Molecular pathology in bone and soft tissue tumors: a multifunctional key for diagnosis and prediction

Lam, S.W.

Chapter 8

NTRK fusions are extremely rare in bone tumors

Suk Wai Lam, Inge H. Briaire-de Bruijn, Tom van Wezel, Arjen H.G. Cleven, Pancras C.W. Hogendoorn, Anne-Marie Cleton-Jansen and Judith V.M.G. Bovée

Histopathology. 2021 Jun 20. doi: 10.1111/his.14432.

Abstract

Introduction

Due to the efficacy of tropomyosin receptor kinase (TRK) inhibitor therapy in tumors with rearrangements of neurotrophic tyrosine kinase receptor (*NTRK*) genes, there has been a surge in demand for *NTRK* fusion screening. To date, most studies involving mesenchymal tumors have been focusing on soft tissue tumors, while data on bone tumors are sparse. Hence, we aimed to explore the frequency of *NTRK* fusions in a large series of primary bone tumors.

Materials and methods

Immunohistochemical expression of pan-Trk was successfully assessed in 354 primary bone tumors using tissue microarrays (TMAs). In a selection of positive cases, additional molecular analysis for *NTRK* fusion analysis was performed using anchored multiplex PCR-based targeted NGS.

Results

Positivity was encountered in nineteen cases (5%) and included Ewing sarcoma (n=6, 33%), osteosarcoma (n=11, 13%) and giant cell tumor of bone (n=2, 3%). In all, except one case, cytoplasmic staining was observed. Weak staining was most often observed (n=13), while five cases showed moderate staining and one case showed focal strong staining. Molecular analysis was successful in six cases, which were all negative for *NTRK* fusions.

Conclusion

The likelihood of finding a *NTRK* fusion in bone tumors in clinical practice is extremely low. This may imply that, if more comprehensive large scale molecular studies confirm this, routine predictive *NTRK* testing in bone tumor patients with advanced disease may be reconsidered.

Introduction

The tropomyosin-related/receptor kinase (Trk) family consists of three transmembrane neurotrophin receptors TrkA, TrkB and TrkC and are encoded by *NTRK1*, *NTRK2* and *NTRK3*, respectively [1]. Oncogenic gene fusions involving these genes lead to a constitutive activation of Trk receptors and are targetable using small molecule inhibitors. Larotrectinib showed significant and durable antitumor activity in patients with *NTRK* fusion positive cancer, regardless of age or tumor type [2-4]. This has led to specific interest for *NTRK* testing, especially since clinical trials have shifted away from site-of-origin and histology-dependent designs towards basket trials, in which targeted therapy is evaluated in different diseases that share molecular alterations [5].

NTRK fusions have been found at high frequency and to be characteristic for several rare cancer types including sarcomas (i.e., infantile fibrosarcoma, secretory breast carcinoma and congenital mesoblastic nephroma). Moreover, there is an emerging group of mesenchymal tumors defined by *NTRK* fusions displaying a wide morphologic spectrum, variable risk of malignancy, and a nonspecific immunoprofile [6]. This also includes CD34-positive fibrosarcoma of bone in which *NTRK3* fusions were recently described in two cases [7]. In this specific category *NTRK* fusions are diagnostic and *NTRK* fusion detection should be performed [8].

In addition, there is an increased demand for *NTRK* fusion testing as predictive biomarker for potential treatment with TRK-inhibitors, irrespective of the tumor type. More common cancers have a low but significant frequency of *NTRK* fusions [1] and thus represent a sizeable at-risk patient population worth testing of *NTRK* fusions [5]. For sarcoma patients with locally advanced and unresectable or metastatic disease, the World Sarcoma Network (WSN) advises *NTRK* fusion testing using pan-Trk immunohistochemistry pre-screening only for those sarcoma types known to harbor a complex genome (e.g., osteosarcoma). In sarcomas with recurrent gene fusions (e.g., Ewing sarcoma) or amplifications as driver alterations, *NTRK* fusion testing should be restricted to research [8] since *NTRK* fusions are typically mutually exclusive with other drivers [5].

Since the proposed screening system is mainly based on the current knowledge of *NTRK* fusions in soft tissue sarcoma [8, 9], we aimed to explore the frequency of *NTRK* fusions in a large series of different bone tumors. According to WSN recommendations, we used immunohistochemistry as a first screening method, followed by molecular analysis using anchored multiplex PCR (AMP)-based targeted next-generation sequencing (NGS) for fusions in selected cases.

Materials and Methods

Case selection

Tissue microarrays (TMAs) of previously published cohorts were used to screen for *NTRK* fusions and included conventional chondrosarcoma (n=137), dedifferentiated chondrosarcoma (n=36), clear cell chondrosarcoma (n=20), mesenchymal chondrosarcoma (n=19), osteochondroma (n=9), enchondroma (n=11), osteosarcoma (n=123), angiosarcoma (n=26), Ewing sarcoma (n=20), giant cell tumor of bone (n=74) and aneurysmal bone cyst (n=6) [10-15]. Most TMAs contained at least three cores of a 1.5mm diameter of each sample to outweigh intratumoral heterogeneity. Samples were handled according to the ethical guidelines described in "code for Proper Secondary Use of Human Tissue in the Netherlands" in a coded (pseudonymized) manner, as approved by the LUMC ethical board (B17.020, B17.036, B20.064).

Immunohistochemistry

Immunohistochemistry was performed as described previously [11, 16]. For the titration of the antibody several dilutions were used on both neural tissue and a molecularly proven *NTRK*-fusion positive tumor of the parotid gland. In our study, a dilution of 1:200 showed the best signal to noise ratio. All slides were manually stained in one session. Microwave antigen retrieval in TRIS-EDTA (pH 9.0) was performed using deparaffinized sections preincubated with PBS/1% BSA/5% non-fat dry milk, followed by overnight incubation with the pan-Trk antibody (Abcam, USA, clone ERP17341, rabbit, 1:200) in PBS/1%BSA/5% non-fat dry milk. Detection using power vision poly-HRP (ImmunoLogic, the Netherlands) and visualization with a DAB+ substrate chromogen system (Dako, Glostrup, Denmark) followed. Lastly, slides were counterstained with hematoxylin, dehydrated and mounted.

For NTRK expression a previously published semi-quantitative scoring system was used [16]. Immunoreactivity was scored according to the location (cytoplasmic or nuclear), the intensity, (1 = weak, 2 = moderate, or 3 = strong) and the percentage of positive cells (1+ = 1%-25%, 2+ = 25%-50%, 3+ = 50%-75% and 4+ = >75%). Positivity of any intensity in ≥1% of cells was considered as positive. All slides were scored by two independent observers (SWL and JVGMB).

Fusion analysis

For selected cases, additional molecular analysis for *NTRK* fusion analysis was performed using AMP-based targeted NGS for fusion analysis. From frozen section, RNA was isolated using TRizol reagent (Life technologies, Carlsbad, CA) as per manufacturer's description. The cDNA library was prepared with the Archer® FusionPlex comprehensive thyroid and lung panel, which included primers for *NTRK1* (exon 1-14, 16), *NTRK2* (exon 4-17) and *NTRK3* (exon 1-12, 14-17), followed by

sequencing using the Ion S5™ system. The Archer analysis software (version 6.2.3) was used to analyze the produced libraries for presence of *NTRK* fusions.

Results

Pan-Trk immunohistochemistry

Immunohistochemistry was successful in 354 cases. In the remaining cases, TMA cores were lost during processing. Nineteen cases (5%) showed staining of any intensity in ≥1% of the cells and included Ewing sarcoma (n=6, 33%), osteosarcoma (n=11, 13%) and giant cell tumor of bone (n=2, 3%). In all, except one case cytoplasmic staining was observed. Most of the positive cases showed weak staining (n=13), five showed moderate staining and one showed strong staining (**Figure 1**). In twelve cases only staining in 1-25% of cells was observed, positivity in 25-50% and 50-75% of cells was seen in respectively four and two cases and in one case >75% of the cells were positive. The 335 remaining cases were negative (**Table 1**).

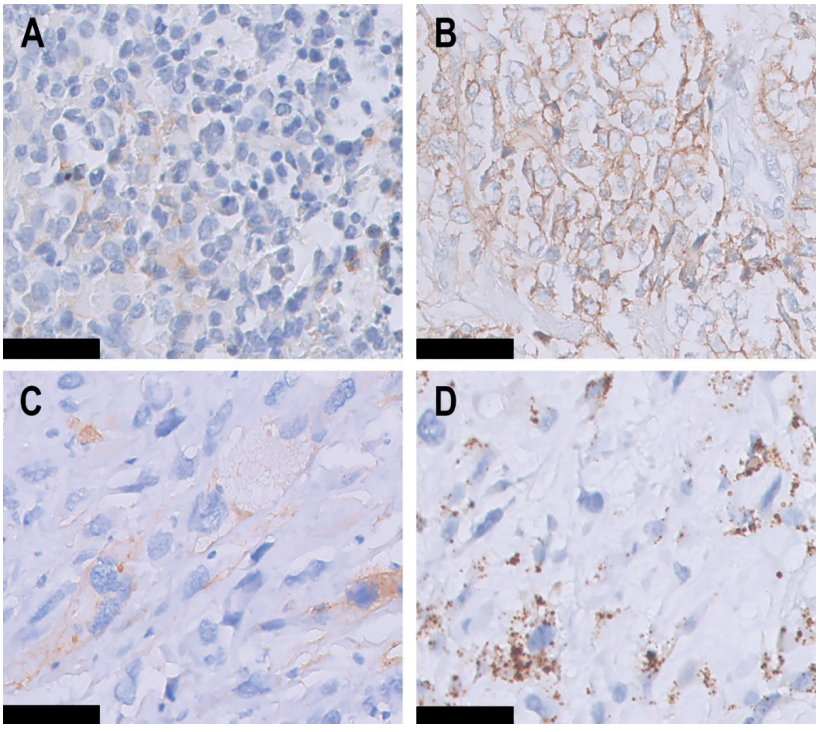

Figure 1. Immunohistochemical staining of pan-TrK in bone tumors. Weak (**A**) and moderate (**B**) cytoplasmic staining is seen in Ewing sarcoma. Moderate (**C**) and strong (**D**) cytoplasmic staining was seen in osteosarcoma. Molecular analysis for *NTRK* fusions was successful in case B and C, which were both negative for *NTRK* fusions. Scale bar: 50µm.

Molecular analysis for NTRK fusion

Molecular analysis was performed in cases with weak staining in >25% of cells and all cases with moderate or strong staining, which was successful in six cases and included two Ewing sarcoma, three osteosarcoma and one giant cell tumor of bone (**Table 1**). In three cases suitable material for molecular analysis was absent. All quality criteria were met, the coverage of *NTRK1-3* was sufficient and none of the cases showed a *NTRK* fusion. The relative RNA expression of *NTRK1-3* was low. Since *NTRK* fusions were absent in cases with moderate and strong staining, cases with weak staining in <25% of the cells were not further analyzed.

Table 1. Summary of immunohistochemical staining for pan-Trk

Tumor type	Total Cases	Positive (%)	Location	Intensity	Percentage
Osteosarcoma	88	11 (13)			
		1	N	weak	1+
		4	C	weak	1+
		2*	C	weak	2+
		1*	C	moderate	1+
		2**	C	moderate	2+
		1**	C	strong	1+
Ewing sarcoma	18	6 (33)			
		3	C	weak	1+
		1	C	weak	3+
		1*	C	moderate	1+
		1*	C	moderate	4+
Giant cell tumor of bone	61	2 (3)			
		1	C	weak	1+
		1*	C	weak	3+
Conventional chondrosarcoma	95	0			
Angiosarcoma	13	0			
Dedifferentiated chondrosarcoma	34	0			
Clear cell chondrosarcoma	16	0			
Mesenchymal chondrosarcoma	11	0			
Osteochondroma	7	0			
Enchondroma	6	0			
Aneurysmal bone cyst	5	0			

1+, 1%-25%; 2+, 25%-50%; 3+, 50%-75%; 4+ >75%; C, cytoplasmic; N, nuclear;
Pan-Trk positivity was defined as staining in ≥1% of cells of any intensity.
* Cases in which molecular analysis for *NTRK* fusions was successful and negative.
** Cases in which molecular analysis for *NTRK* fusions was unsuccessful.

Discussion

This study provides a comprehensive immunohistochemical evaluation of pan-Trk expression as a surrogate marker for *NTRK* fusions in a large series of bone tumors, including osteogenic, chondrogenic tumors and Ewing sarcoma, thereby representing the three most common bone sarcomas. Following WSN recommendations [8], we used pan-Trk immunohistochemistry as a screening method for *NTRK* fusions to explore the frequency of *NTRK* as a targetable therapeutic option in well characterized bone tumors and showed that *NTRK* fusions are virtually none-existing.

NTRK fusions were not identified in 354 bone tumors after pre-screening with immunohistochemistry, which is in line with the low frequency in literature reporting only a handful of anecdotal cases [8]. Besides one *NTRK*-fusion positive bone sarcoma that was found among a diverse set of pediatric malignancies (1.1%), of which the subtype was not further specified [17], two other *NTRK*-fusion positive bone sarcomas were described. These osteosarcoma and dedifferentiated chondrosarcoma patients were enrolled in a clinical trial and received Larotrectinib [4]. Interestingly, in another study of 113 osteosarcoma patients that were sequenced, three cases harbored a *NTRK* fusion of which the chimeric transcript appeared to be non-functional and likely represented randomly occurring passenger alterations [18].

Several caveats should be considered when using pan-Trk immunohistochemistry as a first screening method for *NTRK* fusions, including a variable staining pattern and intensity. While the antibody appears to have 100% specificity in carcinoma of colon, lung and thyroid, the specificity in sarcoma is much lower. False positive staining is especially frequent in tumors with smooth muscle and neural differentiation [5, 19]. In our study, positivity was encountered in 5% of all cases, mostly in osteosarcoma and Ewing sarcoma, while *NTRK* fusions were absent in all sequenced tumors. Although immunoreactivity of pan-Trk in osteosarcoma has not been studied by others, false positivity in Ewing sarcoma was previously described: pan-Trk expression was often present in tumors within the small blue round cell category, including desmoplastic small round cell tumors (100%), Ewing sarcoma (20-33%) and sarcomas with *BCOR* genetic abnormalities (60%-100%) [19, 20]. For the latter category of tumors, it was shown that pan-Trk expression was caused by *NTRK3* gene upregulation [20].

Our cohort includes a large proportion of sarcomas with complex genome (osteosarcoma, high-grade and dedifferentiated chondrosarcoma) for which the WSN recommends *NTRK* fusion testing with immunohistochemistry pre-screening in patients with advanced disease. Our results indicate that the subgroup of sarcoma patients that may become eligible to *NTRK* inhibition is extremely small or even none-existent. However, it should be noted that not all bone tumor types were assessed

for pan-Trk immunohistochemistry and thus that the frequency of *NTRK*-fusions in these tumors remain unknown. Also, since the reported sensitivity of pan-Trk immunohistochemistry in sarcoma is around 80%, the possibility of false negativity in our series cannot be completely ruled out, since molecular data on *NTRK* fusions in our cohort is not available [19]. The false negativity rate may be even higher in tumors with *NTRK3* fusions [19]. Another limitation of this study is that rare oncogenic activating splice variants of the *NTRK1* gene, which have been described in neuroblastoma and acute myeloid leukemia could potentially be missed, since the variant calling pipeline used for *NTRK* fusion analysis is not able to pick this up [1]. Lastly, the effect of decalcification on pan-Trk expression was not studied and therefore false-negative results due to decalcification cannot be ruled out completely. However, TMAs were shown to generate positive staining in previous studies [10, 11, 14, 16, 21, 22] and cases that were scored as pan-Trk positive were also decalcified.

To conclude, the likelihood of finding a *NTRK* fusion in bone tumors in clinical practice, even in tumors with complex genome lacking driver alterations such as osteosarcoma, is extremely low. This may imply that, if more comprehensive large scale molecular studies confirm this, routine predictive *NTRK* testing in bone tumor patients with advanced disease may be reconsidered.

Acknowledgements

L.G. Sand, D. Meijer, J. van Oosterwijk, D. van der Geest, S. Verbeke and Y.T. Sundara are acknowledged for contributing to construction of TMAs.

Author contributions

The study was designed, written and reviewed by S.W. Lam and J.V.M.G. Bovée. All authors contributed to the data collection, data analysis and interpretation. The manuscript was approved by all authors.

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