Pathol. Oncol. Res. (2017) 23:863–871
DOI 10.1007/s12253-017-0200-z

ORIGINAL ARTICLE

Unusual Signal Patterns of Break-apart FISH Probes Used in the Diagnosis of Soft Tissue Sarcomas

Gergő Papp[1] · **Dóra Mihály**[1] · **Zoltán Sápi**[1]

Received: 4 January 2017 / Accepted: 17 January 2017 / Published online: 20 January 2017

Abstract Break-apart FISH probes are the most popular and reliable type of FISH probes used to confirm certain pathological diagnoses. The interpretation is usually easy, however, in some instances it is not so unequivocal. Our aim was to reveal and elucidate the problems occurring in the process of evaluation of the break-apart probe results. Altogether 301 soft tissue sarcomas with confirmed molecular tests using break-apart probes were assessed to reveal the frequency and type of unusual signal pattern. Among 89 synovial sarcoma (*SS18*) 11%, 12 alveolar rhabdomyosarcoma (*FOXO1*) 50%, 53 myxoid liposarcoma (*DDIT3*) 7.5%, 6 low grade fibromyxoid sarcoma (*FUS*) 67%, 93 Ewing sarcoma (*EWSR1*) 3%, 12 clear cell sarcoma (*EWSR1*) 8%, 5 desmoplastic small round cell tumor (*EWSR1*) 0%, 9 extraskeletal myxoid chondrosarcoma (*EWSR1*) 0%, 2 myoepithelial carcinoma (*EWSR1*) 50%, 14 dermatofibrosarcoma protuberans (*COL1A1*) 86% and 6 nodular fasciitis (*USP6*) 17% atypical break-apart signals were detected. Despite the unusual signal pattern type, the fusion genes were detected using either metaphase FISH, interphase FISH with translocation/TriCheck probe or RT-PCR methods. Although the interpretation problems in the process to evaluate the break-apart probe results is well known from sporadic case reports, a systemic overview to detect their frequency has not been performed so far. In our work we highlighted the relative frequency of this problem and pinpointed those signal-patterns which, despite their unusual appearance, can still confirm the diagnosis.

Keywords Atypical FISH pattern · Break-apart probe · Soft tissue sarcoma

✉ Zoltán Sápi
sapi.zoltan.dr@gmail.com

Gergő Papp
gergo.papp@gmail.com

Dóra Mihály
dora.mihaly@gmail.com

[1] 1st Department of Pathology and Experimental Cancer Research, Semmelweis University, Üllői út 26, Budapest 1085, Hungary

Introduction

Molecular studies have become substantially important in the diagnosis of soft tissue neoplasms. Many diagnostic entities have characteristic molecular alterations and, as such, methods that allow detection of these alterations are valuable for diagnostic confirmation [1]. Several methods exist to evaluate these genetic events; including conventional cytogenetics, reverse transcription-polymerase chain reaction (RT-PCR) and fluorescence in situ hybridization (FISH) [2]. Diagnostically useful chromosomal translocations (with commercially available break-apart probes) and their associated chimeric genes are summarized in the Table 1. The focus of this study was to evaluate the utility of the break-apart probes for FISH, which have become one of the most important tools of molecular testing at many institutions. The dual color break-apart probes hybridize to targets which flank the most common breakpoints in a given gene. Separation of the two differently, often green and orange labeled probes displays a rearrangement of the gene which in turn indicates the presence of a translocation related to that gene. The high degree of specificity of probes generally makes their application and interpretation unequivocal. However, it is not uncommon to have an atypical abnormal interphase FISH result. Our study highlights the incidences of the different atypical break-apart FISH patterns in the daily routine diagnostics of soft tissue tumors and also calls the pathologists' and/or molecular

Table 1 Molecular alterations (translocations) in soft tissue neoplasms

Diagnostic entity	Chromosome abnormality	Chimeric gene	Split-signal probe
Synovial sarcoma	t(X;18)(p11.2;q11.2)	SS18-SSX1 SS18-SSX2 SS18-SSX4	SS18
Alveolar rhabdomyosarcoma	t(2;13)(q35;q14) t(1;13)(p36;q14)	PAX3-FOXO1A PAX7-FOXO1A	FOXO1
Myxoid/round cell liposarcoma	t(12;16)(q13;p11) t(12;22)(q13;q12)	FUS-DDIT3 EWSR1-DDIT3	DDIT3, FUS
Low-grade fibromyxoid sarcoma	t(7;16)(q34;p11) t(11;16)(p11;p11)	FUS-CREB3L2 FUS-CREB3L1	FUS
Ewing sarcoma/ PNET	t(11;22)(q24;q12) t(21;22)(q22;q12)	EWSR1-FLI1 EWSR1-ERG	EWSR1
Clear cell sarcoma	t(12;22)(q13;q12) t(2;22)(q34;q12)	EWSR1-ATF1 EWSR1-CREB1	EWSR1
Desmoplastic small round cell tumor	t(11;22)(p13;q12)	EWSR1-WT1	EWSR1
Extraskeletal myxoid chondrosarcoma	t(9;22)(q22;q12)	EWSR1-NR4A3	EWSR1
Myoepithelial carcinoma	t(6;22)(p21;q12) t(1;22)(q23;q12)	EWSR1-POU5F1 EWSR1-PBX1	EWSR1
DFSP	t(17;22)(q22;q13)	COL1A1-PDGFB	COL1A1
Nodular fasciitis	t(16;17)(q22;p13)	USP6-CDH11	USP6

PNET primitive neuroectodermal tumor, *DFSP* dermatofibrosarcoma protuberans

biologists' attention to these problems. We also want to share our experiences with the pathological community to help them when they have to cope with similar interpretation problems.

Materials and Methods

Tissue Samples and Pathology

Altogether 89 synovial sarcomas, 53 myxoid/round cell liposarcomas, 6 low-grade fibromyxoid sarcomas, 12 alveolar rhabdomyosarcomas, 93 Ewing sarcomas/peripheral neuroectodermal tumors, 12 clear cell sarcomas, 5 desmoplastic small round cell tumors, 9 extraskeletal myxoid chondrosarcomas, 2 myoepithelial carcinomas, 14 dermatofibrosarcoma protuberanses and 6 nodular fasciitis were included in the study from the archives of Semmelweis University First Department of Pathology, Budapest, Hungary. All cases were classified according to the World Health Organization (WHO) classification of tumors of soft tissue [3]. Hematoxylin and eosin stained formalin-fixed, paraffin-embedded tissue sections and immunohistochemical stains were re-evaluated in all cases.

Fluorescence in Situ Hybridization (FISH)

FISH analysis was performed on interphase nuclei of paraffin embedded 3-μm-sections using LSI dual color break-apart probes, specific for *SS18* (*SYT*) at 18q11.2, *DDIT3* (*CHOP*) at 12q13, *FUS* at 16p11, *FOXO1* (*FKHR*) at 13q14, *EWSR1* at 22q12 (Vysis, Abbott Park, IL, USA) and ZytoLight SPEC dual color break-apart probes for *COL1A1* at 17q21 and *USP6* at 17p13.2 (ZytoVision, Bremerhaven, Germany). One end of the probes was labeled with spectrum orange™ (telomeric, 5′ to the breakpoint) and the other with spectrum green™ (centromeric, 3′ to the breakpoint). The SS18/SSX1 TriCheck Probe (ZytoVision) was also used for confirmation. After deparaffinization in xylene and rehydration in a series of ethanol, sections were microwaved for 20 min in 10 mM citric acid buffer and incubated in 2× saline-sodium citrate (SSC) in waterbath at 37 °C for 15 min. Enzymatic digestion was carried out with a 10% pepsin solution at 37 °C for 15 min. In all, 4.5 μl diluted probe was co-denatured with the tissues at 82 °C for 10 min and hybridized at 37 °C for overnight using the ThermoBrite Denaturation/Hybridization System (Abbott Molecular). Post-hybridization wash was performed in 0.4× SSC/0.1% NP-40 (73 °C, 2 min) followed by a wash in 2× SSC/0.1% NP-40 (room temperature, 2 min). The slides were air dried in the dark, then counterstained with 10 μl of 4′.6′-diamidino-2-phenylindole from Vector Laboratories (Burlingame, CA, USA). For metaphase FISH analysis a piece of a resected synovial sarcoma was minced and cultured in RPMI 1640 medium (Sigma-Aldrich, St Louis, MO, USA) supplemented with 20% fetal bovine serum (FBS, from Sigma-Aldrich) for 72 h. Chromosome preparation was made following standard procedures including a 3 h colcemid treatment, hypotonization, and fixation in 3: 1 methanol:acetic acid. 50ul of cell suspension was dropped and air-dried on a slide where the above described FISH procedure was performed using *SS18* specific probe. The FISH signals were visualized by using filter sets and a Nikon Eclipse E600 epifluorescence microscope. Image analysis was performed by using Lucia Cytogenetics image acquisition system (Laboratory Imaging, Republic of Czech). For each case and probe, a minimum of 100 non-overlapping nuclei, which were clearly identified and contained unequivocal signals, were

counted. A probe was considered to be split (break-apart) when the orange and the green signals were separated by two times distance greater than the size of one hybridization signal. These break-apart rearrangements were interpreted as "typical" FISH pattern (1fusion, F; 1 red, R and 1 green, G signal) while any other motifs, mainly with extra (also called as isolated) signals were considered to be "atypical".

Results

In the present study, altogether 301 soft tissue sarcomas, including 89 (29.57%) synovial sarcomas, 53 (17.6%) myxoid/round cell liposarcomas, 6 (1.99%) low-grade fibromyxoid sarcomas, 12 (3.98%) alveolar rhabdomyosarcomas, 93 (30.89%) Ewing sarcomas/peripheral neuroectodermal tumors, 12 (3.98%) clear cell sarcomas, 5 (1.66%) desmoplastic small round cell tumors, 9 (2.99%) extraskeletal myxoid chondrosarcomas, 2 (0.66%) myoepithelial carcinomas, 14 (4.65%) dermatofibrosarcoma protuberanses and 6 (1.99%) nodular fasciitis, were analyzed for their characteristic break-apart FISH patterns. The summarized results of atypical break-apart FISH are shown in Table 2. These atypical signal patterns were homogenous for each tumor since more than 80% of the tumor nuclei showed the distinctive break-apart motif.

A total of 89 synovial sarcomas were screened for *SS18* gene rearrangement. A great majority of the cases displayed the typical split pattern (1F1R1G) while (10/89) 11.2% of the tumor samples were considered to be atypical (Table 2). This atypical pattern consisted of two fusions and one red isolated signal (2F1R) as illustrated in an interphase tumor cell in Fig. 1b. The SS18-SSX1 fusion was confirmed by using both the TriCheck FISH probe (Fig. 1c) and RT-PCR method (Fig. 1e). The same 2F1R signal pattern was seen in another 5 synovial sarcoma cases. We revealed that in the case of 2F1R, a translocation of the 5′ telomeric region of *SS18* could be seen on metaphase preparation (from the same synovial sarcoma case as in Fig. 1b) as this appeared as an extra red signal located on an X chromosome beside the two fusion signals located on chromosomes 18 (Fig. 1d). On the other hand, as illustrated in Fig. 1g, an atypical pattern consisted of two fusions, two red and one green signals (2F2R1G) were found in a poorly differentiated synovial sarcoma. Furthermore, a monophasic type showed another variant of the atypical pattern which was comprised of one fusion, two red and one green signals (1F2R1G, Fig. 1i). Two further synovial sarcomas displayed extra red signal patterns, one fusion, one red (1F1R) and two fusions, two red signals (2F2R), specifically. However, in all 89 synovial sarcoma cases, the specific *SS18-SSX1*/*SSX2* fusion gene could be detected by RT-PCR.

Regarding the *FOXO1* rearrangement in 12 alveolar rhabdomyosarcomas, the typical-atypical patterns were shared equally among 50/50% of the cases (Table 2). The atypical FISH pattern demonstrated one pair of juxtaposed fusion signals for *FOXO1* locus, one red signal for the telomeric region for *FOXO1*, and 10–15 copies of green signals for the centromeric region of *FOXO1* indicating that one allele of the *FOXO1* gene was rearranged and its centromeric region subsequently amplified (Fig. 2b). A lower amplification of the centromeric *FOXO1* region is shown in Fig. 2d. However, patterns with higher (10–20) copies of green signals were the most frequently observed alterations among the *FOXO1* rearrangements including six alveolar rhabdomyosarcoma cases. Polyploidy was found in 41.6% (5 of 12 cases) and 83.3% (5 of 6 cases) had an atypical (amplified) *FOXO1* pattern.

53 myxoid liposarcomas were submitted for evaluation using the *DDIT3* and *FUS* break-apart rearrangement probes and 6 low-grade fibromyxoid sarcomas using *FUS* break-apart probe. Among myxoid liposarcomas, (4/53) 7.5% of the cases harbored atypical *DDIT3* translocations while unusual split patterns were not identified using the *FUS* break-apart probe. Three tumors displayed one (or two) isolated red signals, hybridizing for the centromeric side of *DDIT3*, beside

Table 2 Summary of atypical break-apart FISH results

Diagnosis (no.cases)	FISH probes						
	SS18	FOXO1	DDIT3	FUS	EWSR1	COL1A1	USP6
Synovial sarcoma (n = 89)	(10/89) 11.2%*	-	-	-	-	-	-
Alveolar rhabdomyosarcoma (n = 12)	-	(6/12) 50%	-	-	-	-	-
Myxoid/round cell liposarcoma (n = 53)	-	-	(4/53) 7.5%	(0/53) 0%	-	-	-
Low-grade fibromyxoid sarcoma (n = 6)	-	-	-	(4/6) 66.7%	-	-	-
Ewing sarcoma/ PNET (n = 93)	-	-	-	-	(3/93) 3.2%	-	-
Clear cell sarcoma (n = 12)	-	-	-	-	(1/12) 8.3%	-	-
Desmoplastic small round cell tumor (n = 5)	-	-	-	-	(0/5) 0%	-	-
Extraskeletal myxoid chondrosarcoma (n = 9)	-	-	-	-	(0/9) 0%	-	-
Myoepithelial carcinoma (n = 2)	-	-	-	-	(1/2) 50%	-	-
Dermatofibrosarcoma protuberans (n = 14)	-	-	-	-	-	(12/14) 85.7%	-
Nodular fasciitis (n = 6)	-	-	-	-	-	-	(1/6)16.7%

FISH indicates fluorescence in situ hybridization, *PNET* primitive neuroectodermal tumor

*Numbers represent the proportion/percentage of cases demonstrating atypical rearrangement of that particular gene region by FISH break-apart probes

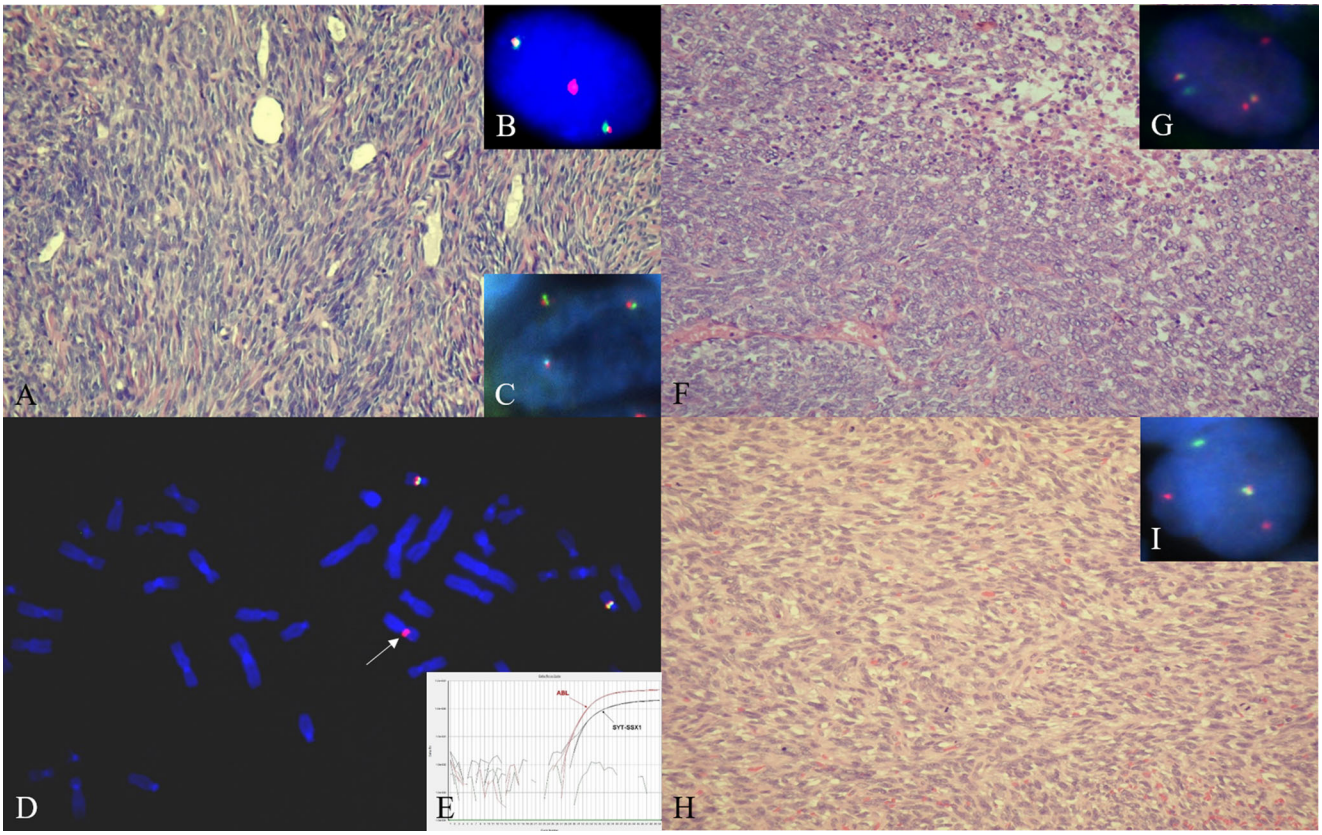

Fig. 1 **a** Monophasic synovial sarcoma (hematoxylin-eosin, original magnification ×200). **b** FISH using dual color break-apart probe for *SS18* demonstrating two fusion (yellow) and one extra red signal (2F1R, original magnification ×1000). **c** Inset demonstrating FISH using the SS18/SSX1 TriCheck Probe. SS18-SSX1 fusion is indicated by one separate orange signal co-localizing with one blue signal. **d** Partial picture of metaphase spread of the same monophasic synovial sarcoma; arrow indicates translocation of the 5′ telomeric region of *SS18* to the X chromosome (original magnification ×600). **e** RT-PCR revealed the amplification of *SS18* (*SYT*)-*SSX1* fusion gene (black); *ABL* gene (red) was used as internal control. **f** Poorly differentiated synovial sarcoma (hematoxylin-eosin, original magnification ×200). **g** Inset showing *SS18* FISH with atypical pattern consisted of two fusion, two red and one green signals (2F2R1G, original magnification ×1000). **h** Monophasic synovial sarcoma (hematoxylin-eosin, original magnification ×200). **i** Inset demonstrating *SS18* FISH with one fusion, two red and one green signals (1F2R1G, original magnification ×1000)

the two not-rearranged loci (2F1R or 2F2R, Fig. 3b). The fourth case showed one fusion, a two red and one green signal pattern (1F2R1G). Of the 6 low-grade fibromyxoid sarcomas, 4 (66.7%) had atypical split pattern working with *FUS* break-apart probe. Two types of signal motifs, composed of two fusions with one or two isolated green signals (2F1G or 2F2G, Fig. 3d), were observed in these 4 tumors.

Concerning the *EWSR1* gene rearrangements, altogether 121 tumors were analyzed, including 93 Ewing sarcomas/peripheral neuroectodermal tumors, 12 clear cell sarcomas, 5 desmoplastic small round cell tumors, 9 extraskeletal myxoid chondrosarcomas and 2 myoepithelial carcinomas. The atypical split patterns were quite rare among the abovementioned entities and, interestingly, all the tested desmoplastic small round cell tumors and extraskeletal myxoid chondrosarcomas demonstrated the typical break-apart motifs (Table 2). Three (3.2%) Ewing sarcomas showed an atypical *EWSR1* FISH pattern (Table 2) with one fusion, two red and one green signal (1F2R1G) or one fusion, one red and two green signals (1F1R2G), and two fusions with one extra red signal (2F1R), respectively. The same 2F1R pattern was observed in the case of one clear cell sarcoma as illustrated in Fig. 4b. The *EWSR1-ATF1* fusion was proved by RT-PCR as depicted in Fig. 4c. The Ewing sarcoma case with a 1F1R2G pattern showed intensive and diffuse ERG immunopositivity and FLI1 negativity; proving the atypical translocation of *EWSR1-ERG* genes (data not shown). An interesting myoepithelial carcinoma case was diagnosed that displayed both amplification of *EWSR1* locus at 22q12 and clear *EWSR1* translocations in the majority of tumor cells (Fig. 4f), however, some cells showed an atypical *EWSR1* break-apart pattern with one fusion, two red and one green signal (1F2R1G, Fig. 4e).

Among dermatofibrosarcoma protuberans cases, only 2 out of 14 (14.3%) patient samples showed the "classical" pattern i.e. 1F1R1G using the *COL1A1* break-apart probe. The remaining

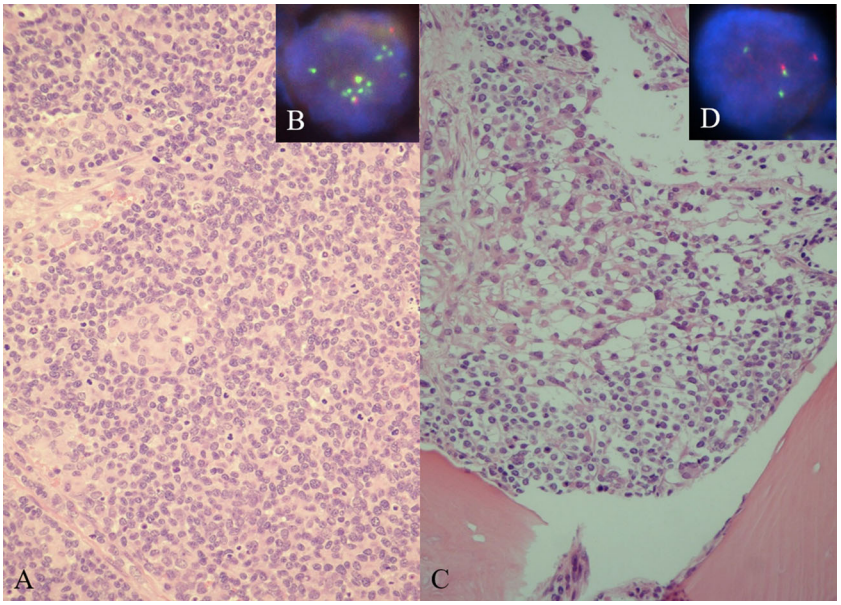

Fig. 2 **a** Alveolar rhabdomyosarcoma (hematoxylin-eosin, original magnification ×200), which contained one normal fusion signal (yellow), one split signal for the telomeric region of *FOXO1* (red), and ~10–15 copies of the split signal for the centromeric region of *FOXO1* (green, original magnification ×1000). This result in inset **b** indicate rearrangement at *FOXO1* locus, as well as amplification of its centromeric region. **c** Alveolar rhabdomyosarcoma (hematoxylin-eosin, original magnification ×200) showing a lower amplification of the centromeric *FOXO1* region as seen in inset **d** (original magnification ×1000)

12 cases (85.7%) were considered as atypical (Table 2), characterized by fusion signals (2F or 3F) and extra copies of the 5′ telomeric region of *COL1A1* (2R to 6R) (Fig. 5b).

Relating to the *USP6* rearrangement in 6 nodular fasciitis, one case (16,7%) displayed an unusual break-apart pattern (Table 2) with two fusions and one extra red signal (2F1R) (Fig. 6b).

Discussion

Soft tissue sarcomas are rare and heterogeneous neoplasms of mesenchymal tissues with diverse morphologies and clinical behavior. In the last few years, the discovery of specific genetic aberrations in these tumors has allowed better

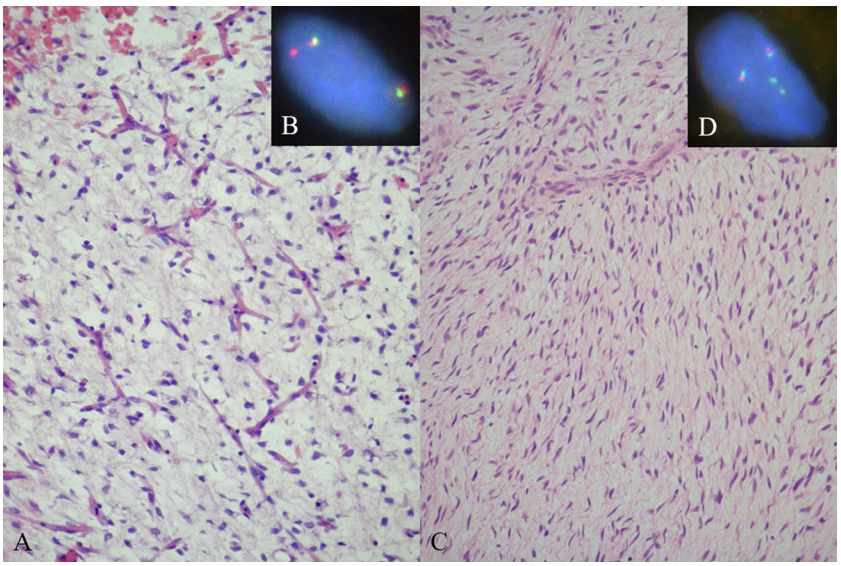

Fig. 3 **a** Myxoid liposarcoma (hematoxylin-eosin, original magnification ×200). **b** Inset showing atypical FISH pattern of *DDIT3* gene comprising of two fusion and one isolated red signals (2F1R, original magnification ×1000). **c** Low-grade fibromyxoid sarcoma (hematoxylin-eosin, original magnification ×200). **d** Inset demonstrating the *FUS* dual color break-apart FISH pattern consist of two fusion and two green signals (2F2G, original magnification ×1000)

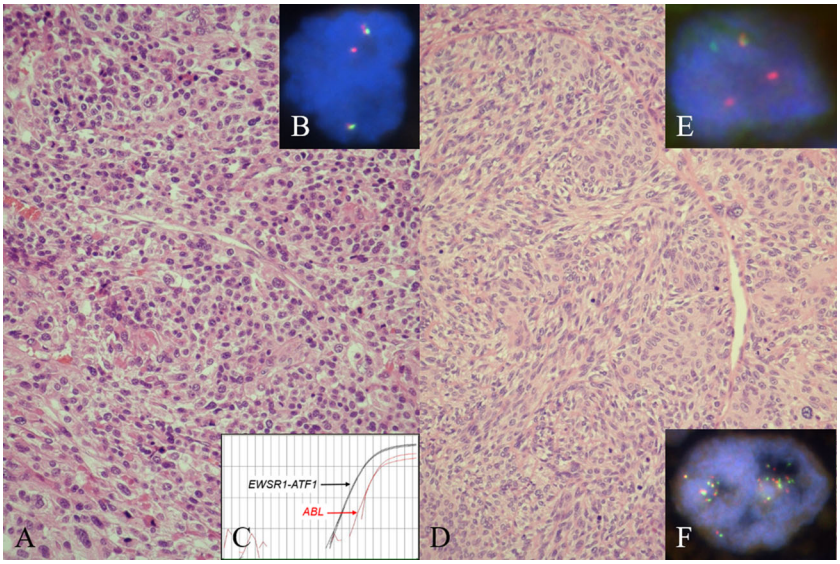

Fig. 4 **a** Clear cell sarcoma (hematoxylin-eosin, original magnification ×200). **b** Inset showing atypical *EWSR1* break-apart FISH pattern with two fusion and one red signals (2F1R, original magnification ×1000). **c** RT-PCR revealed the amplification of *EWSR1-ATF1* fusion gene (black); *ABL* gene (red) was used as internal control. **d** Myoepithelial carcinoma (hematoxylin-eosin, original magnification ×200) displaying two types of *EWSR1* rearrangements: **e** Inset demonstrating a tumor cell with one fusion, two red and one green signal pattern (1F2R1G, original magnification ×1000) using *EWSR1* dual color break-apart FISH. **f** Lower inset showing both amplification of *EWSR1* locus at 22q12 and clear *EWSR1* split (marked by arrows) using the same FISH probe

classification and understanding of their pathogenesis. Numerous technological developments have contributed to the better understanding of cell biology and have shed light on the molecular mechanisms of malignant transformation. Chromosomal karyotyping is the classic methodology for demonstrating translocations, however, it requires the rarely available and often difficult cell-culture from fresh tumor tissue. Moreover, in cases with complex karyotypes, demonstration of characteristic translocations can be more challenging. FISH can be used to identify abnormalities in cases where conventional karyotypic analysis is inconclusive or when metaphase spreads are not available; for example, in cases when only formalin-fixed, paraffin-embedded tissue is available. In practice nowadays, the break-apart FISH methodology is the most common one and many commercially available probes are used for soft tissue sarcoma diagnosis. However, it has been supposed that dual fusion probes are superior to break-apart ones regarding sensitivity because their false positive rate on normal tissue is virtually zero [4]. Despite this fact, a broad spectrum of break-apart probes is commercially available, while the range of dual fusion probes is considerably less. The break-apart (also known as split-apart) strategy is helpful in situations in which one translocation partner is largely conserved but the second translocation partner varies. A good example is the Ewing sarcoma breakpoint region 1

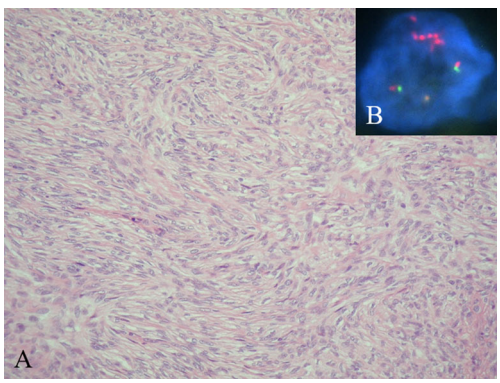

Fig. 5 **a** Dermatofibrosarcoma protuberans (hematoxylin-eosin, original magnification ×200). **b** Inset showing two fusion signals and extra copies (amplification on supernumerary ring chromosome) of the 5′ telomeric region of *COL1A1* (original magnification ×1000) using *COL1A1* dual color break-apart FISH

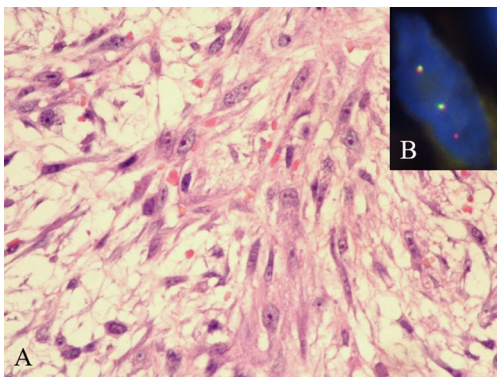

Fig. 6 **a** Nodular fasciitis (hematoxylin-eosin, original magnification ×400) **b** Inset displaying unusual *USP6* break-apart pattern with two fusions and one extra red signal (2F1R, original magnification ×1000) using *USP6* dual color break-apart FISH

(*EWSR1*; also known as *EWS*) which is involved in a broad variety of mesenchymal lesions which include the Ewing sarcoma/peripheral neuroectodermal tumor, desmoplastic small round cell tumor, clear cell sarcoma, angiomatoid fibrous histiocytoma, extraskeletal myxoid chondrosarcoma, myoepithelial carcinoma and a subset of myxoid liposarcoma. In these entities, more than ten fusion partners of *EWSR1* were discovered, including *FLI1*, *ERG*, *ETV1*, *FEV*, *E1AF*, *ATF1*, *WT1*, *CREB1*, *NR4A3*, *NFATC2*, *SP3*, *ZNF278*, *POU5F1* and *DDIT3* [5]. Regarding the interpretation of the split-apart style, the results are clearly visible and readily recognized, as stated by Motoi et al. [6].

Consequently, there are very few data in the literature about the description of atypical break-apart patterns in sarcomas. Actually, one interesting article was found which described 2 cases of malignant melanotic epithelioid renal neoplasms bearing *TFE3* gene fusions. The first case demonstrated unbalanced *TFE3* rearrangement with one fusion and one isolated red signal using split FISH assay. The second case showed one dislocated red and green signal per nucleus which referred to a balanced *TFE3* rearrangement and loss of the second X chromosome [7]. Molecular examinations of synovial sarcoma *SS18-SSX* fusion transcripts discovered some potentially problematic questions concerning the interpretations of break-apart FISH results [8]. This study revealed, for the first time, the loss of one spectrum green signal in more than 70% of neoplastic cells in six synovial sarcoma cases in which the *SS18-SSX* fusion gene was detected by RT-PCR. The speculated cause of atypical pattern was the deletion of the *SSX-SS18* fusion gene which the spectrum green probe labels. The authors suggest the interpretation of FISH results in borderline cases should always be supported by RT-PCR. Moreover, the same study reported, as the second problem during FISH analysis, the presence of multiple copies of *SS18* region in some cases of synovial sarcoma [8].

In our study, we retrospectively evaluated the split-apart FISH results of 301 formalin-fixed, paraffin-embedded soft tissue tumor samples. Altogether, 42 (13.95%) out of the 301 tumors harbored a sort of atypical pattern according to the summarized details of Table 2. A synovial sarcoma case was found first with an isolated red SS18 signal in the majority of the interphase tumor cells. For excluding the possibility of an artifact signal, *SS18* FISH was also performed on the metaphase preparation of the same synovial sarcoma case. The chromosome region of the isolated red signal was found to be translocated to the X chromosome and supposedly fused with *SSX1* as it was validated by interphase FISH with translocation/TriCheck probe and RT-PCR. These results can validate not only the above mentioned 2F1R pattern (which was found in altogether 5 synovial sarcomas) but indirectly also confirm further discovered atypical patterns (2F2R1G, 1F2R1G, 1F1R and 2F2R) concerning another 5 synovial sarcomas.

Atypical/abnormal *FOXO1* rearrangements are well known and are common genetic alterations in alveolar rhabdomyosarcoma [9, 10]. Cytogenetic aberrations demonstrate that one allele of *FOXO1* is rearranged and its centromeric region amplified afterwards. The 50% (6 of 12) incidence of *FOXO1* amplification we found in our alveolar rhabdomyosarcoma cases is in a good correlation with the results of Matsumura et al. [9] who observed the same amplification in 53.8% (7 of 13) of their cases.

Regarding the atypical *DDIT3* break-apart pattern in myxoid/round cell liposarcoma we are not aware of any published data documenting isolated red signal or signals, however, it was found in 7.5% by us. Willmore-Payne et al. observed *DDIT3* probe signal amplification in all 5 well-differentiated liposarcomas and 1 (4.5%) out of 22 myxoid liposarcomas. The authors mentioned that the finding of amplification of either the green fluorophore-labeled probe or both probes was an interesting and initially unsuspected finding, however, data and a detailed description can be found regarding the green telomeric probe part [11]. The explanation of the amplification was that these probes contain a sequence encompassing the *SAS* and *CDK4* genes and these segments of chromosome 12 are frequently amplified in a variety of human sarcomas [12–14].

Explanations for the atypical *FUS* patterns (the telomeric green-labelled part of the probe was exclusively present as extra signal or signals) found in 66.7% of low-grade fibromyxoid sarcomas are the already discovered supernumerary ring chromosomes harboring the *FUS-CREB3L2* fusion gene. Studies with FISH and RT-PCR verified that the ring contained material from chromosome 7 and 16 and, in addition, that the *FUS* gene was present in two rearranged copies and that is expressed by the abovementioned fusion [15, 16].

The *EWSR1* gene, concerning genetic rearrangements, mostly represents balanced translocations with several distinct partners in a broad variety of mesenchymal lesions [17]. Our results with low percentages (3.2% in Ewing sarcoma and 8.3% in clear cell sarcoma) of unusual break-apart patterns for *EWSR1* also support the previous fact. However, a Ewing sarcoma harboring complex rearrangement and amplification of the proximal *EWSR1* region was described by Szuhai et al. [18]. While Jinawath et al. demonstrated a Ewing sarcoma with a normal signal on both apparently normal chromosomes 22, and an additional *EWSR1*–5′ signal on the derivative chromosome 21 using metaphase fluorescence in situ hybridization and *EWSR1* break-apart probe [17]. Furthermore, the presence of *EWSR1* related pseudogenes identified on

chromosomes 1 (annotation NC_000001.10) and 14 (annotation NC_000014.8) put forth the proposal of extra caution in the interpretation of the results of the molecular techniques [5].

The previously observed amplification of chromosome regions and the formation of the ring chromosome are similar to the ones described in dermatofibrosarcoma protuberans which involve *COL1A1* and *PDGFB* on chromosome 17q21.31 ~ q22 and 22q12.3 ~ 13.1, respectively [19]. Our result confirms the demonstrability of the unbalanced translocation as atypical break-apart patterns in dermatofibrosarcoma protuberanses.

FISH studies about atypical break-apart *USP6* rearrangements in nodular fasciitis were not found in the literature. Chen et al. investigated altogether 29 nodular fasciitis using FISH and RT-PCR, finally 83% of the cases were considered as positive for *USP6* gene rearrangement, however only one tumor displayed USP6 deletion [20]. Geiersbach et al. were the first to report a primary aneurysmal bone cyst with rearrangements of both *USP6* and *SS18* [21]. While the *USP6* showed a typical break-apart pattern, *SS18* rearrangement by FISH resulted from a complex rearrangement of 18q11.2 with a deletion of the *SS18* gene. Since evidence of *SS18-SSX* fusion was not verified by RT-PCR, the diagnostic relevance of the discovered rearrangement was also not found accordingly [21].

Conclusions

Although the interpretation problems in the process to evaluate the break-apart probe results is well known from sporadic case reports, a systemic overview to detect their frequency has not been performed so far. In our work, we highlighted the relative frequency of this problem and pinpointed those signal-patterns which, despite their unusual appearance, can still confirm the diagnosis.

Authors' Contributions GP carried out the molecular genetic studies and drafted the manuscript. DM collected the data. ZS conceived of the study, and participated in its design and helped to draft the manuscript. All authors read and approved the final manuscript.

Compliance with Ethical Standards

Conflicts of Interest The authors declare that they have no conflicts of interest.

Funding Hungarian Scientific Research Fund (OTKA), http://www.otka.hu/en, No: K-112993.

Ethics Approval and Consent to Participate The study protocol was approved by the Ethics and Scientific committee of the participating institution. TUKEB 155/2012

References

1. Bridge JA (2014) The role of cytogenetics and molecular diagnostics in the diagnosis of soft-tissue tumors. Modern pathology: an official journal of the United States and Canadian Academy of Pathology, Inc 27(Suppl 1):S80–S97. doi:10.1038/modpathol.2013.179
2. Bridge JA, Cushman-Vokoun AM (2011) Molecular diagnostics of soft tissue tumors. Arch Pathol Lab Med 135(5):588–601. doi:10.1043/2010-0594-rair.1
3. Fletcher CD, Organization WH, Cancer IAfRo (2013) WHO classification of tumours of soft tissue and bone. IARC press
4. Ventura RA, Martin-Subero JI, Jones M, McParland J, Gesk S, Mason DY, Siebert R (2006) FISH analysis for the detection of lymphoma-associated chromosomal abnormalities in routine paraffin-embedded tissue. The Journal of molecular diagnostics: JMD 8(2):141–151. doi:10.2353/jmoldx.2006.050083
5. Romeo S, Dei Tos AP (2010) Soft tissue tumors associated with EWSR1 translocation. Virchows Archiv: an international journal of pathology 456(2):219–234. doi:10.1007/s00428-009-0854-3
6. Motoi T, Kumagai A, Tsuji K, Imamura T, Fukusato T (2010) Diagnostic utility of dual-color break-apart chromogenic in situ hybridization for the detection of rearranged SS18 in formalin-fixed, paraffin-embedded synovial sarcoma. Hum Pathol 41(10):1397–1404. doi:10.1016/j.humpath.2010.02.009
7. Argani P, Aulmann S, Karanjawala Z, Fraser RB, Ladanyi M, Rodriguez MM (2009) Melanotic Xp11 translocation renal cancers: a distinctive neoplasm with overlapping features of PEComa, carcinoma, and melanoma. Am J Surg Pathol 33(4):609–619. doi:10.1097/PAS.0b013e31818fbdff
8. Amary MF, Berisha F, Bernardi Fdel C, Herbert A, James M, Reis-Filho JS, Fisher C, Nicholson AG, Tirabosco R, Diss TC, Flanagan AM (2007) Detection of SS18-SSX fusion transcripts in formalin-fixed paraffin-embedded neoplasms: analysis of conventional RT-PCR, qRT-PCR and dual color FISH as diagnostic tools for synovial sarcoma. Modern pathology: an official journal of the United States and Canadian Academy of Pathology, Inc 20(4):482–496. doi:10.1038/modpathol.3800761
9. Matsumura T, Yamaguchi T, Seki K, Shimoda T, Wada T, Yamashita T, Hasegawa T (2008) Advantage of FISH analysis using FKHR probes for an adjunct to diagnosis of rhabdomyosarcomas. Virchows Archiv: an international journal of pathology 452(3):251–258. doi:10.1007/s00428-007-0554-9
10. Liu J, Guzman MA, Pezanowski D, Patel D, Hauptman J, Keisling M, Hou SJ, Papenhausen PR, Pascasio JM, Punnett HH, Halligan GE, de Chadarevian JP (2011) FOXO1-FGFR1 fusion and amplification in a solid variant of alveolar rhabdomyosarcoma. Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc 24(10):1327–1335. doi:10.1038/modpathol.2011.98
11. Willmore-Payne C, Holden J, Turner KC, Proia A, Layfield LJ (2008) Translocations and amplifications of chromosome 12 in liposarcoma demonstrated by the LSI CHOP breakapart rearrangement probe. Arch Pathol Lab Med 132(6):952–957. doi:10.1043/1543-2165(2008)132[952:taaoci]2.0.co;2
12. Smith SH, Weiss SW, Jankowski SA, Coccia MA, Meltzer PS (1992) SAS amplification in soft tissue sarcomas. Cancer Res 52(13):3746–3749
13. Berner JM, Forus A, Elkahloun A, Meltzer PS, Fodstad O, Myklebost O (1996) Separate amplified regions encompassing CDK4 and MDM2 in human sarcomas. Genes Chromosom Cancer 17(4):254–259. doi:10.1002/(sici)1098-2264(199612)17:4<254::aid-gcc7>3.0.co;2-2

14. Dei Tos AP, Doglioni C, Piccinin S, Sciot R, Furlanetto A, Boiocchi M, Dal Cin P, Maestro R, Fletcher CD, Tallini G (2000) Coordinated expression and amplification of the MDM2, CDK4, and HMGI-C genes in atypical lipomatous tumours. J Pathol 190(5):531–536. doi:10.1002/(sici)1096-9896(200004)190:5<531::aid-path579>3.0.co;2-w
15. Bartuma H, Moller E, Collin A, Domanski HA, Von Steyern FV, Mandahl N, Mertens F (2010) Fusion of the FUS and CREB3L2 genes in a supernumerary ring chromosome in low-grade fibromyxoid sarcoma. Cancer Genet Cytogenet 199(2):143–146. doi:10.1016/j.cancergencyto.2010.02.011
16. Chien YC, Karolyi K, Kovacs I (2016) Paravertebral low-grade fibromyxoid sarcoma with supernumerary ring chromosome: case report and literature review. Ann Clin Lab Sci 46(1):90–96
17. Jinawath N, Morsberger L, Norris-Kirby A, Williams LM, Yonescu R, Argani P, Griffin CA, Murphy KM (2010) Complex rearrangement of chromosomes 1, 7, 21, 22 in Ewing sarcoma. Cancer Genet Cytogenet 201(1):42–47. doi:10.1016/j.cancergencyto.2010.04.021
18. Szuhai K, IJszenga M, Tanke HJ, Taminiau AH, de Schepper A, van Duinen SG, Rosenberg C, Hogendoorn PC (2007) Detection and molecular cytogenetic characterization of a novel ring chromosome in a histological variant of Ewing sarcoma. Cancer Genet Cytogenet 172(1):12–22. doi:10.1016/j.cancergencyto.2006.07.007
19. Sirvent N, Maire G, Pedeutour F (2003) Genetics of dermatofibrosarcoma protuberans family of tumors: from ring chromosomes to tyrosine kinase inhibitor treatment. Genes Chromosom Cancer 37(1):1–19. doi:10.1002/gcc.10202
20. Chen J, Ye X, Li Y, Wei C, Zheng Q, Zhong P, Wu S, Luo Y, Liao Z, Ye H (2014) Chromosomal translocation involving USP6 gene in nodular fasciitis. Zhonghua bing li xue za zhi = Chinese journal of pathology 43(8):533–536
21. Geiersbach K, Rector LS, Sederberg M, Hooker A, Randall RL, Schiffman JD, South ST (2011) Unknown partner for USP6 and unusual SS18 rearrangement detected by fluorescence in situ hybridization in a solid aneurysmal bone cyst. Cancer genetics 204(4):195–202. doi:10.1016/j.cancergen.2011.01.004