a human colon cancer.[2]

This work led to the identification of an oncogenic fusion transcript comprising the 5' exons of a tropomyosin gene (TPM3) and a sequence encoding an unknown protein tyrosine kinase.

Accordingly, this protein was called a "tropomyosin (related) receptor kinase", a TRK. Subsequently it was shown that the coding sequences partnering with TPM3 were 3' exons of NTRK1. The proteins resulting from the transcription of NTRK1, 2 and 3 are named tropomyosin (related) receptor kinase A, B and C respectively (TRKA, TRKB, TRKC).

It is now well established that chromosomal rearrangements involving NTRK1, 2 and 3 genes lead to functional gene fusions that act as oncogenic drivers in a broad range of tumour types.[3] These fusions have now been shown to be actionable genomic events, predicting response to therapy directed against TRK kinases. Larotrectinib, a selective pan-TRK inhibitor, and entrectinib, an inhibitor of TRK but also of ROS1 and ALK, are available.[4] D.S. Hong and colleagues reported on the updated analysis of the phase I and II studies of larotrectinib.[5] Their data further confirm the promising activity of larotrectinib (79% of patients achieved an objective response according to the investigator assessment), with a medium duration of 35,2 months and a medium PFS of 28,3 months. New inhibitors, such as repotrectinib, selitrectinib and DS-6051b, aimed at overcoming acquired resistance to larotrectinib and entrectinib are further expanding the therapeutic landscape of NTRK-rearranged tumours.

In almost all cases, the 5' region of a partner gene fuses with the 3' region of the NTRK genes. Driven by the active promotor of the 5' partner, the fusion transcript encodes for a fusion protein comprising the N-terminus of the 5' fusion partner and the C-terminal tyrosine kinase domain of the TRK receptor. Generally speaking, the 5' fusion partner contains dimerisa-

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tion domains. The resulting structure leads to a ligand independent dimerisation and constitutive activation of the kinase domain and associated downstream signalling.

Currently, nearly 80 different 5' NTRK fusion partners have been identified. In some solid tumours, gene fusion variants seem to affect the sensitivity of tyrosine kinase inhibitors. However, data for the differential activity of TRK inhibitors on various NTRK fusion partners are relatively scarce and this concept should be evaluated in further studies.

INCIDENCE OF NTRK GENE FUSIONS IN CANCER[6]

NTRK fusions are frequent oncogenic drivers in a very rare subset of tumours.

Infantile fibrosarcoma, the most common non-rhabdomyosarcoma soft tissue tumour seen in the first year of life, shows an incidence of up to 90% of NTRK fusions, mostly, but not exclusively, ETV6-NTRK3.

Secretory carcinomas of the breast and salivary gland harbour similar ETV6-NTRK3 fusions in > 90% of cases, making NTRK fusion detection also a diagnostic test.

In addition, in congenital mesoblastic nephroma, a rare spindle cell tumour of the kidney in new-borns or young infants, ETV6-NTRK3 fusions can be found in a high incidence.

Looking at thyroid carcinoma and brain tumours, the younger the patient, the higher the incidence of NTRK fusions. NTRK fusions have been detected in 26% of paediatric papillary thyroid carcinomas, while in adults, less than 10% show NTRK fusions. In an analysis of 112 paediatric high grade gliomas, *Wu et al.* reported 8 (7%) with NTRK fusions, while in a large study of 390 predominantly adult gliomas, NTRK fusions were identified in eight tumours (2%).[7,8]

In the more common cancers such as breast cancer, colorectal and non-small cell lung cancers, the incidence is less than 1%. Regarding colorectal cancer, a study of 4569 cases showed that only 0.2% were positive when screening with TRK IHC.[8] When excluding MSI positive patients, this number is further reduced to less than 0.02%. By restricting testing to MSI positive, RAS and *BRAF* wild type colorectal cancers, the percentage can be augmented to 40%.[9]

A study of 7,008 colon cancers screened for TRK expression also showed immunohistochemical TRK positivity in sixteen (0.23%) cases. A DNA mismatch repair-deficient phenotype was seen in thirteen of these sixteen cases. There was a clear female predominance.[10]

In a recent paper; Antonescu *et al.* reported a particular subtype of uterine non-leiomyomatous, non-endometrial stromal sarcoma characterised by the presence of NTRK fusions. The authors state that this presents a new entity, which is reminiscent of the subdivision of anaplastic large cell lymphoma in ALK positive, and ALK negative cases.[11]

DETECTION OF NTRK FUSIONS BY NGS[6,12,13]

The NTRK genes are located on different chromosomes.

While NTRK1 is located on chromosome 1 (1q23.1), NTRK 2 is located on chromosome 9 (9q21.33) and NTRK3 on chromosome 15 (15q25.3).

NTRK1 is the smallest of the three NTRK genes.

What makes the other two NTRK genes molecularly challenging is the fact that they include several exceptionally large introns, covering genomic regions seventeen to eighteen times longer than that of NTRK1. Incomplete intron coverage in DNA NGS assays for NTRK2 and NTRK3 could explain why a higher number of fusion partners have been identified for NTRK1 compared with NTRK2 and NTRK3.

Moreover, the large size but also the high repetitive element content and the high GC content of certain NTRK2 and NTRK3 introns make a DNA hybridisation capture design to achieve optimal sensitivity technically infeasible.

More than half of the NTRK1 gene fusion partners are localised to chromosome 1, consistent with intrachromosomal rearrangement, where for NTRK2 and 3, the fusion events are more interchromosomal.

DNA sequencing examines the exonic regions of many genes simultaneously for mutations.

The platforms, chemistry and bioinformatic pipelines used in these DNA sequencing assays are variable. The first question to be asked is if NTRK1, 2 and 3 are covered and how well they are covered, in particular at breakpoints since the test needs to detect fusions.

As an example, for both the FoundationOne CDx and MSK-IMPACT assays, only the exonic regions of NTRK3 were covered, and because fusion breakpoints usually occur within introns, inadequate coverage of these introns resulted in false negatives. This situation has been (partially) corrected.

One advantage of current DNA-NGS testing is that mutation, deletions, insertions and to a certain level, fusions (depending on the panel used) and amplifications can be detected. If oncogenic drivers such as activating mutations in EGFR, *BRAF*, RAS, etc. can be detected, the probability that an NTRK fusion is present is near zero.

Therefore, due to mutually exclusivity, it may be possible to narrow down the cohort of common tumours that require fusion screening.

One caveat needs to be addressed. DNA-level rearrangements may not result in an expressed fusion protein. Comes in IHC,

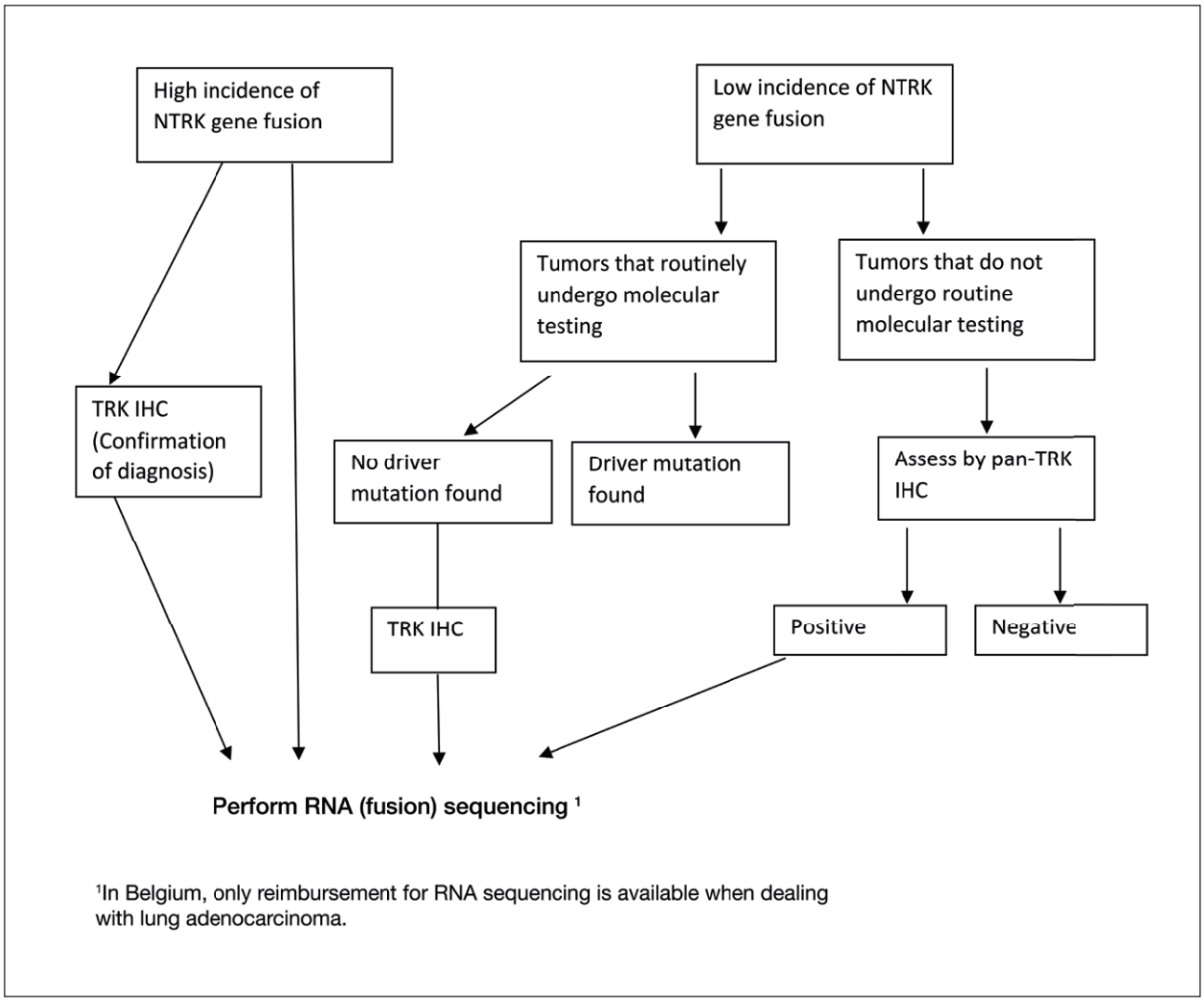

FIGURE 1. Diagnostic algorithm for the identification of NTRK fusion cancer in patients with advanced (unresectable or metastatic) solid tumours.

where expression of TRK protein is demonstrated. Other considerations for DNA-based NGS assays are that they require adequate DNA input (platform dependent) and an adequate amount of tumoral DNA.

An alternative approach is provided by RNA-based NGS. The benefit is that the intronic regions raising technical issues in the DNA-based NGS have been removed by splicing, allowing for more straightforward capture and/or amplification of all fused regions, particularly those involving NTRK genes. RNA sequencing can provide information about genes that are transcribed (at least as mRNA), but also on the different partners involved in the rearrangement.

One refined targeted amplicon-based approach is based on anchored multiplex PCR. Here, target enrichment is achieved through nested, unidirectional gene-specific primers allowing for the detection of fusion transcripts without knowledge of the 5'fusion partners and breakpoints. High quality RNA is needed, but in Belgium, the frequency of dropouts due to poor quality material seems to be limited. Adequate quality control measures are important to assess both the amount and quality of the RNA obtained. Metrics can include distribution of RNA fragment sizes, proportion of sequencing reads that are RNA versus DNA, and average sequencing coverage and depth.

Recently, platforms able to assess both DNA and RNA extracted from the same FFPE sample have been developed.

DETECTION OF TRK BY IMMUNOHISTOCHEMISTRY[6,14,15]

A high degree of homology exists between TRK proteins. Each has an extracellular region, including leucine-rich repeats, Ig-like C2 type I and Ig-like C2 type 2 domains, a transmembrane region and an intracellular region ("cytoplasmic tail").

This intracellular region contains the tyrosine kinase domain and ten evolutionarily conserved tyrosines. A pan-TRK rabbit monoclonal antibody EPR 17341 (Abcam, Cambridge, UK) was developed that recognises an undisclosed epitope close to the C-terminus of the three TRK proteins. This epitope is expected to be present in all functionally expressed TRK fusion proteins. Recent studies indicated a fairly high sensitivity and a high specificity. However, several caveats need to be formulated:

1. In a recent Belgian ring trial, it has been shown that technical concordance between participating labs was excellent.[16] Since more and more experience is gained, it is noted that interpretation of immunohistochemistry data may be more challenging than previously appreciated. In particular, it must be remembered that TRK can be physiologic expressed in neural and smooth muscle tissue. As such, IHC expression can be detected in fusion negative tumours with neural or smooth muscle differentiation. In addition, neuroendocrine tumour (like the pheochromocytoma case included in the ring trial) seem to express TRK in approximately 50% of cases in the absence of NTRK fusions.
2. The staining can be membranous, cytoplasmic, nuclear, dot like, faint. This problem was discussed in two Belgian NTRK meetings.
   The suggestion is that further meetings should be held where cases with RNA-NGS data and immunohistochemical data can be discussed so that pathologist can gain interpretation experience in a short time.
3. Decreased sensitivity has been mentioned for NTRK3 fusions. In a large study of pan-TRK IHC 4,138 cases were examined, including 28 confirmed NTRK fusion positive cases. Although sensitivity was 88% and 89% for NTRK1 and NTRK2 fusions, respectively, only six of eleven cases with NTRK3 fusions were positive with clone EPR 17341. Personal experience confirms this finding.

Regardless of these caveats, the fact remains that IHC has the advantage of being inexpensive, having a rapid turnaround time of approximately one day, requiring as little as one unstained slide and working independent of tumour purity.

As from April 1st, there is reimbursement for TRK immunochemistry testing.

BELGIAN DIAGNOSTIC ALGORITHM FOR THE DETECTION OF NTRK GENE FUSIONS

Several guidelines have been published.[6,17,18]

Ideally, all patients with metastatic cancer should benefit from DNA and RNA sequencing. Due to the abominable reimbursement for NGS in Belgium, this is not realistic. In addition, the NGS convention is DNA-based, without considering the possibilities of RNA sequencing.

Recently, RNA sequencing is reimbursed, but only in lung cancer if and only if DNA sequencing does not demonstrate presence of an oncogenic driver.

A functional distinction has to be made between tumours with a low incidence of NTRK fusions and those with high incidence. The last group contains tumours that are very rare: infantile fibrosarcoma, secretory carcinoma of breast and of salivary gland, congenital mesoblastic nephroma. In these tumours, sequencing is mandatory, in first instance also as a (confirmatory) diagnostic test. In those tumours with a low incidence, a distinction has to be made between tumours that are routinely sequenced, and others.

If a driver mutation is found, then there is no need for further testing due to mutual exclusivity. We acknowledge reported cases where driver mutations and NTRK fusions have been described, but those cases seem to be very rare and we do not know much about the clinical benefit of NTRK inhibition in these cases. In case of tumours in which no routine clinical molecular testing is planned, screening with a pan-TRK IHC can be performed. If positive, RNA sequencing should be used because demonstration of an NTRK fusion is mandatory (vide supra).

Particular attention should be given to the clinical setting. One of the first questions to be addressed should be if there are adequate treatments available.

For example, regarding the case of metastatic follicular cell derived thyroid carcinoma. Since radioactive iodine treatment is an effective treatment, NTRK testing could be postponed until the tumour is resistant to this therapy.

In cases of other tumours, such as metastatic breast cancers, NTRK testing should be considered if the oncologist is running out of options (in cases were no HER2 amplifications or PIK3CA mutations or other drivers have been detected).

Ideally, in the future, every (metastatic) tumour will be tested by NGS for mutations and fusions with panels, which include rare fusions like NTRK also.

CONCLUSION

Due to the marked and durable responses of TRK inhibitors, such as larotrectinib and entrectinib, an active search for NTRK fusions is needed. NTRK fusions can occur in many different tumour types, but unfortunately, in common cancers, the incidence may be 0,1% to 2% of tumours. NTRK fusions can occur regardless of the age of the patient (but are more frequent in young patients) and responses are equal in both age groups. We have therefore proposed a diagnostic algorithm to facilitate the identification of patients with TRK fusion cancer, taking into account the Belgian situation.

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