Establishment of multiplex RT-PCR to detect fusion genes for the diagnosis of Ewing sarcoma

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Abstract

Background: Detection of the tumor-specific EWSR1/FUS-ETS fusion gene is essential to diagnose Ewing sarcoma. Reverse transcription–polymerase chain reaction (RT–PCR) and fluorescence in situ hybridization are commonly used to detect the fusion gene, and assays using next-generation sequencing have recently been reported. However, at least 28 fusion transcript variants have been reported, making rapid and accurate detection difficult.

Methods: We constructed two sets of multiplex PCR assays and evaluated their utility using cell lines and clinical samples.

Results: EWSR1/FUS-ETS was detected in five of six tumors by the first set, and in all six tumors by the second set. The fusion gene detected only by the latter was EWSR1-ERG, which completely lacked exon 7 of EWSR1. The fusion had a short N-terminal region of EWSR1 and showed pathologically atypical features.

Conclusions: We developed multiplex RT–PCR assays to detect EWSR1-ETS and FUS-ETS simultaneously. These assays will aid the rapid and accurate diagnosis of Ewing sarcoma. In addition, variants of EWSR1/FUS-ETS with a short N-terminal region that may have been previously missed can be easily detected.

Keywords: Ewing sarcoma, Multiplex RT–PCR, Genetic diagnosis, Fusion gene, EWSR1, Transcription factor, Breakpoint

Background

Ewing sarcoma primarily occurs in the bones and soft tissues of children and young adults. It is characterized by fusion genes between a gene of the RNA-binding FET family (EWSR1 or FUS) with a gene of the ETS-transcription factor family (FLI1, ERG, ETV1, ETV4 (E1AF), and FEV) [1–5], which are called EWSR1/FUS-ETS fusion genes. EWSR1-FLI1, generated by t(11;22)(q24.3;q12.2), occurs most frequently, followed by EWSR1-ERG, which is generated by t(21;22)(q22.2;q12.2) [6]. Similarly, EWSR1-ETV1, EWSR1-ETV4, EWSR1-FEV, FUS-ERG, and FUS-FEV are rarely formed in Ewing sarcoma, and these are generated by t(7;22)(p21.2;q12.2), t(17;22)(q21.31;q12.2), t(2;22)(q35;q12.2), t(16;21)(p11.2;q22.2) and t(2;16)(q35;p11.2), respectively [7, 8]. Additionally, various exon combinations exist in EWSR1/FUS-ETS fusion genes. In EWSR1-FLI1, the combination of EWSR1 exon 7 and FLI1 exon 6 occurs most commonly, followed by the combination of EWSR1 exon 7 and ERG exon 7 or EWSR1 exon 7 and ERG exon 9 are common exon combinations in EWSR1-ERG. These fusion genes contain the ETS consensus sequence in-frame [9, 10]. The conserved ETS consensus sequence recognizes the ETS

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motif, competes with wild type ETS-transcription factors, and consequently contributes to Ewing tumorigenesis [11].

Ewing sarcoma is composed of dense and diffuse proliferation of small round blue cells with fine chromatin [12]. Generally, it lacks immunohistochemical evidence of differentiation lineages, such as muscle, bone, cartilage, fibroblast and endothelium. Diffuse membranous CD99 immunoreactivity is a hallmark of this tumor and more than 90% of tumors were reported to have EWSR1/FUS-ETS. Therefore, detection of the fusion gene is important to diagnose Ewing sarcoma. Fluorescence in situ hybridization (FISH), reverse transcription-polymerase chain reaction (RT–PCR), including multiplexed assay, and targeted next-generation sequencing have been reported. FISH using an EWSR1 or a FUS break-apart probe is commonly used in clinical settings, but FISH using formalin-fixed paraffin-embedded tissue may sometimes be challenging [13]. Additionally, fusion partners cannot be determined by a single break-apart assay. Many tumors, such as desmoplastic small round cell tumor, myxoid liposarcoma, clear cell sarcoma of tendons and aponeuroses, angiomatoid fibrous histiocytoma, and myoepithelioma, have a fusion gene related to tendons and aponeuroses, angiomatoid fibrous histiocytoma, and myoepithelioma, have a fusion gene related to EWSR1 and FUS [14–18]. Therefore, we believe that the determination of the fusion partner gene is important for the differential diagnosis. RT–PCR detection is sensitive and specific, and it can determine the fusion partner. However, assays for seven different fusion genes are needed for Ewing sarcoma. Multiplex RT–PCR assay is an efficient technique. Nevertheless, multiplex RT–PCR assays for EWSR1-ETS have been reported, those for both EWSR1-ETS and FUS-ETS fusion genes have not [19–22]. Next-generation sequencing is a robust technique, but it is too expensive. Therefore, we aimed to create a multiplex RT–PCR system that can simultaneously detect known EWSR1/FUS-ETS fusion genes. Moreover, we confirmed the utility using clinical samples and plasmids.

Materials and methods
Clinical samples
The pathological diagnosis was confirmed by H.O., A.N., and/or T.Y. based on morphological observations and existing RT–PCR and/or FISH analysis. The clinical samples other than tumor 4 used in this study had already been identified for fusion variants by existing RT–PCR. Furthermore, the multiplex RT–PCR and sequencing analysis were performed as blind for experimenter, and the result was collated with that of existing method. Immunostaining was performed using HISTOSTAINER (NICHIREI BIOSCIENCES, Tokyo, Japan) or the BOND-III automated stainer (Leica Biosystems, Nussloch, Germany). Detailed information about the antibodies used in this study is listed in Supplementary Table S1.

RT–PCR
Tumor tissue for genetic analyses was evaluated by frozen sections, and neoplastic cells accounted for 30–80% of viable cells. The total tumor RNA was extracted using the RNaseasy kit (Qiagen, Hilden, Germany), according to the manufacturers’ protocols. The concentrations of DNA and RNA were assessed using an absorption spectrometer. NCR-EW2, WES, and NCR-EW3 are Ewing sarcoma cell lines, and express EWSR1-FLI1 (fusion variant 8 in Fig. 1), EWSR1-ERG (fusion variant 19 in Fig. 1) and EWSR1-ETV4 (fusion variant 22 in Fig. 1), respectively [23]. NRS-1 (rhabdomyosarcoma cell line) and HEK293 total RNA were used as negative controls [24]. Total RNA was extracted from cells using ISOGEN (NIPPON GENE CO., LTD., Tokyo, Japan). The entire coding sequences of the EWSR1-ETV1, EWSR1-FEV, FUS-ERG, and FUS-FEV were constructed and subcloned into the pGEM-T vector (Promega, Madison, WI). The exon combinations of the control plasmids were EWSR1 (NM_001163285.2) exon 7 – ETV1 (NM_001163148.1) exon 11 (fusion variant 21 in Fig. 1), EWSR1 exon 10 – FEV (NM_017521.2) exon 2 (fusion variant 23 in Fig. 1), FUS (NM_004960.3) exon 7 - ERG exon 11 (fusion variant 25 in Fig. 1), and FUS exon 10 – FEV exon 2 (fusion variant 28 in Fig. 1), respectively. Reverse transcription was performed using the Transcriptor First Strand cDNA Synthesis Kit with Oligo dT primers and random hexamers (Roche Diagnostics, Mannheim, Germany). The reaction temperature and time were applied in accordance with the protocol for long length mRNA recommended by the manufacturer’s protocol. Multiplex RT–PCR was performed using the Qiagen Multiplex PCR Plus Kit (Qiagen, Hilden, Germany). Conventional RT–PCR was performed using the QIAGEN HotStarTaq Plus Master Mix Kit (Qiagen, Hilden, Germany). Two sets of primers for multiplex RT–PCR were designed to detect fusion transcript variants. The primers are shown in Table 1. β-Actin primers used as the control were described elsewhere [25]. NCBI Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) was used to design the primers, and the search conditions were set, so that the Tm of the primers was within 60 ± 3°C, the maximum Tm difference was within 3°C and an amplicon of the longest variant was within 1000 bp for Set A. As the forward primer of Set B, the most suitable primer was selected in combination with all reverse primers. It was confirmed by the Multiplex Primer Analyzer (Thermo Fisher SCIENTIFIC) that dimer formation did not theoretically occur in multiplex method. The optimal conditions for multiplex RT–PCR were as follows (based on the
Fig. 1  Fusion transcript variants of EWS/FUS-ETS. Previously described fusion transcripts are indicated. The arrows indicate the primer position listed in Table 1. ND represents “cannot be detected”.

Table 1  Primer sequences for multiplex RT–PCR

| Primer name | 5′ —— 3′ | RT-PCR | Sequencing |
|-------------|----------|--------|------------|
| EWSR1ex7_F (AF1) | gaacacctatgggcaaccga | ✔ | ✔ |
| EWSR1ex4_F (BF1) | agaccgcctatgcaacttct | ✔ | ✔ |
| FL1ex9_R (R1) | ctcactcggtggctgctatt | ✔ | ✔ |
| ERGex12_R (R2) | cgctcgctgaaactccccgt | ✔ | ✔ |
| ETV1ex11_R (R3) | atctctcgcttggttggttg | ✔ | ✔ |
| ETV4ex11_R (R4) | gagccctctctgctgtatgtt | ✔ | ✔ |
| FVex2/3_R (R5) | gatctctctgctgtatgtt | ✔ | ✔ |
| FUSex5_F (AF2) | ggacacgagacacagtacaca | ✔ | ✔ |
| FUSex3_F (BF2) | ggacacgagacacagtacaga | ✔ | ✔ |
recommendation by the manufacturer’s protocol): final concentration of each primer is 0.2 μM, initial PCR activation at 95 °C for 5 min, 30–40 cycles of PCR consisting of denaturation at 95 °C for 30 s, annealing at 60 °C for 90 s and extension at 72 °C for 90 s, and final extension at 68 °C for 10 min. RT–PCR products of Set A and Set B were detected by electrophoresis using 2 and 1% agarose gel, respectively.

Genomic PCR
The tumor genomic DNA was extracted using the QIAamp DNA Mini kit (Qiagen, Hilden, Germany), according to the manufacturers’ protocol. Genomic PCR was performed using AccuPrime Taq DNA Polymerase, high-fidelity (Invitrogen, Carlsbad, CA). The primers are listed in Supplementary Table S2.

Sequencing analyses
Sequencing analysis was performed using ABI3130xl and ABI3500 (Applied Biosystems, Foster City, CA). The sequencing of the multiplex RT–PCR product was also performed using multiplex primers with 3.2 pmol each primer.

Results
Generation of the multiplex RT–PCR method to detect EWSR1/FUS-ETS

Design of the multiplex RT–PCR primers
We aimed to set up a multiplex RT–PCR system to detect all EWSR1/FUS-ETS fusion variants for rapid and practical genetic diagnosis. However, the exon combinations of the fusion gene are quite broad, and at least 28 types of variants have been reported (Fig. 1) [3, 5, 7–10, 23, 26–28]. There are 14 reported exon combinations of EWSR1-FLI1, all of which include exon 7 of EWSR1. Six combinations were reported in EWSR1-ERG. Five of them contained the complete exon 7 of EWSR1, whereas a single case only partially contained exon 7 [27]. One type of each was reported in EWSR1-ETV1 and EWSR1-ETV4. Two types were reported for EWSR1- FEV. In summary, EWSR1-ETS contained the entire exon 7 of EWSR1 except for one case. Therefore, a forward primer was designed to bind exon 7 of EWSR1 as primer Set A. Reverse primers were designed to bind a common region in each fusion gene; that is, an FLI1 primer was designed for exon 9, an ERG primer for exon 12, an ETV1 primer for exon 11, and an ETV4 primer for exon 11. The FEV primer spanned exon 2 to exon 3. Among FUS-ERG and FUS-FEV variants, the variant that fuses FUS exon 5 to ERG exon 9 has the shortest 5′-terminal side sequences of FUS. Therefore, a forward primer was designed in exon 5. Because all FUS-ERG variants include exon 12 of ERG, and FUS-FEV variants include exon 2 of FEV, the same reverse primers for EWSR1-ETS were used. The deduced size of each PCR product was 221 to 940 base pairs.

Next, we generated primer Set B that could detect fusion with a shorter 5′-terminal side sequence. The EWSR1 forward primer was designed within exon 4 to detect the fusion gene that partially lacks exon 7. The FLI1 forward primer was designed within exon 3 to detect unusually short fusion genes, although no fusion with a shorter FUS 5′-terminal side sequence were reported in Ewing sarcoma. These forward primers were designed to match the reverse primer of Set A. Set A primers detect most variants, and the Set B primers cover all variants reported thus far (Table 1).

Sensitivity for the detection of EWSR1/FUS-ETS
First, we performed PCR using primer Set A and cDNA from cell lines expressing either EWSR1-FLI1 (NCR-EW2), EWSR1-ERG (WES), or EWSR1-ETV4 (NCR-EW3) and diluted plasmid vectors (10^4 molecules) containing EWSR1-ETV1, EWSR1-FEV, FUS-ERG, and FUS-FEV (Fig. 2a). A PCR product with expected length was identified in each reaction without recognizable background. Similarly, fusion genes with the expected length were amplified by PCR using Set B primers (Fig. 2b). In all cases, only a single band was detected with low background. All PCR products amplified with either Set A or Set B were sequenced successfully using the forward or reverse primer mix. Sequence analysis showed that all PCR products were the expected sequences of the EWSR1/FUS-ETS variants.

Next, we examined the sensitivity of the PCR. We performed RT–PCR using Set A primers and cDNA from cell lines expressing either EWSR1-FLI1, EWSR1-ERG, or EWSR1-ETV4. We detected the respective fusion genes from cDNA equivalent to 100 pg of RNA using 35 cycles, according to the cycle number of the existing analysis (Supplementary Fig. S1). RT–PCR using Set B primers had comparable sensitivity with RT–PCR using Set A primers (Supplementary Fig. S1). For fusion transcripts without cell lines, we used a dilution series of plasmid vectors. Using Set A primers, positive results were obtained with 10^2 plasmid molecules for EWSR1-ETV1 and FUS-ERG, and 10^3 molecules for EWSR1-FEV and FUS-FEV (Supplementary Fig. S2). The sensitivity of Set B was comparable with that of Set A. Similarly, we performed the PCR at 40 cycles, and obtained a clear band with less template (data not shown).

Detection of EWSR1/FUS-ETS in clinical samples
We examined the usefulness of these primer sets using clinical samples. We utilized frozen material from Ewing sarcoma diagnosed morphologically, immunohistochemically, and genetically. Specifically, six small round cell tumors with membranous CD99-positivity and
Fig. 2 Specificity of multiplex primers to each fusion gene. Multiplex PCR for EWSR1/FUS-ETS transcript variants using Set A (a) and Set B (b) primers. Lane M: Trackit 100-bp ladder marker (a) or Trackit 1-kbp plus ladder (b). Molecular sizes are indicated in the right with yellow arrowheads (a) and red arrowheads (b), respectively; lane 1: NCR-EW2 cDNA (EWSR1-FLI1); lane 2: WES cDNA (EWSR1-ERG); lane 3: EWSR1-ETV1 plasmid; lane 4: NCR-EW3 cDNA (EWSR1-ETV4); lane 5: EWSR1-FEV plasmid; lane 6: FUS-ERG plasmid; lane 7: FUS-FEV plasmid; lane 8: HEK293 cDNA; lane 9: no template control. The plasmid samples contained the same amount of HEK293 cDNA as the Ewing cell lines.

Fig. 3 Multiplex RT-PCR in tumor samples. Multiplex (top and middle panels) and control (bottom panel) RT-PCR were performed in six tumor samples. Lane M: Trackit 100-bp ladder marker (top panel) or Trackit 1-kbp plus ladder (middle and bottom panels). Molecular sizes are indicated in the right with yellow arrowheads (a) and red arrowheads (b), respectively; lane 1: NCR-EW2 cDNA for the positive control; lane 2: tumor 1; lane 3: tumor 2; lane 4: tumor 3; lane 5: tumor 4; lane 6: tumor 5; lane 7: tumor 6; lane 8: NRS-1 cDNA for the negative control; lane 9: no template control.
confirmed that tumors 1 and 2 had EWSR1-FLI1 in all six cases (Fig. 3). We detected this fusion gene in detail and reviewed the clinicopathological features of this case.

**A unique case of an EWSR1-ERG-expressing tumor**

**Clinical and pathological characteristics**

We reviewed the clinical and pathological characteristics. The patient was a 15-year-old male with a history of acute lymphoblastic leukemia who presented with a mass measuring 1.5 × 1.0 × 0.7 cm in his nasal vestibule. The tumor was subjected to excisional biopsy. Histologically, the tumor exhibited diffuse proliferation of undifferentiated cells (Fig. 4a) with round to oval nuclei and a moderate amount of cytoplasm with a clear cell border. Focally, the tumor cells proliferated with fibrous to myxoid stroma (Fig. 4b) and were positive for Periodic acid–Schiff (PAS) staining in the cytoplasm (Fig. 4c). Immunohistochemically, tumor cells demonstrated membranous positivity for CD99, positivity for Nkx2.2 (Fig. 4d and e), focal positivity for S100 and negativity for desmin, myogenin, MyoD1, cytokeratin (AE1/AE3), CD31, CD34, CD3, CD20, and CD1a. FISH analysis of fresh-tissue touch preparations detected EWSR1 split signals in most tumor cells but not FUS split signals (Fig. 4f). The tumor had consistent features of Ewing sarcoma based on CD99 positivity and EWSR1 rearrangement, although the histological picture was somewhat unusual in that it showed focal myxoid stroma.

**Table 2** Fusion transcripts identified in tumor samples

| Tumor     | Fusion Transcripts                     |
|-----------|----------------------------------------|
| 1         | EWSR1 exon 7 - FLI1 exon 6 (fusion variant 11) |
| 2         | EWSR1 exon 7 - FLI1 exon 6 (fusion variant 11) |
| 3         | EWSR1 exon 7 - FEV exon 2 (fusion variant 24), EWSR1 Δ exon 8 (c.794 to 943)-FEV exon 2 |
| 4         | EWSR1 Δ exon 6 (c.414 to 522) - intron 6 - intron 6 / ERG intron 8 - exon 9 |
| 5         | EWSR1 exon 7 - ERG exon 12 (fusion variant 19) |
| 6         | EWSR1 exon 7 - ERG exon 12 (fusion variant 19) |

*aOnly the in-frame fusions are described here. The fusion variant numbers in Fig. 1 are shown in parentheses.*

**Detailed analysis of the fusion transcript**

Sequence analysis of the multiplex PCR product revealed that the fusion gene was EWSR1-ERG. To determine the sequence of the individual products, we performed another PCR assay using EWSR1 exon 4 and ERG exon 12 primers, and the products were subcloned into the pGEM-T vector and sequenced. We identified four transcript variants, and the most frequent one was in-frame (Fig. 5). The major in-frame EWSR1-ERG fusion transcript variant included a partial sequence of exon 6 (c.414 to c.522), two cryptic exons (c.581 + 55 to + 90, c.581 + 227 to + 369) in intron 6 of EWSR1, a cryptic exon in intron 8 of ERG (c.767–214 to –198), and exon 9 of ERG (Fig. 5 and Supplementary Fig. S3a, b and c). All four variants had identical sequences from ERG. Three out-of-framework variants were thought to be produced by differential splicing within EWSR1 (Fig. 5 and Supplementary Fig. S3a, b and c).

**Genomic structure of the EWSR1-ERG fusion**

To clarify whether the rare variants were derived from alternative splicing or different breakpoints in genomic DNA, we performed genomic PCR and identified a single fused sequence showing that intron 6 of EWSR1 was joined to intron 8 of ERG (Fig. 6). The genomic fusion point was identical to the fusion points in transcripts, indicating a part of EWSR1 intron 6 and a short sequence of ERG intron 8 formed a cryptic exon collectively. Notably, all flanking sequences of all cryptic exons followed the GU/AG mRNA splicing rule, and all four transcript variants were supposed to be derived from alternative splicing.

**Discussion**

We developed a novel RT–PCR assay that can efficiently detect both EWSR1-ETS and FUS-ETS observed in Ewing sarcoma. We identified EWSR1/FUS-ETS fusion transcripts in all cell lines and pathologically defined Ewing sarcoma tumors that were tested. The appropriately designed primers enabled the detection of various fusion variants in a single round of PCR. Additionally, the identified transcripts were successfully sequenced by mixed forward or reverse primers in each case. Among 28 EWS/FUS-ETS variants reported so far, we detected five using cell lines and tumor tissues, and additionally, we identified a novel variant.

We were able to detect the fusion genes from 100 pg of total RNA from cell lines. In addition, we detected 1000 molecules of the fusion gene in a PCR reaction. For clinical samples, we use 1/40 of the cDNA synthesized with 1 μg of total RNA as the PCR template. Assuming that the amount of total RNA per cell is 0.01 ng, the template used for PCR is theoretically equivalent to 2500 cells. Thus, although the number of samples...
analyzed was small, our method is theoretically applicable to clinical tumor samples.

In 1995, Downing et al. reported multiplex RT-PCR for the detection of EWSR1-FLI1 and PAX3-FOXO1 to differentiate Ewing sarcoma and alveolar rhabdomyosarcoma [19]. However, many fusion variants were discovered afterwards. In addition, the primers they used cannot detect fusion variants with a short N-terminal sequence. In 2001, Peter et al. used a real-time PCR system to discriminate Ewing sarcoma, alveolar rhabdomyosarcoma, synovial sarcoma, and small round cell desmoplastic tumor [20]. They used primers only for EWSR1-ETS. As they utilized a common EWSR1 probe for the detection of the amplicon, they were unable to differentiate fusion gene combinations. Moreover, the product sizes were too large for the real-time PCR method. Yoshino et al. reported the simultaneous detection of EWSR1-ETS in 2003 [21]. They used Bioanalyzer
to confirm the product length, which made it possible to predict the gene and exon combinations, but they utilized EWSR1 exon 7 primer and were unable to detect fusion with a short N-terminal sequence. Thus, none of these assays were able to detect FUS-ETS fusions. In routine pathological practice, Ewing sarcoma with atypical morphology or small round cell sarcoma without typical EWSR1/FUS-ETS fusions is occasionally observed. Therefore, rapid detection of all Ewing sarcoma related fusions is useful in clinical settings. The advantages of conventional RT–PCR with gel electrophoresis are that it is inexpensive and not laborious, and the length of the product can be recognized. In addition, all the processes can be performed without special equipment such as a real-time PCR system or next-generation sequencer. The standard break-apart FISH method takes 2 days to determine EWSR1 and FUS rearrangement [29], and it takes another 2 days to determine the fusion partner. However, our method enables confirmation of the presence of the EWSR1/FUS-ETS fusion gene in 5.5 h and determination of the sequence in approximately 10 h.

Furthermore, we reported a case of Ewing sarcoma with atypical histological features whose fusion transcript completely lacked exon 7 of EWSR1. By our methods, the use of the EWSR1 upstream forward primer enabled the detection of a transcript variant with an unexpectedly shorter N-terminal region of EWSR1. Many laboratories detect fusion genes by RT–PCR, but most primers reported thus far cannot amplify fusion transcripts lacking EWSR1 exon 7. Thus, false-negative results may occur with EWSR1-ETS and EWSR1-ERG. Therefore, when Ewing sarcoma is pathologically suspected, but all seven types of known fusion genes are negative, analysis using an EWSR1 primer upstream of exon 7 should be considered.

Conclusions
We developed a multiplex PCR assay method that is simple, accurate, and efficient to detect fusion genes observed in Ewing sarcoma. Our assay will aid in the rapid and accurate diagnosis of Ewing sarcoma. We also identified a novel fusion variant with a short N-terminal region that may have been previously overlooked. This highlights why the RT–PCR primers for the genetic diagnosis of Ewing sarcoma should be optimized.

Abbreviations
RT-PCR: Reverse transcription–polymerase chain reaction; FISH: Fluorescence in situ hybridization; PAS: Periodic acid–Schiff

![Fig. 6 Genomic fusion point of novel EWSR1-ERG translocation. Sequences of the genomic fusion point. The blue boxes indicate cryptic exons in the in-frame transcript. The black arrows indicate primer sites. In the upper part of the sequences, the chromosome positions of the fusion site are indicated.](image-url)
Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13000-021-01164-6.

Additional file 1: Supplementary Table S1. List of antibodies.

Additional file 2: Supplementary Table S2. Sequences of genomic PCR and sequencing primers.

Additional file 3: Supplementary Fig. S1. Detection sensitivity of primers for EWSR1-ETS3 plasmids. Serial dilutions of cDNA from Ewing sarcoma cell lines were amplified by Set A (upper panel) or Set B primers (lower panel). Lane M: Trackit100-bp ladder marker (upper panel, yellow arrowhead) or Trackit1-kbp plus ladder (lower panel, red arrowhead), molecular marker sizes are indicated insethefe.; lane 1: template cDNA corresponding to 10 ng to total RNA; lane 2: 1 ng; lane 3: 100 pg; lane 4: 10 pg; lane 5: 1 pg; lane 6: 0.1 pg; lane 7: no template control.

Additional file 4: Supplementary Fig. S2. Detection sensitivity of primers for EWSR1-ETS plasmids. Serial dilutions of EWSR1/FUS-ETS-containing plasmids were amplified using primer Set A (upper panels) and Set B (lower panels). We estimated the molecular weight from the size of the respective plasmids and used 105 to 10 molecules as a starting template in 25 ml PCR reaction mix. Lane M: Trackit100-bp ladder marker (upper panel, yellow arrowhead) or Trackit1-kbp plus ladder (lower panel, red arrowhead); molecular marker sizes are indicated in the left.; lane 1: 105 molecules; lane 2: 104 molecules; lane 3: 103 molecules; lane 4: 102 molecules; lane 5: 101 molecules; lane 6: 100 molecules; lane 7: no template control.

Additional file 5: Supplementary Fig. S3. Scheme of alternative splicing and sequence in novel EWSR1-ETSk transcripts. (a) Scheme of exon 6 and first 369 bases of intron 6 of EWSR1. The splicing patterns of the in-frame variant are represented by red lines and a red number. The splicing sites shared by some variants are denoted by black numbers. The two blue bars under intron 6 are cryptic exonic regions of the in-frame variant. The splicing patterns of the two out-of-frame variants are shown in purple and green, respectively. No sequence was spliced out in one variant. (b) Sequences of EWSR1 exon 6 and intron 6 (c.381 + 1 to + 393) and alternative splicing sites. The genomic location numbers are based on the GRCh37/hg19 version. The boldface sequences represent truncated exon 6 (c.414 to c.522). The sequences corresponding to the cryptic exon in intron 6 are underlined. GT (green character); splice donor sites, ag (red character); splice acceptor sites. Sites involved in splicing are numbered in the upper part of the sequences. (c) Sequences of ERG intron 8 (c.767–1 to –230) and exon 9 and alternative splicing sites. The genomic location numbers and sites involved in splicing are shown as in (b).

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Authors’ contributions

HU-Y and HO contributed to the conception, design, data curation, visualization of this work and writing the manuscript. HU-Y established methodology and performed investigation. KN supported the experiment. HO, TY and AN performed the evaluation of pathology. CK, MK and KM contributed to the collection of samples. NK provided laboratory infrastructure. All authors read and approved the final manuscript.

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Availability of data and materials

Not applicable.

Declarations

Ethics approval and consent to participate

All specimens and clinical data were obtained with either informed written consent or a formal waiver of consent under protocols approved by the ethics committee/IRB at the National Center for Child Health and Development (No. 1035).

Consent for publication

Not applicable.

Competing interests

The authors declare no competing financial interests.

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