A malignant inflammatory myofibroblastic tumor of the hypopharynx harboring the 3a/b variants of the *EML4-ALK* fusion gene

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Abstract. Inflammatory myofibroblastic tumors (IMT) in the head and neck region are rare neoplasms that generally mimic benign/low-grade neoplasms. Overexpression of anaplastic lymphoma kinase (*ALK*) has been reported in 50% of IMT cases, secondary to *ALK* activation by structural rearrangements in the *ALK* gene, which results in a fusion protein with echinoderm microtubule associated protein like 4 (*EML4)* in ~20% of cases. The present study describes a case of a 74-year-old woman with a malignant IMT in the right posterior hypopharynx harboring a previously unreported chromosomal rearrangement resulting in *EML4* and *ALK* gene fusion. Strong ALK immunoreactivity was observed in neoplastic cells, while fluorescent *in situ* hybridization combined with fluorescent fragment analysis and direct sequencing identified the first case of the 3a/b variants of the *EML4-ALK* fusion gene in IMT. The results of the current study highlight the uncommon occurrence of ALK-positive IMT in the head/neck region and demonstrate the importance of integrating different molecular methodologies to identify unequivocal gene fusion characterization.

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Introduction

Inflammatory myofibroblastic tumors (IMT) are distinctive tumor entities described in almost all anatomical sites under several definitions, including inflammatory pseudotumor and plasma cell granuloma. IMT generally shows a benign clinical outcome and is common in children and young adults, although the age of occurrence is extremely broad (1). Presenting symptoms are related to the site of the primary tumor origin, although fever, weight loss, malaise and night sweats are also reported. Surgical resection is the mainstay of treatment, while chemoradiotherapy is generally ineffective (1).

Approximately half of IMT patients carry rearrangements of the anaplastic lymphoma kinase (*ALK*) gene (2-5). ALK is a tyrosine kinase receptor, which is typically expressed in the central nervous system (3,4). Fusion of the *ALK* gene with different partners may result in the overexpression of ALK and activation of its kinase domain (6-8). Identification of *ALK* fusion genes may support a diagnosis of IMT, and additionally allow initiation of an effective treatment regimen with ALK inhibitors (6,7). IMTs occurring in the head and neck region are extremely rare and are more commonly located in the larynx, orbit, paranasal sinus, trachea and parotid gland. The present study describes the characterization of the 3a/b variants of the echinoderm microtubule associated protein like 4 (*EML4)-ALK* gene fusion, which has not been reported previously in IMT of the hypopharynx.

Materials and methods

Case description. A 74-year-old, non-smoking woman was hospitalized after presenting with weight loss, progressive dysphagia, odynophagia and globus sensation. The patient had an unremarkable medical past and routine laboratory tests, serum tumor markers, a QuantiFERON® tuberculosis test and antineutrophil cytoplasmic antibodies test came back negative. A total-body computed tomography scan revealed a 5-cm
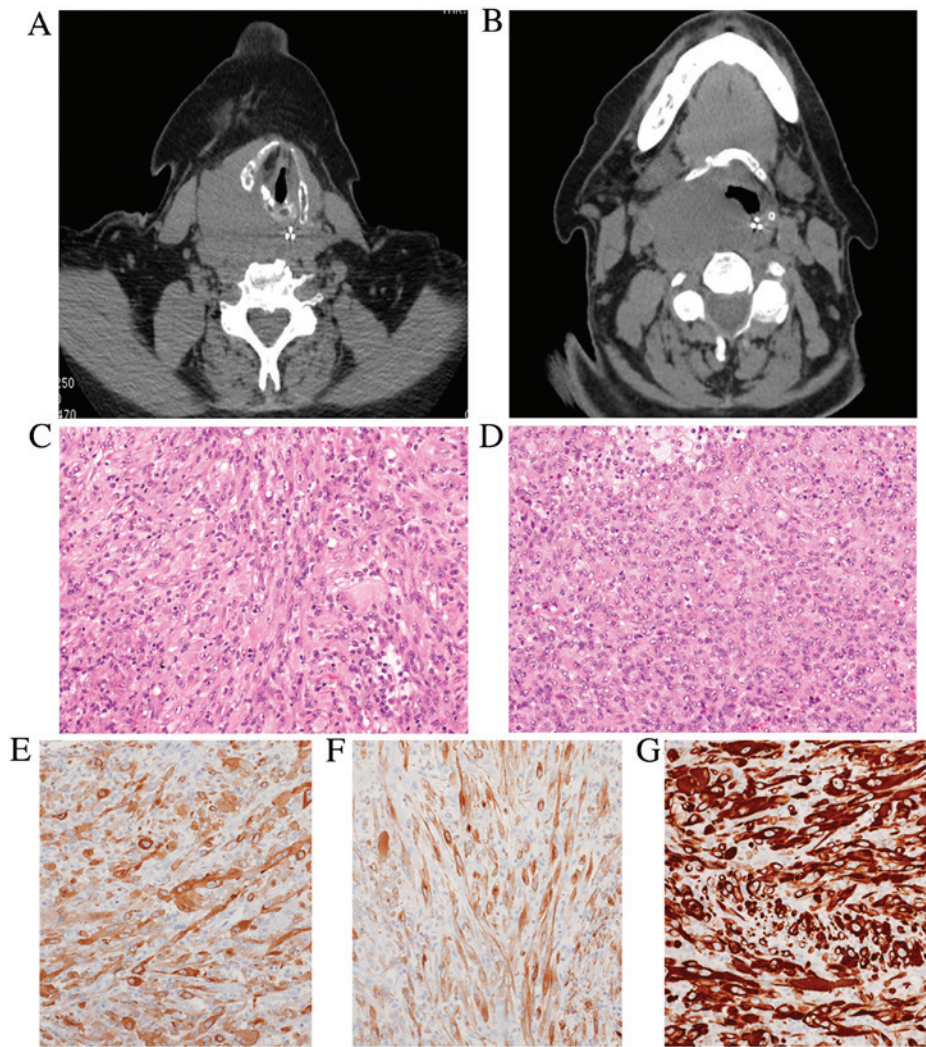
Figure 1. Computed tomography scans of the (A) head and (B) neck region revealed a poorly-circumscribed mass (maximum diameter, 5 cm), which involved the posterior wall of the right hypopharynx, dislocating the oesophagus and tracheal axis and infiltrating to the soft palate. Hematoxylin and eosin staining of tumor tissue sections revealed that the tumor was composed of (C) spindle-shaped or (D) epithelioid myofibroblasts arranged in fascicular or storiform-like patterns, admixed with scattered lymphocytes, plasma cells and foamy histiocytes (magnification, x150). Immunohistochemistry demonstrated that the tumor cells expressed (E) smooth muscle actin, (F) desmin and (G) ALK (magnification, x150). ALK, anaplastic lymphoma kinase.

mass situated at the right posterior wall of the hypopharynx (Fig. 1A). No other lesions were identified.

Radiological examinations demonstrated the impossibility to perform a complete surgical resection since the tumor exhibited infiltrative margins and involved adjacent structures. However, the patient was symptomatic due to the progressive occlusion of the pharynx, which could result in a life-threating condition.

Diagnostic and palliative surgical resection was performed, and the specimen was fixed in 10% neutralized formalin for 24 h at room temperature and embedded in paraffin blocks, which were subsequently cut into 3-μm sections for routine hematoxylin-eosin staining. Examination under a light microscope revealed the proliferation of spindle-to-epithelioid cells intermingled with a mixed inflammatory infiltrate represented by lymphocytes, plasma cells and foamy histiocytes (Fig. 1B-F). Mitotic rate was extremely high (>5 mitoses/high-power field) and focal ulceration of the over lining mucosa was observed. Tumor cells strongly expressed smooth-muscle actin, desmin and ALK (clones

ALK1, 5A4 and D5F3), while there was no immunoreactivity for pan-cytokeratins, S100 protein, p63, cluster of differentiation (CD) 21, CD35, CD68 and human herpes virus (HHV)-8. *In situ* hybridization with an Epstein-Barr virus-encoded small RNA probe came back negative. Subsequently, a diagnosis of IMT with epithelioid features was confirmed. In accordance with the guidelines of the Hospital Institutional Review Board, consent for anonymous research use of the tumor specimen and data collection was obtained. The patient refused enrollment in a clinical trial, which included treatment with ALK inhibitor, and subsequently succumbed to disease progression at 11 months post-diagnosis.

Tissue specimen and cell lines. A representative neoplastic formalin-fixed paraffin-embedded (FFPE) tissue sample from the 74-year-old woman was used as biological material. NCI-H2228 and MRC5 cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA) and Sigma-Aldrich (Merck Millipore, Darmstadt, Germany), respectively. The cell lines were cultured under

Table I. *ALK* fusion partners in IMT.

Authors, year	IMT location	Patient age/gender	ALK fusion	Refs.
Lawrence *et al*, 2000	Abdomen	23 years/F	*TMP3-ALK*	(3)
	Lung	30 years/F	*TMP3_ALK*	(3)
	Abdomen	1 year/M	*TMP4-ALK*	(3)
Bridge *et al*, 2001	Neck	3 years/F	*CLTC-ALK*	(16)
	Pelvis	37 years/M	*CLTC-ALK*	(16)
Cools *et al*, 2002	Abdomen	4 months/M	*CARS-ALK*	(17)
Ma *et al*, 2003	Abdomen	1 year/M	*RANBP2-ALK*	(18)
	Abdomen	7 months/M	*RANBP2-ALK*	(18)
Debiec-Rychter *et al*, 2003	Bladder	46 years/M	*ATIC-ALK*	(19)
Panagopoulos *et al*, 2006	Abdomen	23 years/M	*SEC31L1-ALK*	(20)
Takeuchi *et al*, 2011	Lung	45 years/M	*PPFIBP1-ALK*	(21)
	Lung	34 years/F	*PPFIBP1-ALK*	(21)
Wang *et al*, 2012	Neck	7 years/F	*DCTN1-ALK*	(22)
Lovly *et al*, 2014	Lung	38 years/F	*EML4-ALK*	(12)
	Mesentery	11 years/F	*LMNA-ALK*	(12)
	Shoulder	1 year/F	*PRAKAR1A-ALK*	(12)
	Bladder	26 years/F	*FN1-ALK*	(12)
	Pelvis	14 years/M	*TFG-ALK*	(12)

ALK, anaplastic lymphoma kinase; IMT, inflammatory myofibroblastic tumors; F, female; M, male.

the recommended conditions. Briefly, the NCI-H2228 cell line was cultured in RPMI-1640 medium containing 1% antibiotics (penicillin/streptomycin) and 10% fetal bovine serum (FBS) at 37˚C in 5% CO_2. The MRC5 cell line was cultured in Eagle's Minimum Essential Medium supplemented with 2 mM glutamine, 1% non essential amino acids and 10% FBS at 37˚C in 5% CO_2. All media were purchased from Euroclone (EuroClone SpA, Milan, Italy). RNA and cell blocks from the H2228 and MRC5 cell lines were utilized as positive and negative controls for fluorescent *in situ* hybridization (FISH) analysis and variants 3a/b *EML4-ALK* fusion.

Immunohistochemistry. Serial 4-μm-thick sections were obtained from FFPE blocks representative of tumor tissue for immunohistochemical analysis. All reactions were performed using a BenchMark XT fully automated immunostainer (Ventana Medical Systems, Inc., Tucson, AZ, USA) and antibody incubation was for 8 h at 37˚C. The following antibodies were used: Pan-cytokeratins (prediluted; cat. nos. 760-2521 and 790-4555 for clones AE1 and CAM5.2, respectively; Ventana Medical Systems, Inc.), smooth-muscle actin (prediluted; cat. no. 760-2833; Ventana Medical Systems, Inc.), desmin (prediluted; cat. no. 760-2513; Ventana Medical Systems, Inc.), p63 (prediluted; cat. no. 790-4509; Ventana Medical Systems, Inc.), CD21 (prediluted; cat. no. 760-4245; Ventana Medical Systems, Inc.), CD35 (prediluted; cat. no. 760-4439; Ventana Medical Systems, Inc.), HHV-8 (prediluted; cat. no. 760-4260; Ventana Medical Systems, Inc.), ALK (prediluted; cat. nos. 790-2918 and 790-4796 for clones ALK1 and D5F3, respectively; Ventana Medical Systems, Inc.), ALK

(1:50 dilution; cat. no. NCL-L-ALK; Novocastra; Leica Microsystems, Inc., Buffalo Grove, IL, USA), S100 (prediluted; cat. no. 760-2523; Ventana Medical System, Inc.) and CD68 (prediluted; cat. no. 790-2931; Ventana Medical Systems, Inc.).

Cytogenetics and ALK break apart FISH assay. FISH assay was performed on 4-μm FFPE sections using Vysis ALK Break Apart FISH Probe kit (Abbott Molecular, Abbott Park, IL, USA). The hybridized slides were reviewed on an Olympus IX-50 microscope (Olympus Corporation, Tokyo, Japan) at x100 magnification.

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR). Total RNA extraction from tumor tissue was performed with the RecoverAll™ Total Nucleic Acid Isolation kit (Ambion; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. A total of 500 ng RNA was reverse transcribed using the SuperScript® III First-Strand Synthesis system (Invitrogen; Thermo Fisher Scientific, Inc.) for RT-PCR reactions. To analyze variants 1 and 2, two specific synthetic DNA double strands of the *EML4-ALK* fusion region were synthesized. Amplified products were characterized by cloning PCR products into the StrataClone™ PCR Cloning Vector pSC-A (Agilent Technologies, Inc., Santa Clara, CA, USA), straight amplifying from crude lysates of single bacterial colonies was performed using the following primers: EML4_exon6_F, 5'-CTGCAGACA AGCATAAAGATG-3'; and ALK_exon20_R, 5'-GCTTGC TCAGCTTGTACTC-3'. Direct sequencing was performed using the BigDye® Terminator v3.1 Cycle Sequencing kit
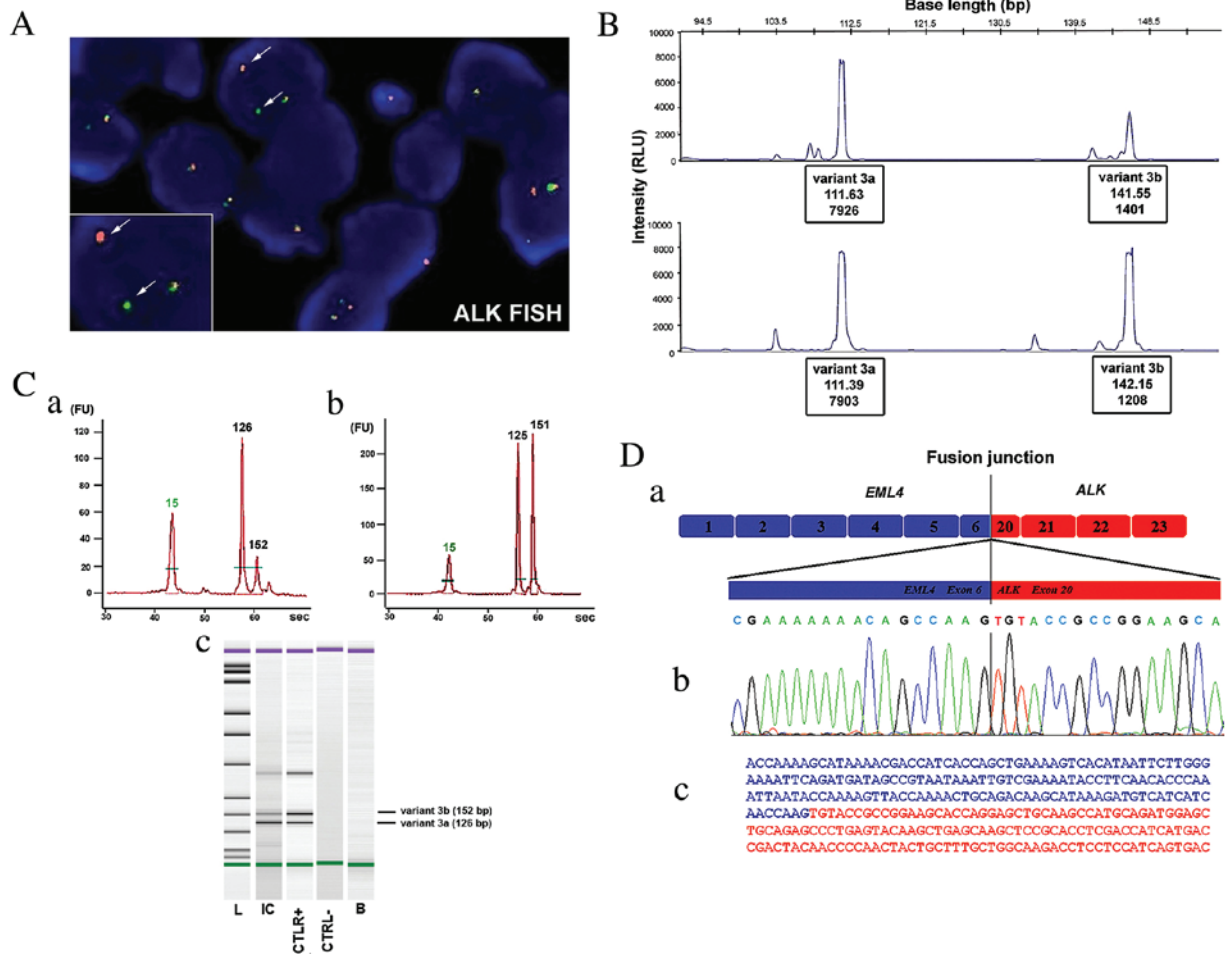
Figure 2. (A) Molecular cytogenetic analysis of the FFPE tumor sample, in which the disrupted *ALK* locus was identified by fluorescent *in situ* hybridization analysis. The white arrows indicate the split of the green and red probe signals (hybridizing to 5' and 3' regions of *ALK*, respectively) in the tumor cells. (B) Fluorescent fragment analysis in the tissue specimen positive for *ALK* translocation. RNA from the FFPE tissue (upper panel) and NCI-H2228 cell line (lower panel) was amplified by RT-PCR using an unlabeled *EML4* exon 6 forward primer and a fluorescein amidite-labeled *ALK* exon 20 reverse primer. Two peaks were observed (at 112 and 142 bp) indicating the presence of the 3a/b variants of the *EML4-ALK* fusion. (C) Fluorescent fragment analysis of the (Ca) inflammatory myofibroblastic tumor tissue and (Cb) control NCI-H2228 cell line using the Agilent 2100 BioAnalyzer. Two different fluorescent peaks were observed at 126 and 152 bp, corresponding to the 3a/b variants of the *EML4-ALK* fusion. (Cc) The peaks were visualized on the gel resulting from the capillary microelectrophoresis. (D) *EML4-ALK* fusion variant 3a characterization. (Da) Schematic of the *EML4-ALK* fusion variant 3. (Db) Sequence electropherogram of the RT-PCR product. (Dc) Fusion junction of *EML4* exon 6 (blue) and *ALK* exon 20 (red) sequences. FFPE, formalin-fixed paraffin-embedded; ALK, anaplastic lymphoma kinase; RT-PCR, reverse transcription-polymerase chain reaction.

(Applied Biosystems; Thermo Fisher Scientific, Inc.) and an ABI PRISM® 3130 Sequence Detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.).

Fluorescent fragment analysis. Fluorescent PCR amplification was performed using a reverse fluorescein amidite-labeled primer specific to exon 20 of *ALK* and forward primers specific to exons 13-20 and 6 of *EML4* to amplify variants 1, 2 and 3a/b, respectively (8). As a control for RNA quality, primers specific to β-2-microglobulin were employed. RT-PCR products were size-fractionated by capillary electrophoresis in an ABI 3130 Genetic Analyzer (Applied Biosystems; Thermo Fisher Scientific, Inc.) and results were analyzed with GeneMapper® software v.4 (Applied Biosystems; Thermo Fisher Scientific, Inc.).

To confirm the results of fluorescent analysis, DNA fragments were analyzed by microchip electrophoresis using an Agilent 2100 Bioanalyzer with the DNA 1000 assay sizing reagent kit (Agilent Technologies, Inc.).

Results

Detection of ALK involvement in IMT. ALK rearrangement was assessed by FISH analysis. Single and multiple copies of the intact *ALK* fusion together with the abnormal split pattern were observed in the tumor cells of the tissues specimen with a frequency of 65% *ALK* rearranged gene (combined *EML4-ALK* fusion and 5'end-*ALK* deletion), (Fig. 2A).

Identification of the EML4-ALK 3a/b fusion variants. Two fragments of the expected length (112 and 142 bp) corresponding to the fusion 3a/b variants were detected during the first round of RT-PCR, which is designed to detect the most frequent 1,2 and 3a/b types of transcript. Using specific primers for the amplification of the *EML4-ALK* 3a/b variants fusion cDNA, the same two pair of PCR products were detected in the NCI-H2228 cell line and in the IMT patient sample. These molecular findings were confirmed using two different fluorescent fragment analysis methods; the first was

previously described by Sanders *et al* (8), (Fig. 2B) and the second uses novel, fast and efficient microchip electrophoresis to carefully detect *ALK* variation through the use of capillary microelectrophoresis and detection (Fig. 2C). Molecular cloning and direct sequencing of each PCR product confirmed the presence of the variant 3a fusion (linking exon 6 of *EML4* to exon 20 of *ALK*) and the 3b fusion, containing an insertion of 33 bp mapped to intron 6 of *EML4* that was located between the same exons of *EML4* and *ALK* (Fig. 2D).

Discussion

The World health organization (WHO) classification of soft tissue tumors defines IMT as the neoplastic counterpart among the wastebasket group of lesions previously known as inflammatory pseudotumors (9). IMT represents a proliferation of myofibroblastic cells, often intermingled with lymphocytes, plasma cells and histiocytes, which eventually forms a highly aggressive lesion (9). IMT is ubiquitous and margin-free surgical resection is considered the only curative treatment. By contrast, aggressive or metastatic lesions have no standard therapy and chemotherapy is usually ineffective.

In the head and neck region, the larynx is the most common location of IMT, while a few cases in the hypopharynx have been reported (10,11). Clinical and radiological differential diagnosis is broad and includes infections (tuberculosis and mycoses), Wegener's granulomatosis, amyloidosis and other malignancies, such as carcinoma, melanoma and salivary gland tumors (10,11).

Up to 70% of IMTs harbour *ALK* gene rearrangements resulting in the formation of a chimeric fusion protein, which is detectable by FISH or immunohistochemistry (2-5). ALK is a receptor-type protein tyrosine kinase that is currently being analysed by oncologists as an essential growth driver, which defines its potential susceptibility to ALK inhibitors (7,12-15). The oncogenic function of ALK occurs as a result of it forming a fusion protein with various gene partners through chromosomal translocations, most commonly *TPM3* or *TPM4* in IMT, resulting in oncogenic activation of ALK (3). Alternatively, a small inversion [inv2(2)(p21p23)] on the short arm of chromosome 2 leads to a functional *EML4-ALK* fusion-type tyrosine kinase (3-22) (Table I). Initially described in non-small cell lung carcinoma (15), this abnormality was also identified in ~20% of *ALK*-rearranged pulmonary IMT cases (5). To date, a total of 10 cases of *EML4-ALK* fusions in IMT have been reported (3,5,9). The present study described a *EML4-ALK* rearranged IMT located in the hypopharynx. By using two different fluorescent fragmentation analysis methods, the specific 3a/b variants were identified within the RNA of the tumor specimen and confirmed by standard Sanger sequencing. This description of the *ALK* fusion 3a/b variants in an IMT case, together with the few cases of *ALK-EML4* fusions reported in literature, corroborates the existing hypothesis that identical *ALK* fusions detected in different tumor types may drive an inappropriate activation of the same kinase signalling pathway, which could be oncogenic in disparate cellular lineages. In addition, the present case also noted the epithelioid appearance of the tumor cells, which could potentially lead to a dismal clinical course. Notably, Mariño-Enríquez *et al* (23) described ALK-positive

intra-abdominal IMT composed predominantly of sheets of round-to-epithelioid cells significantly associated with aggressive course with rapid local recurrences.

In conclusion, to the best of our knowledge, the present study describes for the first time the presence of the *EML4-ALK* 3a/b variant in a case of malignant IMT of the hypopharynx. This was achieved by integrating different methodologies, which is considered the most suitable molecular approach for gene fusion characterization. Furthermore, the identification of *ALK* rearrangement in cases of malignant IMTs may offer a rationale to adopt selective targeted therapies in this 'orphan' tumor.

Acknowledgements

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