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ORIGINAL ARTICLE

The Presence of ALK Alterations and Clinical Relevance of Crizotinib Treatment in Pediatric Solid Tumors

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Abstract Soft tissue sarcomas (STS) and neuroblastomas (NBL), are childhood malignancies still associated with poor prognoses despite the overall improvement in childhood tumor survival of the past decades. Anaplastic lymphoma kinase (ALK) inhibition is promising new strategy to improve the outcome of these pediatric tumors. Eighteen histologic samples of pediatric STS and 19 NBL patients were analyzed for ALK abnormalities using fluorescent in situ hybridization (FISH) with break-apart probes and immunohistochemistry (IHC). ALK alterations were presented in 20 of the 37 sections. The presence of ALK alteration in NBL samples were detected using IHC in 84,2% of all cases compared to 21,1% FISH positivity. In STS cases the results were less different (IHC 16,7% vs FISH 22,2%). The difference can be explained by the different type of molecular alterations. FISH method detected translocation and amplification, but not the point mutation of ALK gene. IHC confirmed the diagnosis by detecting the expression of ALK protein.After ALK positivity was proven, the effectiveness and safety of the crizotinib therapy was examined in 4 patients (1 alveolar rhabdomyosarcoma (RMA), 1 embryonal rhabdomyosarcoma (RME), 1 inflammatory myofibroblastic tumor (IMT), 1 NBL). We observed continuous remission of the IMT patient, all other cases the inhibitor treatment was not curative.Our findings underline the importance of screening the ALK status parallel with both IHC and FISH. Crizotinib treatment had a long-term effect in ALK positive IMT patients, however itwas only temporary efficient in relapsed, progressive STS and NBL.

Keywords ALK · Crizotinib · Soft tissue sarcoma · Neuroblastoma · Inflammatory myofibroblastic tumor

Background

ALK gene was first identified during the exploration of the genetic background of the anaplastic large cell lymphoma (ALCL) [1]. ALK is a tyrosine kinase receptor, localized on the cell membrane. The subcellular mechanism of ALK is not completely elucidated. To our current knowledge, the active ALK plays role in cell growth, differentiation and apoptosis through the signalizationpathways of phosphatidylinositol 3-

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kinase (PI3K) - mammalian target of rapamycin (mTOR), Janus kinase (JAK) – signal transducer and activator of transcription (STAT), Ras and JUN. ALK loses its activity after the embryonic development, it can be reactivated by gene translocation, overexpression or point mutation [2–4]. The ALK locus is a genetic hotspot for translocation events, more than 22 partner genes and 60 fusion proteins are known. ALK alterations are described in various cancer types: in ALCL, non-small cell lung cancer (NSCLC), neuroblastoma, soft tissue tumors such as RMS, IMT, leiomyosarcoma and many other tumor types [5].

ALK status can be tested by IHC, FISH and reverse transcription-polymerase chain reaction (RT-PCR). FISH is the gold standard method approved by the Food and Drug Administration (FDA). FISH is a high-cost, time consuming technique, therefore it is not a routine diagnostic procedure, the only exception being the metastatic NSCLC cases [6, 7]. IHC is a candidate alternative method for screening ALK status [8–10]. The incidence of ALK abnormalities in RMS specimens is highly variant, the most common rearrangement is amplification (59%) [5, 11]. Other reports found 50% ALK positivity in IMT and NBL.In the case of IMT translocation are most commonly reported, and in the case of NBL point mutations are reported as a molecular background [5, 11–14].

Theoretically ALK is an ideal anti-tumor target as it participates in the embryonic development of the nervous system and after embryonal life it is inactivein all tissues [5]. There are multiple ways to block the function of ALK. ALK inhibitors, anti-ALK antibodies, vaccinate are used orthe downstream elements of the signalization are targeted. Clinically the most promising option is using the ALK inhibitor family [13]. The first ALK inhibitor, crizotinib, was approved in 2011. This molecule inhibits not only the ALK, but the cMET, ROS-1 and RON as well [15]. Originally crizotinib was designed for the treatment of the metastatic NSCLC cases, since then it is clinically approved in other indications. In pediatric oncology, it is established in the treatment for ALCL, neuroblastoma and in the case of several soft tissue sarcomas. The main side effects of crizotinib are: hepatotoxicity, interstitial lung disease, bradycardia, QT interval prolongation, embryofetal toxicity and testicular failure with infertility as consequence. Other possible adverse reactions are dizziness, flashes of light, vomiting, nausea, diarrhea, constipation, edema, neutropenia, lymphopenia, hypokalemia, hypophosphatemia. All side effects are reversible [16].

Our aim was to identify the ALK alterations and select the patients who are the most sensitive to ALK inhibitor treatment. We also aimed to investigate the safety and efficacy of ALK inhibitor treatment. We included high-risk (HR) STS and NBL cases with a possible poor outcome according to the metastatic status, relapse rate, response to chemotherapy and unfavorable pathology.

Methods

Patients and Samples

Allchildren diagnosed between 2010 November and 2014 December in the 2nd Department of Pediatrics of the Semmelweis University with HR STS and allwith HR NBLwere examined for ALK rearrangements. The group included 2 ASPS, 2 RMA, 11 RME, 3 IMT and 19 NBL. Patient characteristics are described in Tables 1 and 2. Whole formalin-fixed, paraffin-embedded sections from biopsies of surgical specimens were analyzed. The representative blocks were sectioned at a thickness of 3 μm.

IHC for ALK

The slides were dewaxed with xylene and rehydrated in graded alcohol series followed by 5-min incubation with 3% hydrogen-peroxide solution in order to block the endogenous peroxidase activity The IHC reaction was performed withLeica BOND-MAX™ autostainer (Leica GmbH, Nussloch, Germany). Antigen retrieval was performed at pH 6/ pH 8using Bond Epitope Retrieval 1/2 Solution (Leica Microsystems) for 30 min. Mouse anti-human ALK (monoclonal 5A4 antibody, Novocastra, Leica, 1:10 dilution) was applied for 30 min, followed by the Post Primary application for 40 min. Polymer was incubated for 15 min. The reaction was visualized by diaminobenzidine (DAB) for 10 min, followed by Bond DAB Enhancer for 6 min. All slides were counterstained with hematoxylin. A known positive IMT and NBL histological sample was used as a positive control. The slides were evaluated semiquantitatively by two different investigators independently. The ALK positive tumor cells were counted in 10 fields of vision with 40× magnification. The slide was considered positive, if more than 10% of the tumor cells showed positive reaction. The immunohistochemical results were scored by the following system:+ (10–50%), ++ (50–80%) or +++ (80%<) (Fig. 1). Since the IHC staining intensity was reported to be correlated with the prognosis of NBL, all IHC sampleswere analyzed manually and digitally [17, 18]. The digital evaluation was performed by the 3DHISTECH's DensitoQuant program.

Detection of ALK Abnormalities by Fish

FISH was performed on 3 μm thick slides for ALK rearrangement using ALK Dual Color Break Apart probe (Vysis, Abbott Molecular Inc.). This probe detects rearrangements in the 2p23 region. The telomeric DNA fragment binds to the 3′ end of the ALK (orange fluorophore), the centromeric to the 5′ end (green fluorophore). The probe does not specify the rearrangement gene partner. Dewaxed and rehydrated slides were immersed in a citrate buffer solution and incubated

Table 1 Patient clinical characteristics

Patient No.	Gender	Age at the diagnosis (years)	Localization	Metastasis	Histology	Chemo-therapy	Radio-therapy	Surgery	Relapse
s4	f	4	orbita	no	ASPS			+	0
s13	m	16	musculus vastus medialis	lymph nodes, lung	ASPS	+		+	0
s8	m	10	pararectal region	no	RMA	+	+	+	1
s14	m	8	unknown	multiplex bone	RMA	+			2
s1	f	6	pelvical	no	RME	+	+	+	0
s2	f	3	nose	no	RME	+	+	+	1
s3	f	6	preauricular region	no	RME	+	+		0
s5	m	15	paratesticular region	lymph nodes, lung	RME	+	+	+	0
s6	m	10	orbita	no	RME	+	+	+	0
s7	m	3	paratesticular region	multiplex lung	RME	+	+	+	0
s9	m	4	urine bladder	omental	RME	+		+	1
s10	f	2	pelvical	no	RME	+	+	+	1
s11	m	5	buccal region	no	RME	+	+	+	1
s17	m	1	eardrum	no	RME	+	+	+	0
s18	f	1	frontal region	no	RME	+	+	+	2
s12	m	10	abdominal	no	IMT			+	1
s15	m	9	gluteal region, pelvical	no	IMT	+			0
s16	m	12	pleura	no	IMT			+	1
n1	f	<1	adrenal gland	liver	NBL	+		+	0
n2	f	<1	adrenal gland	liver	NBL	+		+	0
n3	f	4,5	chest cavity	lymph nodes	NBL	+		+	0
n4	m	<1	adrenal gland	no	NBL			+	0
n5	f	1	adrenal gland	no	NBL			+	0
n6	m	3	adrenal gland	bone, chest cavity	NBL	+		+	0
n7	m	3	adrenal gland	no	NBL	+			0
n8	m	<1	adrenal gland	liver	NBL	+			0
n9	f	3	adrenal gland	no	NBL	+	+	+	0
n10	m	1	adrenal gland	lymph nodes	NBL	+		+	0
n11	f	2	adrenal gland	no	NBL	+		+	0
n12	m	8	adrenal gland	no	NBL	+	+	+	1
n13	m	2	adrenal gland	lymph nodes, skull	NBL	+		+	1
n14	m	3	adrenal gland	no	NBL	+			2
n15	m	8	mediastinum	bone	NBL	+			0
n16	f	6	adrenal gland	lymph nodes	NBL	+	+		0
n17	f	4	adrenal gland	lymph nodes, liver	NBL	+			0
n18	f	3	adrenal gland	lymph nodes, bone	NBL	+	+	+	1
n19	f	6	mediastinum	no	NBL	+		+	2

in boiling water for 20 min, then digested by pepsin for 15 min at 37 °C. The slides were incubated in a saline sodium citrate (SSC) solution at 37 °C for 15 min and dehydrated. For each section, 3–6 μl of the probe mixture was applied. They were then denaturalized at 85 °C for 10 min andhybridized overnight at 37 °C. On the second day, the slides were washed at 70 °C in 0,4× SSC for 5 min and in a solution of 2× SSC and 0.1% Nonidet P40 at room temperature for another 5 min. Nuclei were counterstained with 4,6-diamino-2-fenilindol (DAPI). To evaluate the sections, we analyzed 100 tumor nuclei in approximately 8–10 fields of vision with 100× enlargementwith Nikon Eclipse E6000(camera: ProgRes MF, Jenoptik). The tumor was considered positive if more than 15% of the nuclei were positive with FISH. Rearrangements appeared as split 3′ and 5′ signals. Overexpression was detected in cases where the nuclei showed at least quadrupled signs (Fig. 2). Point mutation are not detectable by FISH using this probe.

Table 2 Clinical characteristics per tumor types

Disease	No of cases	Gender	Age		No. of metastatic cases	No. of relapsed cases
		F/M	mean	range		
ASPS	2	1/1	10,5	4–17	1/2	1/2
IMT	3	0/3	10	8–12	0/3	0/3
RMA	2	0/2	8,5	8–9	1/2	2/2
RME	11	5/6	4	1–16	3/11	5/11
NBL	19	9/10	3	1–8	11/19	5/19

Crizotinib Treatment

Patients whose sample was positive for ALK received crizotinib treatment afterchemotherapy resistance was confirmed and total resection was not performable. We started the treatment of 4 patientsaccording to the license by National Institute of Pharmacy and Nutrition. The dosage was 280 mg/m^2 twice daily. We studied the efficacy and the side effects of the therapy. To monitor the response, we used MRI and ultrasound controls and we followed the patients' clinical status by the Karnofsky-Lansky score.

Results

STS

ALK protein expression was observed in 3 out of 18 STS cases(in 2 IMT and 1 RMA). In one IMT, immunohistochemistry displayed a strongly granulated pattern in the cytoplasm with perinuclear acceleration, while the other IMT case showed diffuse intensive cytoplasmic staining. The RMA samples membrane and cytoplasm staining was observed. ALK rearrangement was identified in 4 STS cases using FISH, 1 RME, 1 RMA and 2 IMT cases. Out of these patients 1 ALK positive RMA patient developed metastasis at the time of the diagnosis. The ALK positive RME patient's section was amplified and the other 3 cases were translocated.

NBL

We detected the expression of the fusion protein on 16 NBL section in all 19 cases. Out of these 16 patients, 8 had metastases at the time of the diagnosis and later we observed 3 other cases. The staining was strong in most of these cases. We confirmed + in 2, ++ in 8 and +++ in 6 cases. The evaluation of the slides manually and digitally resulted the same conclusion. ALK rearrangement was confirmed in 4 NBL cases, 3 amplifications and 1 translocation was observed. Two patients already had metastases at the time of their diagnosis while in the other 2 cases metastases developed during the progression.

Concordance between the Results of the IHC and the Fish

With one exception, FISH positive cases were stained with the IHC too. In 12 cases the ALK protein was detected by IHC, but the FISH did not confirm the diagnosis. The difference between the results of the two methods can be explained by the different genetic alterations. FISH cannot detect point mutation of ALK gene, but the IHC can confirm the diagnosis by detecting the

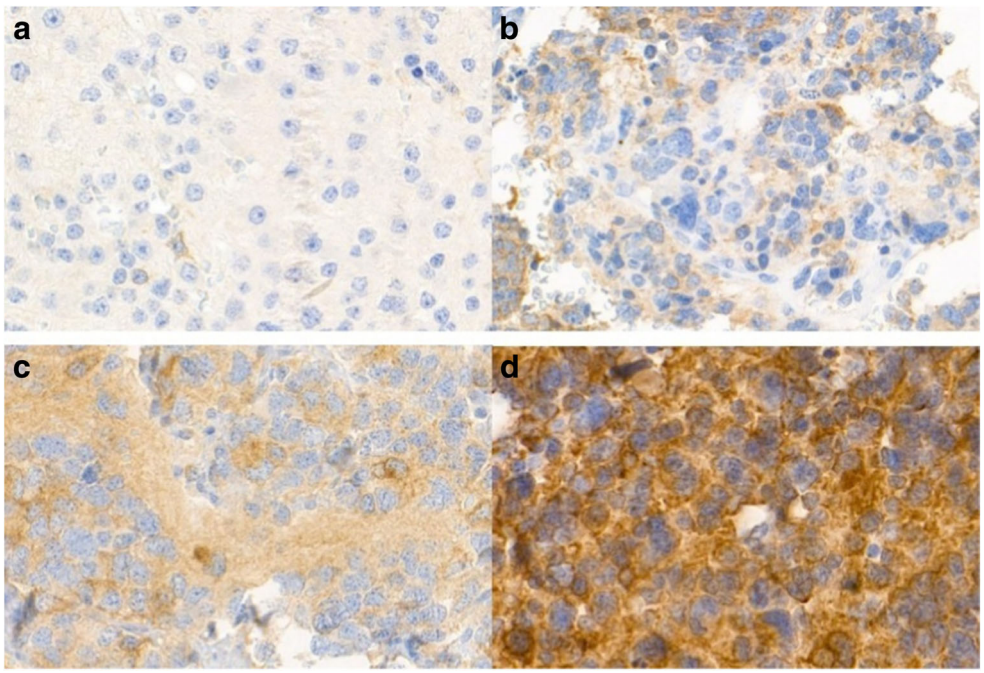

Fig. 1 ALK immunohistochemical staining patterns. Picture **a** negative; **b** weak, faint and focal (+)cytoplasmic staining; **c** moderate and diffuse (++) cytoplasmic staining; **b** strong, granulated and diffuse (+++) cytoplasmic staining

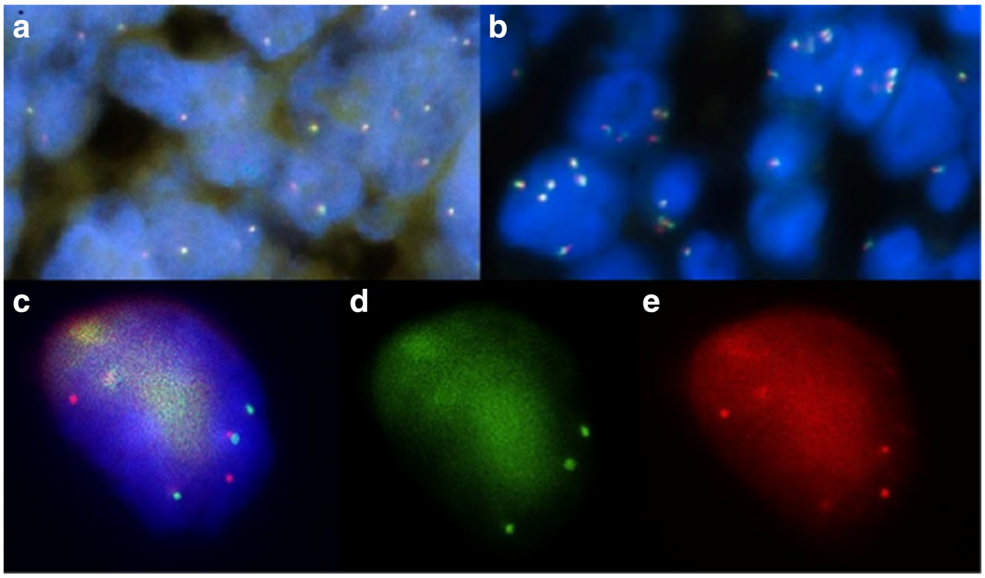

Fig. 2 FISH results. Picture **a**: normal ALK status; **b**: amplified ALK status; **c**: translocated status with merged picture, separated signals are visible through green (**d**) and orange (**e**) filters

expression of ALK protein.The results are described in Tables 3 and 4.

Crizotinib Treatment

Altogether 4 patients (1 IMT,1 RMA, 1RME and 1 NBL) received crizotinib therapy. The therapy reviews are described in Table 5. In the case of the IMT patienttotal resection was not performed due to the infiltrative spread of the tumor. The multilocular pattern tumor had infiltrated the acetabulum, pubic bone, ischium and the gluteal and adductor muscles in the region. We started chemotherapy treatment according to the high-risk protocol of the CWS 2009 guideline. The MRI control showed no signs of regression. At this time, he performed 20–30% on the Karnofsky-Lansky scale. We started crizotinib treatment with 250 mg crizotinib twice daily. The early side effects, flashes of light and tasting problems, bradycardia and electrolyte level abnormalities were all tolerable. One month later the control MRI showed 20% regression in volume and his performance improved to 80% on the Karnofsky-Lansky scale. During the 25-months-long treatment the tumor regressed and no more activity was detectable with the diffusion-weighted whole-body imaging with background body signal suppression (DWIBS) MRI diagnostics (Fig. 3). We decided to stop the therapy in the 25th month. After one month, his pain returned and the MRI showed progression. We restarted the treatment immediately. Since than the patient is without any complaints. The IMT patient is in partial morphological remission, but in complete metabolic remission. The RMA patient had advanced disease. Diagnostics revealed the involvement of the skull, most of the ribs, all vertebra with extraosseal propagation and both femur and tibia. We started chemotherapy according to the guideline of the CWS 2012 metastatic protocol. Good response was established; remission had been achieved. Two

Table 3 Results of the pathological investigation

patient no.	IHC	FISH	patient No.	IHC	FISH
s1	–	+(a)	n1	–	–
s2	–	–	n2	+++	–
s3	–	–	n3	++	–
s4	–	–	n4	+++	–
s5	–	–	n5	++	–
s6	–	–	n6	+++	–
s7	–	–	n7	+++	–
s8	–	–	n8	++	–
s9	–	–	n9	+	–
s10	–	–	n10	++	–
s11	–	–	n11	++	–
s12	+	+(t)	n12	+++	+(t)
s13	–	–	n13	–	–
s14	+	+(t)	n14	++	–
s15	+	+(t)	n15	–	–
s16	–	–	n16	++	+(a)
s17	–	–	n17	+	–
s18	–	–	n18	+++	+(a)
			n19	++	+(a)

Table 4 Results of the pathological investigation per tumor types

	negative	positive	patient No.
RME [11]	10	1	s1
RMA [2]	1	1	s14
IMT [3]	1	2	s12, s15
ASPS [2]	2	0	–

Table 5 Therapy review

Disease	Gender	Age at the diagnosis	Metastasis	Treatment	Relapse	Treatment	Length of crizotinib treatment (months)	Evaluation of crizotinib treatment
ASPS	m	16 y	lymph node, pulmo	CWS 2012 META	–		6	SD (6mo), PD
RMA	m	8 y	multiplex bone	CWS 2009 META	1. 2.	CWS-91-REZ Xalkori, Torisel	8	PR (3mo), PD
RME	f	1 y	–	CWS 2009 SR	1. 2.	CWS 2009 s-line Xalkori	3	SD(3mo), PD
IMT	m	9 y	–	CWS 2009 HR	–		38	CR
NBL	f	6 y	–	SIOP n-myc neg, II. st protocol	1. 2.	rapid COJEC, RIST RIST protocol, Xalkori	3	PD

months after finishing his treatment we confirmed the relapse. He was treated according to the relapse protocol of the CWS 2012 guideline. After confirmation of ALK positivity the tumor board suggested crizotinib treatment. We started the therapy with a dosage of 400 mg twice daily.On the first night, the patient experienced strong nausea and vomiting, he was not able to take medication or eat. He also experienced dizziness, weakness and abdominal pain. Several days later the treatment was continued with a reduced dosage of 200 mg in the morning and 400 mg in the evening, without any complications. In the 3rd month of crizotinib therapy further progression was observed, we added Temsirolimus, an mTOR inhibitor, to his therapy. He showed no response to the therapy (Fig. 4). Crizotinib was part of palliative treatment of the RME patient, where the disease had been observed to be stable for several months. This crizotinib therapy was stopped because of progression and bad compliance.The NBL patient received crizotinib also as late palliation after the second relapse, the tumor was resistant to the first-line and the crizotinib therapy as well. We lost the patient several months later.In summary, only 1 continuous remission was achieved, IMT responded well to the inhibitor therapy. We observed partial response in 1 case (RMA), stable disease in 1 (RME) and progressive disease in 1 (NBL). The early side effects, flashes of light, dysgeusia, bradycardia and electrolyte level abnormalities were all tolerable, the patients experienced them only in the first weeks of the therapy.

Discussion

FISH analysis with the dual colorbreak-apart probe is the gold standard method to determine the ALK status and to enroll patients in off-label crizotinib treatment. On the other hand, FISH has its limitations. As a single screening modality, it is not able to detect all ALK positive cases. The probe was designed to detect the translocated variants and can also visualize amplification, but not point mutation. It is also expensive. The IHC method to detect ALK positivity is becomingmore and more widespread. High-affinity antibodies can provide an effective prescreening technique and lower the number of the unnecessary FISH tests. Point mutations in the ALK status are more common pediatric oncology cases. It is important to standardize the ALK screening by IHC in certain tumor types such as neuroblastoma and the above mentioned soft tissue sarcomas.

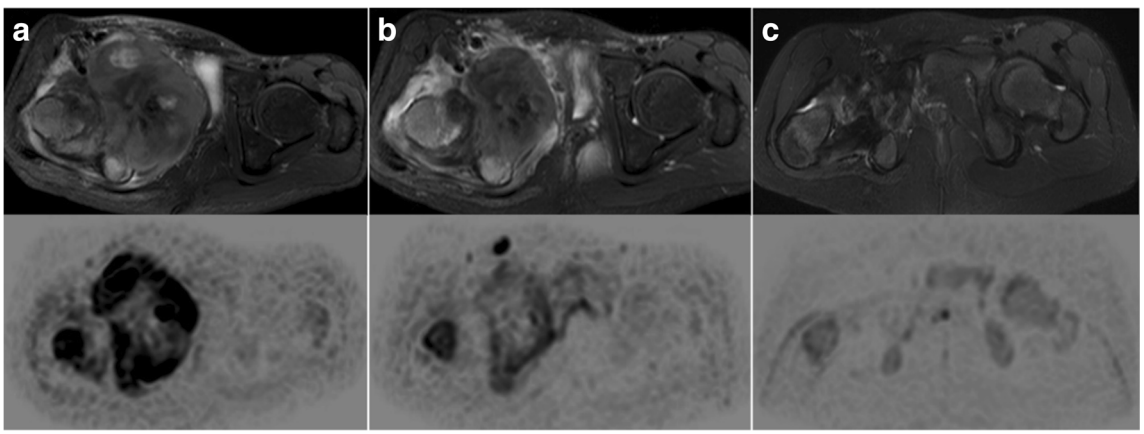

Fig. 3 IMT patient: **a** T2 SPAIR and DWIBS images show the tumor size and the diffusion restriction before crizotinib therapy. **b** The first follow-up examination shows a reduction in tumor size and diffusion restriction after 1 month of crizotinib therapy. **c** On the fifth follow-up examination (in the 25th month of the inhibitor treatment) a small residual tumor is visible without diffusion restriction

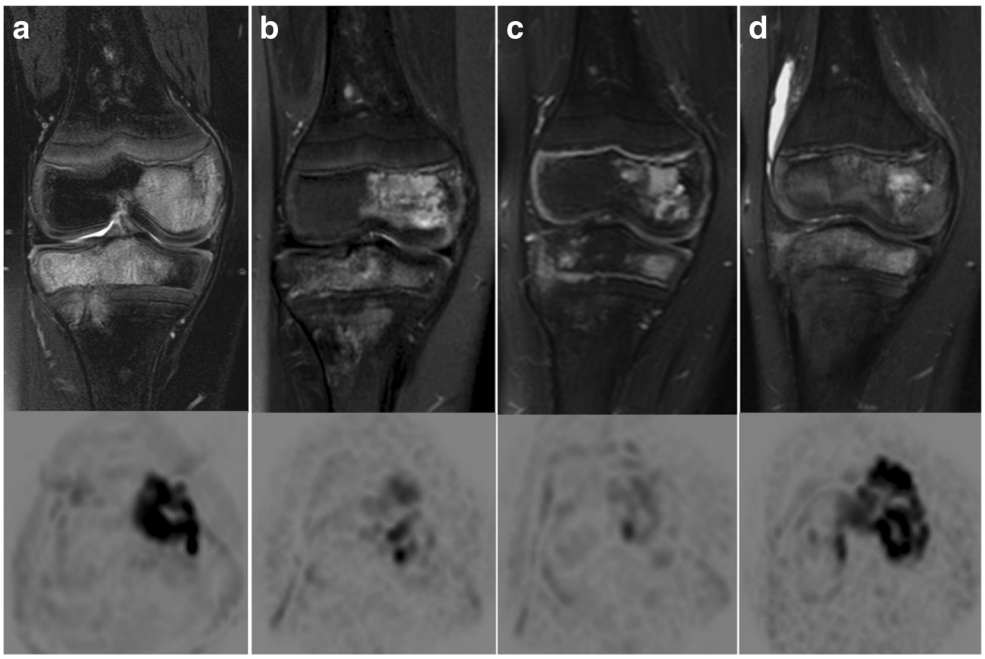

Fig. 4 RMA patient: **a** T2 SPAIR and DWIBS images show the tumor size and the diffusion restriction before crizotinib therapy.After the initiation of the therapy the first (**b**) and second (**c**) follow-up examination shows a continuous reduction in tumor size and diffusion restriction. D. The T2 SPAIR and DWIBS images well demonstrate the recurrence of the disease under the crizotinib therapy

We analyzed the concordance between ALK protein expression and ALK rearrangement. We found ALK alteration in 20 cases; 19 cases were positive with IHC, 8 with FISH. With one exception, FISH positive cases stained with the IHC, too. In 12 cases, the ALK protein expression was detected by IHC, but it was not confirmed by FISH. 4 STS (3 of them were translocated and 1 amplified) and 4 NBL (1 of them was translocated and 3 amplified) samples were FISH positive. The explanation of the difference between the results of the two methods is that FISH pics up the translocated and amplified variants, but the IHC can visualize the presence of the altered ALK protein (because of the gain of function point mutations) in the cytoplasm. However, FISH can be superior to IHC if there is a poor fixation or if there is only a small amount of tumor cells on the slide. Our findings suggest the benefit of IHC prescreening before FISH analysis.

The crizotinib treatment in RMS, ASPS and NBL cases was less effective than expected. Several studies investigated the ALK protein positivity in RMScell cytoplasm. Their results showed that although ALK protein positivity was obvious in the cytoplasm and on cell membranes, it was in an unphosphorylated, inactive form. These findings question the effectiveness of ALK inhibitors in RMS patients and the key role of ALK in the oncogenesis of RMS [19]. The IMT patient response was excellent, other case presentations had promising results with crizotinib treatment as well [20, 21]. Several studies emphasize the prominent role of ALK translocation in the pathogenesis of this tumor [14, 22]. However, our patient did not respond well to the ALK inhibitor treatment, according to the literature crizotinib could be a potent antitumor target in NBL cases. The amplification on the chromosome 2 in NBL cases involves the MYCN oncogene so the dual-mutation leads to an increased oncogene potential [17, 23]. The optimal treatment regimen might be a combination of mTOR inhibitors or anti-ALK antibodies with ALK inhibitors to enhance the cytotoxic effect [13, 24, 25].

It is essential to identify new therapeutic targets and clarify their implication in the tumorigenesis to improve the outcome and diminish the late side effects in pediatric oncology. ALK is a potent target, but its role in the signaling pathway needs further investigation. Besides confirming the presence of ALK alteration, it is mandatory to identify the subcellular events, because they can influence the outcome of ALK inhibitor therapy.To summarize our findings, we call attention to the possible benefit of crizotinib treatment in high risk ALK positive soft tissue sarcoma patients, particularly in inflammatory myofibroblastic tumorcases.

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Compliance with Ethical Standards

Ethics Approval and Consent to Participate Investigations were approved by the Institutional Ethical Review Board. / The study protocol was approved by the Ethics and Scientific committee of the participating institution. TUKEB 7/2006.

Consent for Publication and Competing Interests All of the authors declare that they have no competing interests. All authors have read and approved the final manuscript.

List of Abbreviations *AKT*, akt murine thymoma viral oncogene; *ALCL*, anaplasticlarge cell lymphoma; *ALK*, anaplastic lymphoma kinase; *ASPS*, alveolarsoft part sarcoma; *CWS*, Cooperative Weichteilsarkom Studiengruppe; *cMET*, c mesenchymal epithelial transition growth factor; *DAPI*, 4,6-diamino-2-fenilindol; *DLBCL*; diffuse large B-cell lymphoma; *DWIBS*, diffusion-weighted whole-body imaging with background body signal suppression; *FDA*, Food and Drug Administration; *FISH*, fluorescent in situ hybridization; *HR*, high risk; *IHC*, immunohistochemistry; *IMT*, inflammatory myofibroblastic tumor; *JAK*, Janus-kinase; *mTOR*, mammalian target of rapamiycin; *NBL*, neuroblastoma; *NSCLC*, non-small cell lung carcinoma; *PI3K*, phosphatidylinozitol 3-kinase; *RMS*, rhabdomyosarcoma; *RMA*, alveolar rhabdomyosarcoma; *RME*, mbryonal rhabdomyosarcoma; *RON*, receptor originated from nantes; *ROS1*, ROS protooncogene; *RTPCR*, real-time polymerase chain reaction; *SIOPEN*, International Society of Pediatric Oncology Europe Neuroblastoma, *SSC*, saline sodium citrate; *STAT*, signal transducer and activator of transcription; *STS*, soft tissue sarcoma

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