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ORIGINAL PAPER

Multiple splice variants of *EWSR1*-ETS fusion transcripts co-existing in the Ewing sarcoma family of tumors

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Abstract
Purpose The Ewing sarcoma family of tumors (EFT) is characterized by fusions of the *EWSR1* gene on chromosome 22q12 with either one of the genes encoding members of the ETS family of transcription factors, in the majority of cases *FLI1* or *ERG*. Many alternative *EWSR1*-ETS gene fusions have been encountered, due to variations in the locations of the *EWSR1* and ETS genomic breakpoints. The resulting heterogeneity in *EWSR1*-ETS fusion transcripts may further be increased by the occurrence of multiple splice variants within the same tumor. Here we present a retrospective study designed to detect all of the *EWSR1-FLI1* and *EWSR1-ERG* fusion transcripts in a series of 23 fresh frozen EFT tissues.
Methods RT-PCR and nested fluorescent multiplex PCR were used to amplify *EWSR1-FLI1* and *EWSR1-ERG* transcripts from EFT tissues. Fusion transcripts were identified by laser-induced fluorescent capillary electrophoresis and confirmed by sequence analysis.
Results Nine different *EWSR1-FLI1* fusion transcripts and one *EWSR1-ERG* fusion transcript were identified in 21 out of 23 fresh frozen EFT tissue samples. In five cases multiple fusion transcripts were found to coexist in the same tumor sample. We additionally reviewed previous reports on twelve cases with multiple *EWSR1*-ETS fusion transcripts.
Conclusions Alternative splicing may frequently affect the process of EFT-associated fusion gene transcription and, as such, may significantly contribute to the pathogenic role of EFT-associated chromosome translocations. In a considerable number of cases this may result in multiple splice variants within the same tumor.

Keywords Ewing sarcoma family of tumors · *EWSR1* gene fusions · Alternative splicing · Molecular diagnostics

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1 Introduction

The Ewing sarcoma family of tumors (EFT) encompasses undifferentiated small, round cell tumors of bone and soft tissues, and includes Ewing sarcomas (ES), extra-osseus Ewing tumors and peripheral primitive neuroectodermal tumors (pPNET). EFT tumors share common specific chromosomal translocations, usually involving the *EWSR1* (Ewing sarcoma breakpoint region 1) gene on chromosome 22q12, encoding a TET family member protein, and either one of five genes encoding ETS family transcription factors. The *FLI1* (11q24) [1] and *ERG* (21q22) [2] genes are the most common ETS family translocation partners, and they are involved in approximately 85–90 % and 5–10 % of the cases, respectively [3–7]. Alternative, less frequent, translocation partners are the *ETV*1 (7p22) [8], *ETV4*/*E1AF* (17q12) [9] and *FEV* (2q33) [10] genes.

In recent years, the observed heterogeneity in variant EFT translocations has increased, and there is ample evidence now

that both partners of the EFT-associated translocations are interchangeable. In addition, it has been found that another member of the TET protein coding family, *FUS* (16p11), can act as a translocation partner as well [11, 12]. Furthermore, in the past few years novel sporadic C-terminal fusion partners, i.e., *PATZ1*/*ZSG* [13], *SP3* [14], *NFATc2* [15] and *SMARCA5* [16], not belonging to the ETS family of transcription factors, have been identified in EFT or Ewing-like tumors.

The variations in genomic breakpoints in the *EWSR1* and ETS-related genes may result in a significant number of alternative EFT translocation products, which complicates RNA-based molecular diagnostics. The positions of the genomic breakpoints in the most common *EWSR1-FLI1* translocation were found to be located in intron 7, 8, 9 or 10 of the *EWSR1* gene, and in intron 3, 4, 5, 6, 7 or 8 of the *FLI1* gene [3, 17]. Of the several possible alternative *EWSR1-FLI1* translocation products, 12 are considered to be most prevalent [18]. Of these, the *EWSR1*(ex7)-*FLI1*(ex6) (51 %) and *EWSR1*(ex7)-*FLI1*(ex5) (27 %) translocation products, called type I and type II, are the most frequent ones [19].

The resulting fusion proteins act as aberrant transcription factors, which lead to the disruption of normal transcriptional control, the transformation of cells and, ultimately, the development of EFT [20, 21]. The minimal and also critical components of the EWS-ETS oncoproteins are the transcription activation domain of the EWS protein (encoded by exons 1–7 of *EWSR1*) and the DNA-binding domains of the respective ETS proteins (encoded by exon 9 of *FLI1*) [1, 20, 21]. The full lengths of the active fusion proteins can vary according to the locations of the translocation breakpoints and the transcribed RNA sequences.

Though in the majority of cases only one EFT-associated fusion transcript can be identified in the tumor tissues, it has been reported that more than one fusion transcript may be present [3, 21]. Such cases, however, were considered to be exceptions to the one translocation-one fusion transcript rule, and never gained significant attention.

Here, we report the presence of multiple alternative mRNA products in a retrospective study designed to detect EFT-associated *EWSR1-FLI1* and *EWSR1-ERG* fusion transcripts in fresh frozen tissues, using multiplex fluorescent PCR and laser-induced fluorescence capillary electrophoresis. Nine different *EWSR1-FLI1* fusion transcripts and one *EWSR1-ERG* fusion transcript were identified in 21 out of 23 fresh frozen tissue samples. Multiple fusion transcripts were found to coexist in five cases. Together with a review of previously reported cases, we conclude that alternative splicing may play an important role in the formation of the EFT-associated active onco-proteins.

2 Materials and methods

2.1 Tumor cohort

With the approval of the Ethics Committee of the Ministry of Health of Hungary (No.ad.22-3/2007-1018EKU), 23 fresh frozen tissues from 22 patients with EFT tumors resected between 1996 and 2006 [22], were included. All samples were retrieved from the Tumor Bank at the Department of Orthopaedics, Semmelweis University, Budapest. The histopathological diagnosis of Ewing sarcoma (ES)/peripheral primitive neuroectodermal tumor (pPNET) was based on current histological criteria defined by the WHO [23]. We also included one tissue sample diagnosed as small round cell tumor with a questionable classification of ES/pPNET. Of the 22 EFT patients, 10 were male and 12 were female. The ages ranged from 4 years to 62 years and the median age was 16 years. The tumor location was axial in 6 patients, and in the other 16 patients the tumors were located in the extremities.

2.2 RNA extraction

Total RNA was extracted from fresh frozen tissues using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA), based on the acid guanidinium-phenol-chloroform method, according to the supplier's instructions. The RNA was quantified using a SmartSpec™ Plus spectrophotometer (Bio-Rad, Hercules, CA). The extracted RNA concentrations ranged between 194 and 4,812 ng/μl. Highly concentrated RNA samples were diluted to 200 ng/μl. The RNAs were stored at −80 °C until use.

2.3 Primers

According to the reported breakpoint regions of the *EWSR1*, *FLI1* and *ERG* genes, and the sporadic fusion transcripts [2, 3, 17, 24, 25], two outer (E7.1-F9.1 and E7.1-ERG9) and three inner, previously published, primer sets (EWS B, FLI1, ERG) [22] were used (Table 1). The EWS B and FLI1 primer sets included four forward (E7.2, E8.2, E9.2 and E10.2 for exons 7, 8, 9 and 10 of *EWSR1*, respectively) and reverse (F4.2, F5.2, F7.2, F9.2 for exons 4, 5, 7, 9 of *FLI1*, respectively) primers (Fig. 1). The ERG primer set encompassed two reverse primers (ERG7 and ERG9) designed for *ERG* exons 7 and 9, respectively. Each of the four forward primers of the EWS B primer set was labeled by either one of the 6FAM, VIC, NED or PET fluorescent dyes.

2.4 RT-PCR and PCR

For RT-PCR, 40–60 ng of total RNA was reverse-transcribed and amplified using a GeneAmp RNA PCR

Table 1 Primers used for RT–PCR and nested PCR amplification of *EWSR1-FLI1* and *EWSR1-ERG* transcripts

EWS A1	E7.1 TCCTACAGCCAAGCTCCAAG
Outer primer pair	F9.1 GAGAGCAGCTCCAGGAGGAA
EWS A2	E7.1 TCCTACAGCCAAGCTCCAAG
Outer primer pair	ERG9 AGGAACTGCCAAAGCTGGAT
EWS B 5′ primers	E7.2 *6FAM*-ATATAGCCAACAGAGCAGCAG
Inner sets	E8.2 *VIC*-GAGGCATGAGCAGAGGTG
	E9.2 *NED*-GTGGCTTCAATAAGCCTGGTG
	E10.2 *PET*-GGATGAAGGACCAGATCTTGAT
FLI1 3′ primers	F4.2 TTCTGGAAAAAGGATGTGTCG
Inner sets	F5.2 GTGAGGATTGGTCGGTGTG
	F7.2 CTGTATTCTTACTGATCGTTTGTGC
	F9.2 GCAGCTCCAGGAGGAATTG
ERG 3′ primers	ERG7 GTGGAAGGAGATGGTTGAGC
Inner sets	ERG9 AGGAACTGCCAAAGCTGGAT

Kit (Perkin-Elmer Corp., Norwalk, CT) in a final reaction volume of 7.5 μl. The mixture contained 2 mM $MgCl_2$, 200 μM of each dNTP (dATP, dCTP, dGTP and dTTP), 0.075 μM of primer pairs E7.1-F9.1 or E7.1-ERG11, 1.5U of RNase Inhibitor, 0.375U of AmpliTaq Gold DNA polymerase, and 2.25U of MultiScribe Reverse Transcriptase. The PCR reactions were carried out on a PTC-225 Peltier Thermal Cycler (MJ Research, Watertown, MA, USA), with reverse transcription at 42 °C for 12 min, a polymerase activation step at 95 °C for 10 min, followed by 40 cycles of denaturation (94 °C for 20 s) and annealing (60 °C for 1 min) and, finally, one extension step at 72 °C for 7 min. The nested multiplex PCR was performed on 0.3 μl of RT-PCR product using AmpliTaq Gold DNA Polymerase with GeneAmp Gold Buffer (Applied Biosystems Inc., Foster City, CA, USA) and gene specific primers (fluorescent-labeled inner sets of EWS B primers and FLI1 or ERG sets of primers) in a total volume of 7.5 μl, containing 2 mM $MgCl_2$, 200 mM each dNTP, 0.15 μM of each primer from the primer sets and 0.375U of AmpliTaq Gold DNA polymerase. The multiplex PCR reactions were performed using an AmpliTaq Gold activation cycle at 95 °C for 10 min, followed by 40 cycles of denaturation (94 °C for 20 s) and annealing (60 °C for 1 min) and a final extension step at 72 °C for 7 min.

2.5 Laser-induced fluorescence capillary electrophoresis and sequencing

Multiplex PCR products were visualized by laser-induced fluorescent capillary electrophoresis on an ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). On the electropherograms, analyzed using GeneScan software (Applied Biosystems, Foster City, CA, USA), the PCR products were represented as peaks with accurate sizes. Fusion transcripts were identified on basis of the size of the PCR products compared to the expected sizes of the various possible *EWSR1-FLI1* or *EWSR1-ERG* fusion transcripts, and on basis of the fluorescence of the PCR products, represented on the electropherogram by different colors corresponding to each of the fluorophores. The identification of fluorescent dyes labeled to the forward primers was instrumental for limiting the number of possible fusion transcripts. The sequences of the specific PCR products were always confirmed by sequencing on an ABI PRISM™ 310 sequencer.

For each step of the RNA extraction, reverse transcription and amplification, no-RNA and water were used as negative controls.

3 Results

EWSR1-ETS fusion variants were identified in 21 (91 %) of the 23 fresh frozen EFT tissue samples included (Table 2). In the analyzed samples, 9 distinct *EWSR1-FLI1* fusion transcripts, one *EWSR1-ERG* fusion transcript, and one alternative type II transcript were detected. *EWSR1-FLI1* type I *EWSR1*(ex7)-*FLI1*(ex6) fusion transcripts were found in 11 samples from 10 patients, whereas type II *EWSR1*(ex7)-*FLI1*(ex5) and *EWSR1*(ex7)-*ERG*(ex8) fusion transcripts

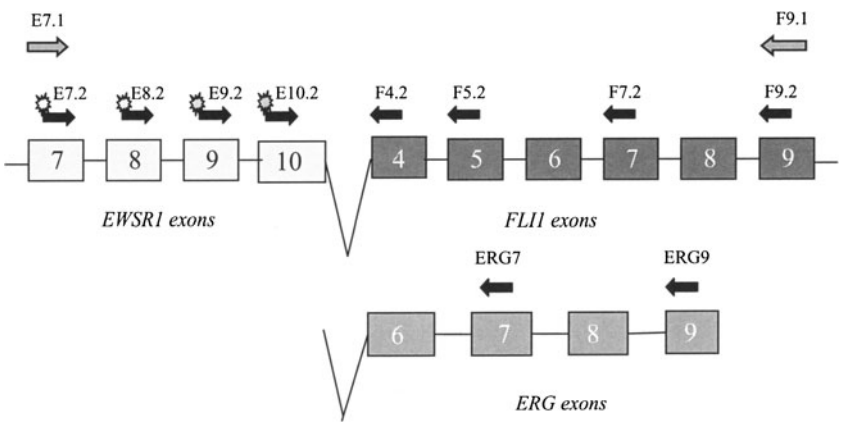

Fig. 1 Primer placement for *EWSR1-FLI1* and *EWSR1-ERG* translocations

Table 2 Data of 23 fresh frozen samples from 22 EFT cases, with additional results of formalin-fixed paraffin-embedded samples from patients no. 2, 4, 7, 19 and 20

Patient no.	Sample no.	Specimen	Translocation	Histological diagnosis[a]
1.	1.	FFT	*EWSR1*(ex 7)-*FLI1*(ex 5)	ES
2.	2.	FFT	*EWSR1*(ex 7)-*FLI1*(ex 6) *EWSR1*(ex 8)-*FLI1*(ex 6) *EWSR1*(ex 8)-*FLI1*(ex 8)	ES
	3.	FFPE	*EWSR1*(ex 7)-*FLI1*(ex 6)	
	4.	FFPE	*EWSR1*(ex 7)-*FLI1*(ex 6)	
3.	5.	FFT	*EWSR1*(ex 7)-*FLI1*(ex 6)	ES
4.	6.	FFT	*EWSR1*(ex 7)-*FLI1*(ex 6) *EWSR1*(ex 7)-*FLI1*(ex 8)	ES
	7.	FFPE	*EWSR1*(ex 7)-*FLI1*(ex 6)	
5.	8.	FFT	*EWSR1*(ex 7)-*FLI1*(ex 6)	ES
6.	9.	FFT	*EWSR1*(ex 7)-*ERG*(ex 8)	ES
7.	10.	FFT	*EWSR1*(ex 7)-*FLI1*(ex 5) *EWSR1*(ex 8)-*FLI1*(ex 5)	ES
	11.	FFPE	*EWSR1*(ex 7)-*FLI1*(ex 5)	
8.	12.	FFT	*EWSR1*(ex 7)-*FLI1*(ex 6)	ES
9.	13.	FFT	*EWSR1*(ex 7)-*FLI1*(ex 6)	ES
10.	14.	FFT	*EWSR1*(ex 7)-*FLI1*(ex 5)	ES
11.	15.	FFT	*EWSR1*(ex 7)-*FLI1*(ex 6)	ES
12.	16.	FFT	*EWSR1*(ex 7)-*ERG*(ex 8)	ES
13.	17. 18.	FFT FFT	*EWSR1*(ex 7)-*FLI1*(ex 6) *EWSR1*(ex 7)-*FLI1*(ex 6)	ES
14.	19.	FFT	*EWSR1*(ex 10)-*FLI1*(ex 6)	ES
15.	20.	FFT	*EWSR1*(ex 7)-*FLI1*(ex 6)	ES
16.	21.	FFT	Negative	ES
17.	22.	FFT	*EWSR1*(ex 7)-*ERG*(ex 8)	ES
18.	23	FFT	*EWSR1*(ex 7)-*FLI1*(ex 6)	SRCT, ES/pPNET?
19.	24.	FFT	*EWSR1*(ex 7)-*FLI1*(ex 7) *EWSR1*(ex 9)-*FLI1*(ex 7)	ES
	25.	FFPE	*EWSR1*(ex 7)-*FLI1*(ex 7) *EWSR1*(ex 8)-*FLI1*(ex 7) *EWSR1*(ex 9)-*FLI1*(ex 7)	
20.	26.	FFT	*EWSR1*(ex 7)-*FLI1*(ex 5) *EWSR1*(ex 7)-*FLI1*(ex 5^8)	ES
	17.	FFPE	*EWSR1*(ex 7)-*FLI1*(ex 5)	
21.	28.	FFT	*EWSR1*(ex 7)-*ERG*(ex 8)	ES
22.	29.	FFT	Negative	pPNET

FFT fresh frozen tissue, *FFPE* formalin-fixed paraffin-embedded tissue, *ES* Ewing's sarcoma, *pPNET* peripheral primitive neuroectodermal tumor, *SRCT* small round cell tumor

[a]Analysis by fluorescence in situ hybridization has not been performed to confirm the diagnosis of EFT

were detected in four cases each. The following seven *EWSR1-FLI1* fusion transcripts were found only in single cases: *EWSR1*(ex7)-*FLI1*(ex7), *EWSR1*(ex7)-*FLI1*(ex8), *EWSR1*(ex8)-*FLI1*(ex5), *EWSR1*(ex8)-*FLI1*(ex6), *EWSR1*(ex8)-*FLI1*(ex8), *EWSR1*(ex9)-*FLI1*(ex7), and *EWSR1*(ex10)-*FLI1*(ex6). In one sample, a type II splice variant of *EWSR1-FLI1* was identified: *EWSR1*(ex7)-*FLI1*(ex5^8). In the latter fusion transcript *FLI1* exons 6 and 7 were excluded.

In 5 of the 23 fresh frozen tumor samples, we identified multiple *EWSR1-FLI1* fusion transcripts. In the tumor sample from patient No. 2, three different fusion transcripts were detected, i.e., *EWSR1*(ex7)-*FLI1*(ex6), *EWSR1*(ex8)-*FLI1*(ex6) and *EWSR1*(ex8)-*FLI1*(ex8) (Fig. 2a). In the tumor sample from patient No. 4 *EWSR1*(ex7)-*FLI1*(ex6) and *EWSR1*(ex7)-*FLI1*(ex8) fusion transcripts were detected (Fig. 2b), and in the tumor sample from patient No. 7 *EWSR1*(ex7)-*FLI1*(ex5) and *EWSR1*(ex8)-*FLI1*(ex5) fusion transcripts were found (Fig. 2c). In the tumor sample of patient No. 19 *EWSR1*(ex7)-*FLI1*(ex7) and *EWSR1*(ex9)-*FLI1*(ex7) fusion transcripts were detected (Fig. 2d). Finally, in the sample of patient No. 20 we identified a *EWSR1*(ex7)-*FLI1*(ex5)

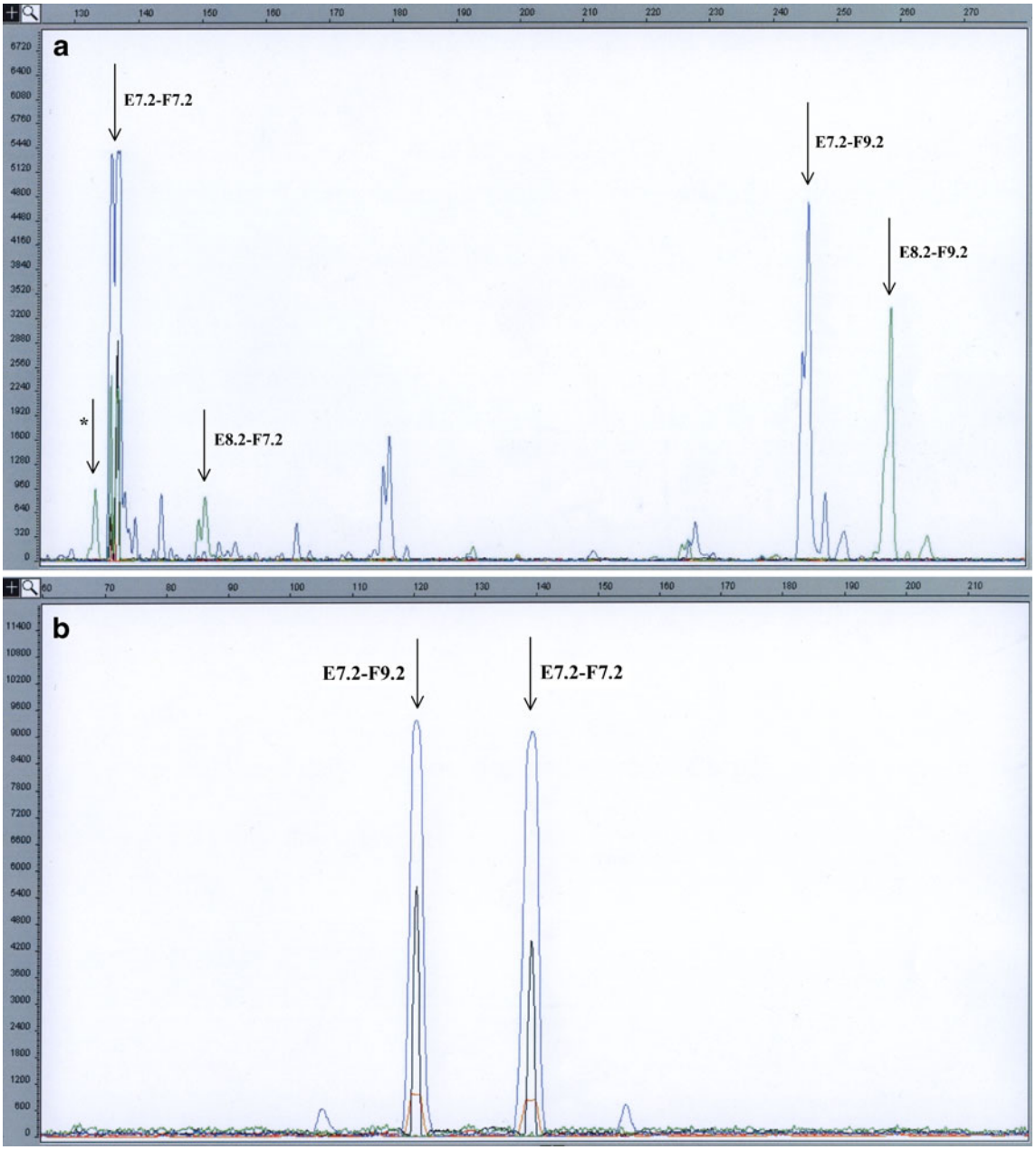

Fig. 2 Electropherograms of PCR products with multiple fusion splice variants. PCR products amplified by a fluorescent dye-labeled forward primer set (EWS B) and a reverse primer set (*FLI1* or *ERG*) were separated by capillary electrophoresis to identify *EWSR1-FLI1* or *EWSR1-ERG* fusion transcripts by color-coded fluorophores and fragment size. PCR products amplified by E7.2, E8.2, E9.2 and E10.2 primers are coded by the colors blue, green, black and red, respectively. **a** Three chimeric transcript isoforms identified in the tumor of patient No. 2: *EWSR1*(ex7)-*FLI1*(ex6) amplified by primer pairs E7.2-F7.2 and E7.2-F9.2; *EWSR1*(ex8)-*FLI1*(ex6) amplified by E8.2-F7.2 and E8.2-F9.2; and *EWSR1*(ex8)-*FLI1*(ex8) amplified by E8.2-F9.2 (labeled by asterisk on electropherogram). **b** Two fusion transcript isoforms are visualized on the electropherogram of PCR products amplified from the tumor sample of patient No. 4: *EWSR1*(ex7)-*FLI1*(ex8) transcript amplified by primer pair E7.2-F9.2; and *EWSR1*(ex7)-*FLI1*(ex6) amplified by E7.2-F7.2. **c** PCR products amplified from the tumor sample of patient No. 7 identified as fusion transcripts *EWSR1*(ex7)-*FLI1*(ex5) and *EWSR1*(ex8)- *FLI1*(ex5) amplified by primers E7.2-F7.2 and E8.2-F7.2, respectively. **d** Two types of fusion transcripts identified in the fresh frozen tumor sample of patient No. 19: *EWSR1*(ex)-*FLI1*(ex7) amplified by E7.2-F7.2; and *EWSR1*(ex9)-*FLI1*(ex7) amplified by primer pairs E8.2-F7.2 and E9.2-F9.2

fusion transcript and, in addition, an alternative transcript of this type, i.e., *EWSR1*(ex7)-*FLI1*(ex5^8). Comparing the fusion transcripts detected in fresh frozen samples with those previously reported [22] in FFPE tumor samples of patients No. 2, 4, 7, 19 and 20 (Table 2), it can be noted that in the FFPE samples of patients No. 2, 4, 7 and 20 only one of the alternative fusion transcripts was detected, the most common one (type I or type II, depending on samples). In the FFPE sample

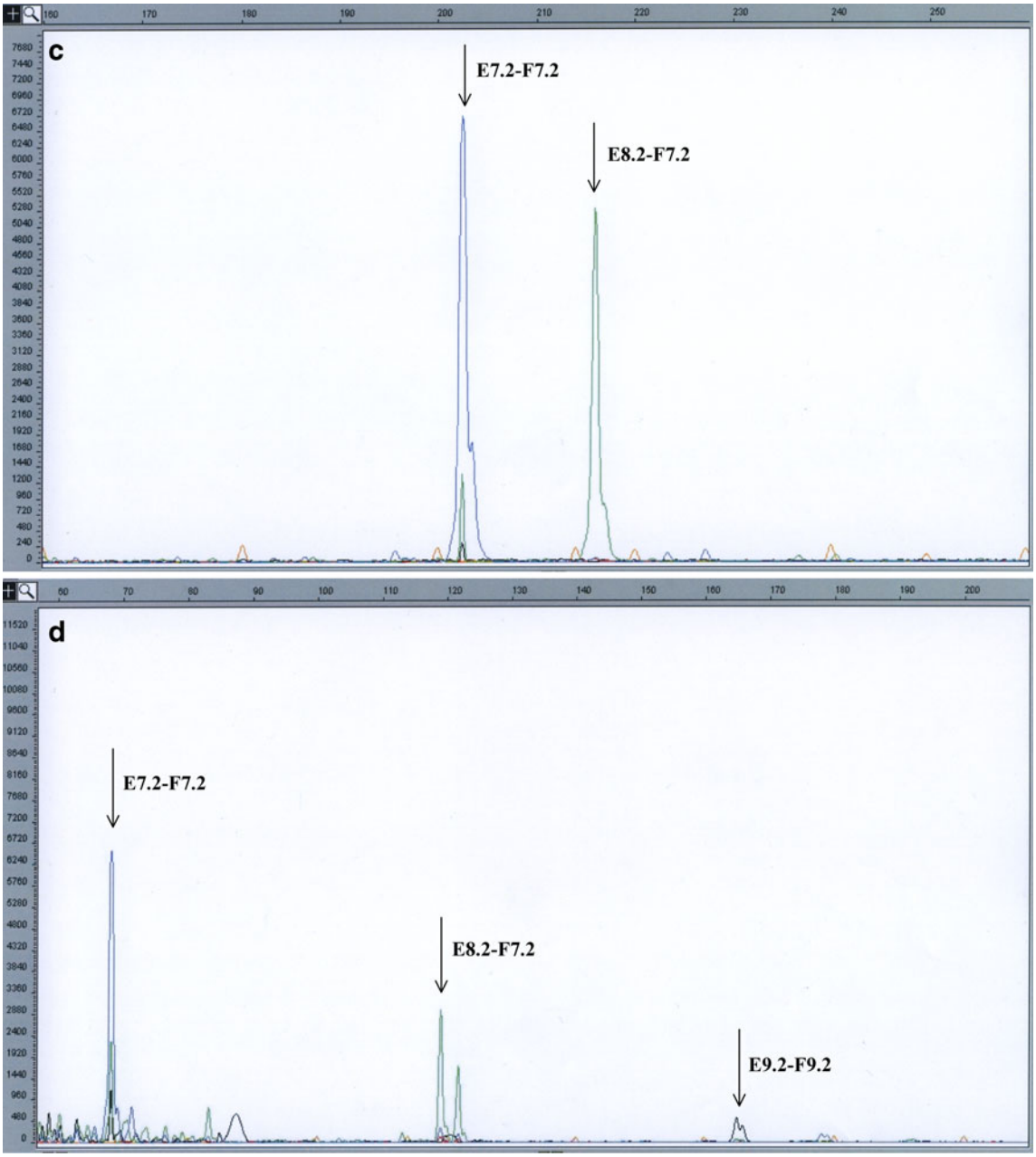

Fig. 2 (continued)

of patient No. 19, however, three different fusion transcripts were detected, i.e., *EWSR1*(ex7)-*FLI1*(ex7), *EWSR1*(ex8)-*FLI1*(ex7) and *EWSR1*(ex9)-*FLI1*(ex7), while in the fresh frozen sample only two were found.

To assess the transcription pattern of the *EWSR1* gene on the other, normal, alleles in our 23 EFT tumor samples, we amplified *EWSR1* mRNA from exon 7 to exon 11 by RT-PCR and nested fluorescent PCR, and visualized the resulting products by laser-induced fluorescent capillary electrophoresis (not shown) and by agarose gel electrophoresis (Fig. 3). By doing so, no alternative transcripts were detected.

4 Discussion

In this study on 23 EFT tumor samples, we have identified 7 of the 12 most prevalent alternative *EWSR1-FLI1* fusion transcripts [18], two fusion transcripts that, to our knowledge, have not been reported before, i.e., *EWSR1*(ex8)-*FLI1*(ex5), *EWSR1*(ex8)-*FLI1*(ex8), one *EWSR1-FLI1* type II splice variant, and one *EWSR1-ERG* fusion transcript. Moreover, different fusion transcripts were found to coexist in five of the tumors. In one of them, three variant fusion transcripts were found, and in four tumors two different fusion transcripts were identified. No evidence was obtained

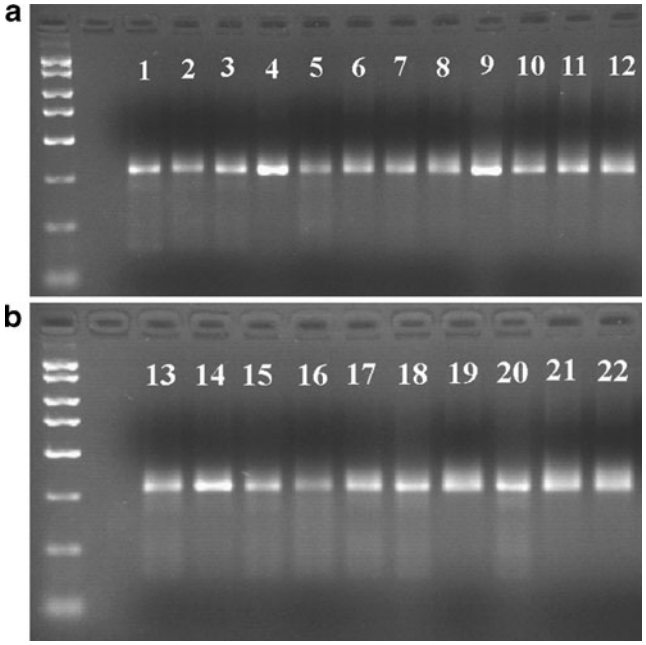

Fig. 3 Agarose gel electrophoresis showing PCR products after the amplification of the *EWSR1* transcript. Amplification of the *EWSR1* transcript from exon 7 to exon 11 by forward primer E7.2 and a reverse primer designed for *EWSR1* exon 11 shows only one PCR product in each of 22 fresh frozen samples

for alternative splicing of the *EWSR1* gene on the other, normal, alleles in the 23 tumor samples analyzed. Thus, multiple alternative fusion transcripts seem to be characteristic for EFT. To optimize our method, we also extracted RNA from the Ewing sarcoma cell line SK-ES-1 (DSMZ, Braunschweig, Germany). Interestingly, the fusion transcript identified in RNA extracted from an early passage was identified as type II, whereas in RNA extracted from a later passage also *EWSR1*(ex8)-*FLI1*(ex5) and *EWSR1*(ex7)-*FLI1*(ex5) transcripts were detected (data not shown). Since we found only sporadic and/or unique fusion transcripts in our tumor samples, and negative controls were used, we do not think that the multiple alternative fusion transcripts detected in our samples are the result of cross contamination or product carry-over.

The occurrence of multiple fusion transcripts in the same tumor sample is a well-known phenomenon in human malignancies, and has e.g. been reported in desmoplastic small round cell tumors [26], synovial sarcomas [27], clear cell sarcomas [28, 29], and *ETV6-ABL1* or *PML-RARA*-positive leukemias [30, 31]. Although in EFT tumors the coexistence of multiple fusion transcripts is not considered to be typical, some cases have been reported previously (Table 3). The first observation was made by May et al. [21], who identified *EWSR1*(ex7)-*FLI1*(ex6) and *EWSR1*(ex7)-*FLI1*(ex7) fusion transcripts in the Ewing sarcoma cell line TC-32. After mapping of the genomic breakpoints of the t(11;22) translocation, Zucman et al. [3] found two distinct *EWSR1-FLI1* fusion transcripts in four tumors, of which one was in-frame and the other was out-of-frame. The out-of-frame transcript encoded a putative truncated inactive EWS protein lacking the DNA binding domain of FLI1. Zoubek et al. [17] reported five alternative *EWSR1-FLI1* fusion transcripts in one tumor sample, with a single DNA breakpoint in *EWSR1* intron 9. De Alava et al. [32] and Yoshino et al. [33] found single cases with two and three different *EWSR1-FLI1* fusion transcripts, respectively. In Yoshino's study, however, the detection of the fusion transcripts was based only on the sizes of the PCR products, and they were not confirmed by DNA sequencing. In a study of Minoletti et al. [34] the presence of two in-frame *EWSR1-ERG* fusion transcripts in the same tumor was reported, and one of them was found to be derived from an insertion of a fragment from an alternative transcript of the *ERG* gene, termed *erg-3*. Wang et al. [14] reported two in-frame *EWSR1-SP3* fusion transcripts in an Ewing-like tumor sample, of which the longer fusion transcript showed again an insertion of a cryptic exon. Finally, Bielack et al. [35] and Lewis et al. [36] reported different fusion transcripts occurring in the primary and secondary tumors and in the primary tumor and metastasis of the same patients, respectively.

Lewis et al. [36] suggested two different explanations for the occurrence of multiple *EWSR1-FLI1*/*ERG* fusion transcripts in the same patients. One explanation might be the occurrence of two different chromosomal rearrangements in the same patient, resulting in either polyclonality of the same tumor or the development of two distinct EFT tumors. Though the probability of two independent chromosomal translocations leading to two distinct EFT tumors is low, it cannot completely be excluded. The case reported by Bielack et al. [35], in which different *EWSR1-ERG* and *EWSR1-FLI1* fusion transcripts were detected in primary and secondary tumors, respectively, could be in support of this explanation. Alternatively, mRNA splicing may underlie the occurrence of multiple fusion transcripts in EFTs, as was first suggested by Zucman et al. [3] based on the above described cases expressing two *EWSR1-FLI1* fusion transcripts with only one type of the *EWSR1-FLI1* translocation identified in each case. In most of the collected cases so far, alternative mRNA splicing offers the most probable explanation for the occurrence of multiple fusion transcript variants.

Of the ten cases reported in the literature [3, 17, 21, 32–34] where multiple fusion transcripts in the same tumor sample were detected, seven exhibited splice variants derived from splicing out of an exon(s) from the *EWSR1* gene. Alternative splicing of *FLI1* was mentioned only in two cases, while in one case an alternative *ERG* gene fragment, *erg-3*, was spliced in. In five cases, alternative transcripts were detected in which *EWSR1* exon 8 and exons of *FLI1* were joined together. These splice variants were out-of-frame and produced putative truncated fusion proteins incapable of DNA binding. Only one fusion transcript containing a cryptic exon [14] proved to be

Table 3 Summary of cases expressing multiple fusion transcripts reported previously or described in this study

Author	Tumor sample	Translocation
May et al. 1993 [21]	1 (TC-32 cell line)	*EWSR1*(ex 7)-*FLI1*(ex 6) *in-frame*
		EWSR1(ex 7)-*FLI1*(ex 7) *in-frame*
Zucman et al. 1993 [3]	4/89 (tumor sample)	*EWSR1*(ex 7)-*FLI1*(ex 6) *in-frame*
		EWSR1(ex 8)-*FLI1*(ex 6) *out-of-frame*
		EWSR1(ex 9)-*FLI1*(ex 7) *in-frame*
		EWSR1(ex 8)-*FLI1*(ex 7) *out-of-frame*
		EWSR1(ex 10)-*FLI1*(ex 5) *in-frame*
		EWSR1(ex 8,10)-*FLI1*(ex 5) *out-of-frame*
		EWSR1(ex 10)-*FLI1*(ex 8) *in-frame*
		EWSR1(ex 8,10)-*FLI1*(ex 8) *out-of-frame*
Zoubek et al. 1994 [17]	1/30 (cell line from a patient's metastasis)	*EWSR1*(ex 9)-*FLI1*(ex 4) *in-frame*
		EWSR1(ex 7)-*FLI1*(ex 4) *in-frame*
		EWSR1(ex 7)-*FLI1*(ex 6) *in-frame*
		EWSR1(ex 7)-*FLI1*(ex 8) *in-frame*
		EWSR1(ex 7)-*FLI1*(ex 9) *in-frame*
de Alava et al. 1998 [32]	1/112 (tumor sample)	*EWSR1*(ex 10)-*FLI1*(ex 5) *in-frame*
		EWSR1(ex 7)-*FLI1*(ex 5) *in-frame*
Minoletti et al. 1998 [34]	1 (tumor sample)	*EWSR1*(ex 7)-*ERG*(ex 6) *in-frame*
		EWSR1(ex 7)-erg3-*ERG*(ex 6) *in-frame*
Yoshino et al. 2003 [33]	1/3 (tumor sample)	*EWSR1(ex 7)-FLI1(ex 7) ? in-frame*
		EWSR1(ex 8)-FLI1(ex 7) ? out-of-frame
		EWSR1(ex 9)-FLI1(ex 7) ? in-frame
Bielack et al. 2004 [35]	1 (tumor sample)	*EWSR1-ERG* (primary tumor)
		EWSR1-FLI1 type 5 (secondary tumor)
Wang, 2007 [14]	1/4 (Ewing-like tumor samples with rare *EWSR1* fusions)	*EWSR1*(ex 7)-*SP3*(ex 6) *in-frame*
		EWSR1(ex 8)-*EWSR1*(intron 8)-*SP3* (ex 6) *in-frame*
Lewis et al. 2007 [36]	1/43 (tumor sample)	*EWSR1*(ex 7)-*FLI1*(ex 5) (primary)
		EWSR1(ex 7)-*FLI1*(ex 6) (metastasis)
Summary	12/284	
Our study	5/23 tumor sample	*EWSR1*(ex 7)-*FLI1*(ex 6) *in-frame*
		EWSR1(ex 8)-*FLI1*(ex 6) *out-of-frame*
		EWSR1(ex 8)-*FLI1*(ex 8) *out-of-frame*
		EWSR1(ex 7)-*FLI1*(ex 8) *in-frame*
		EWSR1(ex 7)-*FLI1*(ex 6) *in-frame*
		EWSR1(ex 7)-*FLI1*(ex 5) *in-frame*
		EWSR1(ex 8)-*FLI1*(ex 5) *out-of-frame*
		EWSR1(ex 7)-*FLI1*(ex 7) *in-frame*
		EWSR1(ex 8)-*FLI1*(ex 7) *out-of-frame*
		EWSR1(ex 9)-*FLI1*(ex 7) *in-frame*
		EWSR1(ex 7)-*FLI1*(ex 5) *in-frame*
		EWSR1(ex 7)-*FLI1*(ex 5^8) *in-frame*

in-frame, in which the insertion of an intronic fragment resulted in the recovery of the reading frame. In six tumor samples, at least two in-frame fusion transcripts coexisted encoding different active EFT-associated onco-proteins.

In our current study, alternative transcripts were derived from the splicing out of *EWSR1* exons in two cases and from the splicing out of *FLI1* exons in two other cases. In one case, both *EWSR1* and the *FLI1* exons were spliced out. In

two of the five cases, there was only one in-frame transcript, and in three cases, there were two variant in-frame transcripts encoding active proteins capable of DNA binding. It must be noted that in cases of out-of-frame fusions, like the ones formed by breaks in *EWSR1* intron 8, alternative splicing provides the only possibility for the expression of active onco-proteins. Thus, alternative splicing can play an important role in tumorigenesis. Moreover, the molecular characterization of the *EWSR1* and *FLI1* chromosomal breakpoints [3, 25] revealed a frequent occurrence of translocation breakpoints in intron 8 of the *EWSR1* gene, with an approximately equal frequency to that in intron 7. In the current studies the frequency of breakpoints in intron 8 encompassed approximately 1/3 of all *EWSR1-FLI1* translocations. Thus, alternative splicing seems to be a frequent phenomenon leading to the formation of active EFT onco-proteins.

The 12 cases with multiple EFT-associated transcripts collected from the literature were detected in 284 EFT tumor samples. In our study, 23 tumor samples from 22 patients were analyzed and 5 cases with multiple fusion transcripts were identified. In our opinion, this relatively high frequency is the result of our detection method, in which nested multiplex PCR and capillary electrophoresis were combined. This approach is in principle more reliable and sensitive for the amplification and detection of fusion transcripts, respectively. We suppose that one of the splice variants detected, the in-frame variant, is predominantly present, whereas the other ones are transcribed at a lower level, which is characteristic for alternative splicing events [37, 38]. This notion is in accordance with previous observations of cases with one prominent amplification product among multiple EWSR1-ETS variants [14, 17]. Our results also confirm this theory, since unlike in the cases of fresh frozen tissue samples where multiple fusion transcripts were identified, in the FFPE tissue samples of the same patients usually only one fusion transcript was detected, invariably the in-frame variant. RNA degradation alone does not provide a satisfactory explanation for these findings, since the differences in size between the fusion fragments amplified by multiplex PCR were small (on average 10–20 nucleotides). Differences in amounts of fusion transcripts may, however, very well explain the results obtained.

In summary, we here report five EFT cases expressing multiple *EWSR1*-ETS splice variants, including two novel out-of-frame variants. In addition, we show that the splicing events only affect the *EWSR1-FLI1* fusion gene, but not the wild-type *EWSR1* gene. Our results, and a review of similar studies, led us to conclude that alternative *EWSR1*-ETS splicing is a frequent event, and in several cases results in multiple splice variants, with at least one in-frame variant, within the same tumor. In cases of out-of-frame gene fusions, alternative splicing can play an important role in the formation of biologically active onco-proteins.

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