Detection of Chimeric Genes in Ewing's Sarcoma and Its Clinical Applications

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Ewing's sarcoma (ES), most commonly an undifferentiated tumor of bone, belongs to the enigmatic diagnostic category of small round cell tumors (SRCT) of childhood. The consistent presence of the translocation t (11; 22) in the vast majority of tumors provides evidence for a common histogenesis in ES and its family of tumors (ESFT), and also provides a unique diagnostic characteristic to discriminate this tumor family from SRCT. Molecular analysis of this translocation has revealed that it forms a chimeric gene between *EWS* on chromosome 22 and *FLI-1* on chromosome 11. Similarly, the variant t (21; 22), t (7; 22), t (17; 22), and t (2; 22) rearrangements also form chimeric genes between regions of *EWS* and the *ETS* gene family (*ERG*, *ETV1*, *E1AF*, and *FEV*). Detection of these specific chimeric genes would provide a method for diagnosis of ESFT. We have developed a procedure for simultaneous detection of the chimeric genes by reverse transcription polymerase chain reaction (RT-PCR) with a mixture of primers. We conclude that the detecting those chimeric genes by this method can be easy and useful for diagnosis of ESFT. Moreover, by defining the specific chimeric gene it is possible to detect the tumor cell contamination in autologous blood stem cell transplantation.

Key words Ewing's sarcoma; chimeric gene; reverse transcription polymerase chain reaction

Ewing's sarcoma (ES), which occurs in bone and soft tissue, is one of the most malignant tumors in children and young adults.[1] ES, neuroblastoma (NB), rhabdomyosarcoma, and malignant lymphoma are classified as small round cell tumors (SRCT). However, it is difficult to distinguish ES from other tumors of SRCT. Similarly, peripheral primitive neuroectodermal tumor (PNET) is also of bone or soft tissue origin, and is also difficult to diagnose. ES, PNET and Askin tumor have some common characteristics, and form the so-called ES family tumors (ESFT).[2] Although several methods for diagnosing ESFT are widely used, for example pathological analysis, ultrastructural analysis or immunohistochemical analysis with a specific antibody (glycoprotein p30/32^{MIC2}),[3] these analyses involve complex and time-consuming procedures.

ESFT are associated with specific chromosomal translocations, which are correlated with the presence of specific chimeric genes. These chimeric genes are considered to play an important role in tumorigenesis and the mechanisms of malignancy in ESFT. The best-known chromosomal translocation in ESFT is t (11; 22),[4] and the *EWS–FLI1* chimeric gene is generated through this translocation. Other chimeric genes in ESFT are *EWS–ERG*, *EWS–ETV1*, *EWS–E1AF*, and *EWS–FEV*, which are generated from the chromosomal translocations t (21; 22),[5,6] t (7; 22),[7] t (17; 22),[8] and t (2; 22),[9] respectively. The partners of these chimeric genes (*FLI1*, *ERG*, *ETV1*, *E1AF*, and *FEV*) are members of the *ETS* gene family, whose products function as transcription factors.[10] Moreover, these *ETS* gene family members generate chimeric genes by using different parts of exons.[11—13]

Here, we describe much more easy and simultaneous detection for these chimeric genes in ESFT and its clinical applications.

MATERIALS AND METHODS

Cell Lines We examined three ES cell lines (NCR-EW2,[12] SCMC-ES-1,[13] and KP-EW-MS[13]), two PNET cell lines (SK-N-MC[13] and SK-N-L0[13]), and one NB cell line (IMR-32). The chimeric gene detected for each cell line is shown in Table 1. IMR-32 has no chimeric gene, so we used it as a negative control. All cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 2 g/l sodium bicarbonate, then incubated in 5% CO_2 at 37 °C.

RNA Extraction Total RNA was extracted from cultured cells with TRIZOL reagent (GIBCO BRL), based on the acid guanidinium-phenol-chloroform method.[14]

Primer Design We designed all primers from outside sequence of break points on the genes, which compose chimeric genes. So we can detect all chimeric genes, which have been recognized in ESFT (Fig. 1).

RT-PCR and PCR Products Analysis cDNA was generated by using ReverTra Ace $-\alpha-$ (TOYOBO) from 0.8 μg of total RNA in a 10 μl reaction mixture. mRNA Selective PCR Kit Ver. 1.1 (TaKaRa) was used for PCR, which was

Table 1. Origin and Detected Chimeric Gene of Cell Line

Name	Origin	Chimeric gene
SK-N-MC	PNET	*EWS* (exon 7)–*FLI1* (exon 6)
SK-N-L0	PNET	*EWS* (exon 7)–*FLI1* (exon 6)
NCR-EW2	ES	*EWS* (exon 7)–*FLI1* (exon 5)
KP-EW-MS	ES	*EWS* (exon 10)–*FLI1* (exon 5)
SCMC-ES1	ES	*EWS* (exon 7)–*ERG* (exon 6)
IMR-32	NB	Not detected

PNET: peripheral primitive neuroectdermal tumor, ES: Ewing's sarcoma, NB: neuroblastoma.

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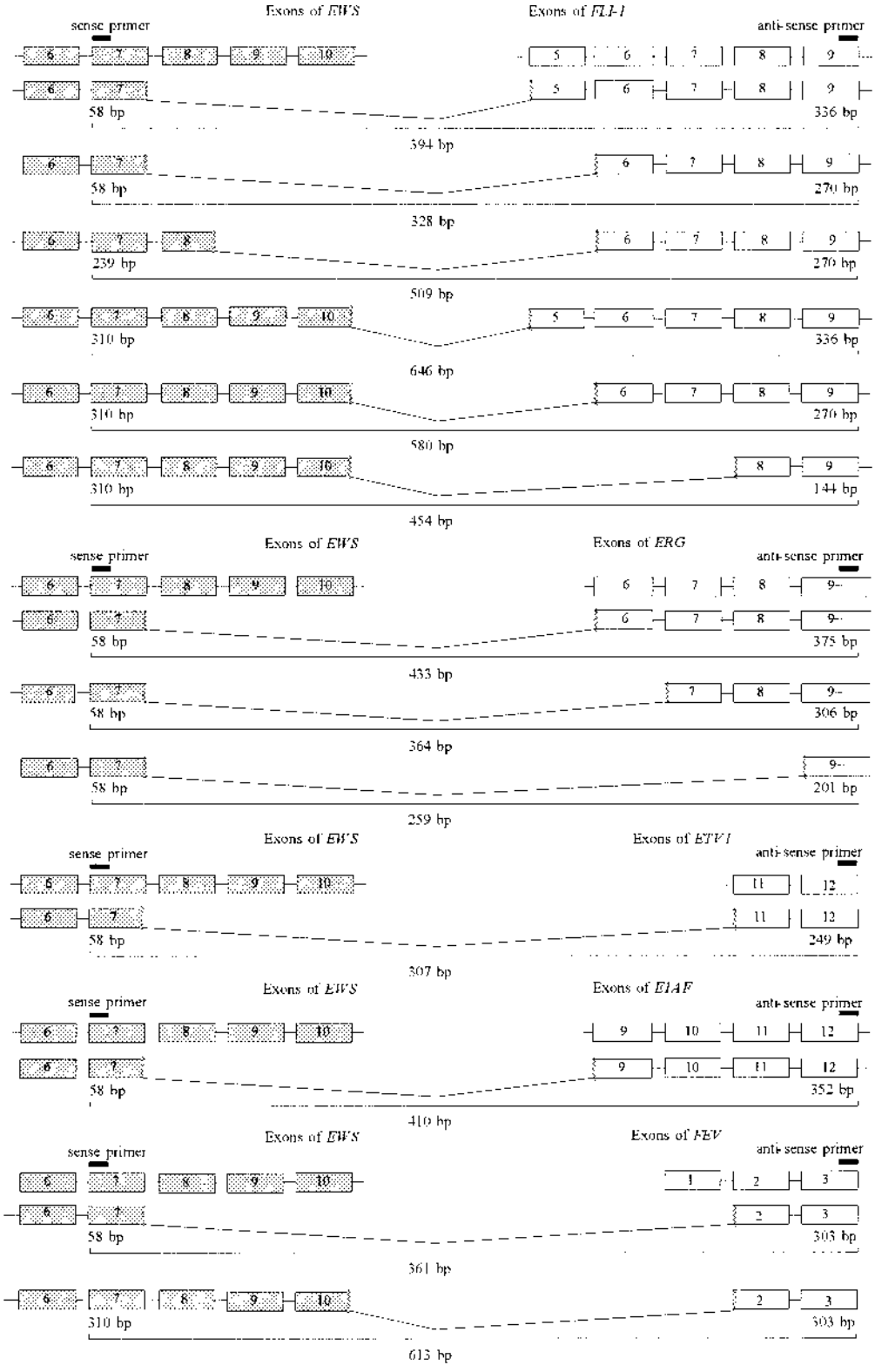

Fig. 1. Location of Sense and Anti-sense Primers and Sizes of Chimeric Transcripts
Primers were designed from the sequence adjoining each chimeric point.

performed in 10 μl of reaction mixture containing 1.2 pmol of all primers, as summarized in Table 2.[8,12,15] The amplification step was preformed in a TaKaRa PCR Thermal Cycler PERSONAL for thirty cycles. The first cycle consisted of 3 min of denaturation at 85 °C, 3 min of annealing at 57 °C and 4 min of extension; the next 29 cycles consisted of 1.5 min of denaturation at 85 °C, 1.5 min of annealing at 57 °C and 2 min of extension. After the last cycle, extension continued for an additional 3 min at 72 °C. We designed the primers to be able to distinguish the fusion points of the chimeric genes. The different band sizes of RT-PCR products arising from chimeric genes between *EWS* and *FLI-1*, *ERG*,

Table 2. Primers Used for PCR

Name	Sequence	Exon
EWS-F	5′-TCCTACAGCCAAGCTCCAAGTC-3′	7
FLI1-R	5′-ACTCCCCGTTGGTCCCCTCC-3′	9
ERG-R	5′-CATAGTAGTAACGGAGGGCGC-3′	X
ETV1-R	5′-TAGTAATAGCGGAGTGAACGGC-3′	12
E1AF-R	5′-GCTGGCCGGTTCTTCTGGATGC-3′	12
FEV-R	5′-TAGCGCTTGCCATGCACCTT-3′	3
GAPDH sense	5′-TCCTCTGACTTCAACAGCGACACC-3′	8
GAPDH anti-sense	5′-TCTCTCTTCCTCTTGTGCTCTTGG-3′	9

ETV, *E1AF*, or *FEV* are shown in Fig. 1. We used glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal marker.[15] The PCR products were electrophoresed on 2% agarose gel, stained with ethidium bromide and visualized under a UV lamp. Moreover, we analyzed the sizes of these products accurately using a Bioanalyzer (Agilent Technologies). Bioanalyzer detects laser fluorescence using Labotip in which capillary was included and it has about 5% of coefficient of variation.

RESULTS AND DISCUSSION

When the RT-PCR products from the six cell lines were analyzed by electrophoresis, the band was observed near the size expected in all cell lines (Fig. 2A). Moreover, using the Bioanalyzer distinct bands of approximately the expected size were observed (Fig. 2B). GAPDH as an internal marker was also observed in all cell lines. The real band sizes as determined by Bioanalyzer were 332 base pairs (bp) (expected size 328 bp) for SK-N-MC, 333 bp (328 bp) for SK-N-L0, 398 bp (394 bp) for NCR-EW2, 651 bp (646 bp) for KP-EW-MS, and 438 bp (433 bp) for SC-MC-ES1 (Fig. 2). No chimeric genes were detected for IMR-32. The difference between the expected band sizes and the real band sizes as determined by the Bioanalyzer was only 4 or 5 bp (less than 1.6% different from the real band size). Real RT-PCR product sizes were always 4 or 5 bp longer than expected RT-PCR product sizes.

As shown in Fig. 1, in clinical tumor samples from ESFT, 6 different chimeric genes were detected for *EWS–FLI1*, 3 for *EWS–ERG*, 2 for *EWS–FEV*, and 1 each for *EWS–E1AF* and *EWS–ETV1*. All chimeric genes had different expected sizes of RT-PCR products. We can detect almost all chimeric gene RT-PCR products by Bioanalyzer because they differ by only 4 or 5 bp from the expected RT-PCR products. For instance, it is possible to distinguish the difference in about 20 bp between *EWS* (exon 10)–*FLI1* (exon 8) (454 bp) and *EWS* (exon 7)–*ERG* (exon 6) (433 bp), which are probably undistinguishable on the gel. However, there is only a 3 bp difference between the RT-PCR products for *EWS* (exon 7)–*ERG* (exon 7) (364 bp) and *EWS* (exon 7)–*FEV* (exon 2) (361 bp), so it is difficult to distinguish between these chimeric genes by Bioanalyzer. Reliable differentiation of these RT-PCR products will require direct sequencing.

It has been reported that in the clinical tumor samples 85% of chimeric genes originated from *EWS* and *FLI-1*, 10% originated from *EWS* and *ERG*, and less than 2% each originated from *EWS* and *E1AF*, *ETV1*, or *FEV*.[16] In practice, distinguishing between the RT-PCR products for *EWS* (exon 7)–*ERG* (exon 7) (364 bp) and for *EWS* (exon 7)–*FEV* (exon 2) (361 bp) may not be a major problem, because the frequencies of both products are very low in the clinical tumor samples.

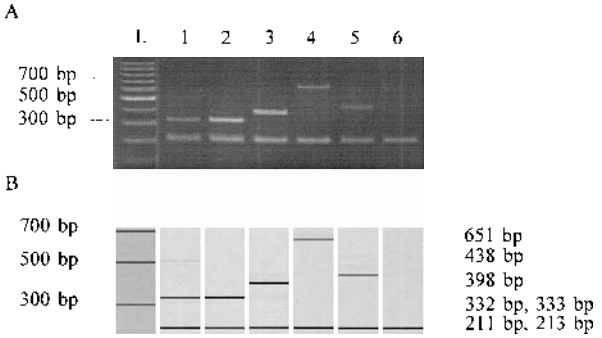

Fig. 2. Detected RT-PCR Products in Cell Lines

L: Ladder, lane 1: SK-N-MC, lane 2: SK-N-L0, lane 3: NCR-EW2, lane 4: KP-EW-MS, lane 5: SCMS-ES1, lane 6: IMR-32. (A) Detected bands by electrophoresis. (B) Detected bands by Bioanalyzer; Chimeric gene's bands were detected at 1: 332 bp, 2: 333 bp, 3: 398 bp, 4: 651 bp, 5: 438 bp. GAPDH's bands were detected at 1: 213 bp, 2: 213 bp, 3: 213 bp, 4: 213 bp, 5: 211 bp, 6: 211 bp.

Table 3. Frequency of Chimeric Transcripts in Clinical Tumor Samples

Chimeric transcripts	Frequency in clinical tumor samples (%)
EWS (exon 7) –*FLI1* (exon 5) –*FLI1* (exon 6) *EWS* (exon 8) –*FLI1* (exon 6) *EWS* (exon 10)–*FLI1* (exon 5) –*FLI1* (exon 5) –*FLI1* (exon 8)	85
EWS (exon 7) –*ERG* (exon 6) –*ERG* (exon 7) –*ERG* (exon 9)	10
EWS (exon 7) –*E1AF* (exon 9)	<2
EWS (exon 7) –*ETV1* (exon 11)	<2
EWS (exon 7) –*FEV* (exon 2) *EWS* (exon 10)–*FEV* (exon 2)	<2

Therefore, we found that we could quickly detect in a single step almost all the specific chimeric genes in ESFT by using a mixture of primers. In the future, we will apply this method to detect the existing of living tumor cell in tumor specimen at second look operation and the diagnosis of ESFT for clinical samples from biopsies and operation. Moreover, we can also apply this method to the detection of tumor cell contamination in bone marrow and peripheral blood stem cells during autologous bone marrow transplantation and autologous peripheral blood stem cell transplantation.

It is reported that the patients with translocation at *EWS* (exon 7)–*FLI1* (exon 6) exhibit a good prognosis.[17] Therefore, our method can be useful for the estimation of prognosis for ESFT patients too.

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