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Molecular Analysis of Ewing's Sarcoma: Another Fusion Gene, *EWS-E1AF*, Available for Diagnosis

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Ewing's sarcoma, one of the most malignant tumors of children and young adults, expresses specific chimeric genes, e.g. *EWS-FLI-1*, *EWS-ERG*, *EWS-ETV1* and *EWS-FEV*. In this paper, we extensively characterized a new fusion gene, *EWS-E1AF* by means of whole cDNA sequencing, RNA blot analysis, DNA blot analysis and chromosomal analysis, and showed it to be available for the diagnosis of Ewing's sarcoma and to participate in the oncogenesis of Ewing's sarcoma. Furthermore, we conducted a genetic analysis of Ewing family tumors in conjuction with immunohistochemical analysis and ultrastructural analysis. Our results demonstrate some limitations of both genetic analysis and histopathological analysis, and establish the relationship between neurogenic phenotypes and chimera genes.

Key words: Ewing's sarcoma — Molecular diagnosis — *E1AF* — Ets — RNA binding protein

Ewing's sarcoma, which occurs in the bone and soft tissue, is one of the most malignant tumors in young people.[1)] This tumor does not have specific morphological phenotypes. By light microscopic analysis, it is sometimes difficult to differentiate Ewing's sarcoma from neuroblastoma, malignant lymphoma, some types of rhabdomyosarcoma and osteosarcoma.[2)] Similarly, peripheral primitive neuroectodermal tumor (PNET), which has similar morphology but has neuronal character, is also hard to diagnose.[3)] Therefore, in order to diagnose these two tumors, several approaches are employed; macroscopic and microscopic analysis, ultrastructural analysis, and immunohistochemical analysis with specific antibodies to vimentin, desmin, neurofilaments, S-100, neuron-specific enolase, MIC2[4)] and 5C11.[5)] However, even such extensive analysis has failed to identify any specific morphological character of Ewing's sarcoma and PNET.

Many human tumors contain characteristic chromosomal translocations. Through the chromosomal translocations, specific fusion genes are generated.[6, 7)] These fusion genes are considered to play an important role in oncogenesis. Ewing' sarcoma and PNET have the same specific chromosomal translocation, t(11;22)[8, 9)] and through this translocation, the *EWS-FLI-1* fusion gene is generated.[10)] *FLI-1*, a partner of the fusion gene, is a member of the Ets family of transcription factors. Other Ets family genes, *ERG* and *ETV1*, are also fused to *EWS* through t(21;22) and t(7;22), respectively.[11–13)] Recently, we identified a new fusion gene, *EWS-E1AF* in a case of Ewing's sarcoma.[14)] Since analysis of the fusion genes revealed that Ewing's sarcoma and PNET have the same fusion transcripts, Ewing's sarcoma and PNET are considered to belong to a common entity.[15)] In this study, we extensively characterized the *EWS-E1AF* fusion gene in the established cell line NCR-EW3, which has t(17;22) (q21;q21). Furthermore, we conducted a genetic analysis of Ewing's sarcoma and PNET in comparison with the accumulated data on clinical history and the results of pathological examination.

MATERIALS AND METHODS

Tumors and cell lines Thirty surgical samples were obtained from Japanese patients. On the basis of the clinical and pathological findings, these tumors were diagnosed as Ewing's sarcoma and PNET. Neuroblastoma, rhabdomyosarcoma, osteosarcoma, malignant lymphoma and Wilms' tumor were also analyzed for comparison. As well as surgical samples, 18 cell lines were also examined; twelve Ewing's sarcoma cell lines, four PNET cell lines and two neuroblastoma cell lines. Chromosomes were analyzed by trypsin G-banding as previously described.[16)] RD-ES and SK-ES1 were purchased from American Type Culture Collection (ATCC, Rockville, MD). They were cultured in Dulbecco's modified Eagle's medium (DMEM) medium supplemented with 10% fetal bovine serum.

Histological and immunohistochemical examination Surgically resected tumors were immediately frozen in OCT-compound (Sakura Finetek U.S.A. Inc., Torrance, CA) for immunohistochemical procedures. Tumors were also fixed in 20% buffered formalin and embedded in paraffin

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for routine histological examination. Antibodies used in this study were as follows: cytokeratin AE1/AE3 (Boehringer Mannheim, Mannheim, Germany), EMA (Dako A/S, Glostrup, Denmark), Vimentin (Dako), Desmin (Dako), nerve growth factor receptor (NGF-R, Boehringer Mannheim), neuron-specific enolase (NSE, Dako), Leu-7 (Becton Dickinson, Mountain View, CA), p30/32^{MIC2} (Signet, Dedham, MA) and 5C11.[5] The tumor blocks were also fixed in 2.5% glutaraldehyde, postfixed with 1% OsO_4, dehydrated in graded alcohol and embedded in Epon 812 for electron microscopic observation.[17]

RNA extraction Total RNA from tumors and cell lines were extracted by the guanidinium thiocyanate method followed by centrifugation in cesium chloride solutions or ISOGEN (Nippon Gene, Tokyo). In the case of ISOGEN, the surgical sample was ground with a small stick in a microcentrifuge tube.

Reverse transcription and polymerase chain reaction (RT-PCR) Complementary DNA was generated by using a first-strand cDNA synthesis kit (Pharmacia Biotech, Uppsala). Approximately 1 to 5 μg of total RNA was transcribed. PCR was carried out in a 100 μl reaction mixture containing 1–7 μl of cDNA template, 200 m*M* deoxynucleotide triphosphates, 0.5 m*M* of each oligonucleotide primer, and 2.5 units of *Taq* polymerase in a 10 m*M* Tris-HCl buffer, pH 8.8, containing 50 m*M* KCl, 1.5 m*M* $MgCl_2$. Oligonucleotide primers used for PCR were EU-5 (poly T), EU-6 (*EWS* specific), EU-10 (*E1AF* specific), EU-11 (*E1AF* specific), EU-12 (*EWS* specific), EU-13 (*ETV1* specific), EU-14 (*ETV1* specific), EU-15 (*ERG* specific), EU-16 (*EWS* specific), ESBP-1 (*EWS* specific), ESBP-2 (*FLI-1* specific)[18] and ERG-1 (*ERG* specific) (Table I). Thirty-five cycles of PCR were performed with the following parameters: denaturation step at 94°C for 1 min, annealing at 65°C for 1 min and elongation step at 72°C for 1 min. Amplified products were analyzed on 0.7 to 2% agarose gel.

Cloning and sequencing of PCR products The amplified products were purified by using MicroSpin Columns (Pharmacia Biotech) and direct sequencing was performed with an AutoCycle Sequencing Kit (Pharmacia Biotech). A primer used for direct sequencing was EFU-C (*EWS* specific, tat gga cag cag agt agc tat ggt c). When multiple bands were observed, they were eluted from the gel and cloned into pGEM-T Vector with the pGEM-T Vector System (Promega, Madison, WI). When the signals were not clear, nested PCR was carried out. Primers used for sequencing were M13-40 Primer and A.L.F. M13 Reverse Primer (Pharmacia Biotech). Sequences were analyzed using the A.L.F. DNA Sequencer (Pharmacia Biotech).

Southern blot analysis Five micrograms of DNA was digested to completion with restriction enzymes, subjected to electrophoresis on a 0.7% agarose gel, and transferred onto Hybond N+ (Amersham, Buckinghamshire, UK) in 1.5 *M* NaCl/0.25 *N* NaOH. The membranes were prehybridized at 65°C for 3 h in 5× SSPE (1× SSPE is 0.18 mol of NaCl, 10 mmol of NaH_2PO_4/Na_2HPO_4 [pH 7.4], 1 m*M* EDTA), 5× Denhardt's solution (1× Denhardt's solution is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% sodium dodecyl sulfate (SDS)), 1% SDS, and 10 μg of poly A per ml, and then hybridized with cDNA probe radiolabeled with [α-^{32}P]dCTP by the random-primer method. After overnight hybridization, blots were washed twice with 1× SSPE, 1% SDS at room temperature, twice with 1× SSPE, 1% SDS at 65°C and twice with 0.1× SSPE, 0.1% SDS at 65°C, and then exposed to X-ray film.

RNA blot analysis Total RNA was electrophoresed in a 1.0% agarose gel, transferred to a nylon membrane and

Table I. Primers Used for RT-PCR Experiments

Name	Sequence		
EU-5	Poly T		Anti-sense
EU-6	TAT GGA CAG CAG AGT AGC TAT GGT C	EWS/ex7	Sense
EU-10	ATC AAG TCC CCT GCC CCT GGT G	E1AF	Sense
EU-11	GCT GGC CGG TTC TTC TGG ATG C	E1AF	Anti-sense
EU-12	TCA AAT CCC CCT CTT CCC CTG C	EWS/ex8	Anti-sense
EU-13	TGT AGG GGT GGG GGT TGC AGC	ETV1	Anti-sense
EU-14	TAG TAA TAG CGG AGT GAA CGG C	ETV1	Anti-sense
EU-15	CAT GTA CGG GAG GTC TGA GGG GT	ERG	Anti-sense
EU-16	TTT CCA TCC TGC GGT CTT GTA	EWS/ex5	Anti-sense
EU-17	GAG GGA AAG CGA GAG GGA GAC GGA	EWS 5′flanking	Sense
ESBP-1	CGA CTA GTT ATG ATC AGA GCA GT	EWS/ex7	Sense
ESBP-2	CCG TTG CTC TGT ATT CTT ACT GA	FLI-1/ex7	Anti-sense
ERG-1	ACT CCC CGT TGG TGC CTT CC	ERG	Anti-sense
22.6	GAA CGA GGA GGA AGG AGA GA	EWS/ex1	Sense
11.3	ACT CCC CGT TGG TCC CCT CC	FLI-1/ex9	Anti-sense

hybridized with cDNA probe radiolabeled with [α-^{32}P]dCTP by the random-primer method at 65°C for 14–16 h in a buffer containing 5× SSPE, 5× Denhardt's solution. The blots were washed with 2× SSC containing 1% SDS at room temperature and 65°C. Final washings were done with 0.1× SSC containing 0.1% SDS at 65°C. The blots were exposed to X-ray film using an intensifying screen.

RESULTS

Detection of the chimera genes in the tumor samples and cell lines of Ewing's sarcoma RNA samples from 30 surgical samples and 16 cell lines of Ewing/PNET were examined to determine if the fusion genes were present. These RNAs were subjected to RT-PCR analysis using *EWS*, *FLI-1*, *ERG* and *ETV1* specific primers (Table I). Fusion transcripts were detected in 23 out of 30 surgical samples (Table II) and 12 out of 16 cell lines (Table II). The absence of the fusion genes was not due to general RNA degradation or inappropriate reaction, since the PCR products using the β-actin primers were obtained from these samples.

To determine the junctions of the fusion transcripts, we sequenced the fusion transcripts around the breakpoints. All cases were found to have junctions with the coding sequences of *EWS* and Ets family genes. *EWS-FLI-1* fusion transcripts had four types of junctions; *EWS* exon 7 to *FLI-1* exon 6, *EWS* exon 7 to *FLI-1* exon 5, *EWS* exon 10 to *FLI-1* exon 6 and *EWS* exon 10 to *FLI-1* exon 8 (Fig. 1A). In one case (cell line SCCH196 in Table III), two different *EWS-FLI-1* fusion transcripts were detected, *EWS* exon 7 to *FLI-1* exon 6 and *EWS* exon 8 to *FLI-1* exon 6. One was found to be in-frame and the other was

Table II. Fusion Transcripts in Ewing Family of Tumors (Surgical Samples)

Case	Age/sex	Origin	Diagnosis	Fusion transcripts
1	2y/M	Metatarsalia (O)	EWING	EWS exon7–FLI-1 exon6
2	3y/F	Chest wall (EO)	EWING	EWS exon7–FLI-1 exon5
3	5y/M	Back (EO)	EWING	EWS exon7–ERG exon7
4	5y/M	Iliac bone (O)	EWING	Not detected
5	6y/F	Thigh (EO)	EWING	EWS exon7–FLI-1 exon6
6	7y/F	Pubic (O)	EWING	EWS exon7–FLI-1 exon6
7	8y/M	?	EWING	EWS exon10–FLI-1 exon8
8	9y/M	Paravertebra (EO)	PNET	Not detected
9	10y/M	Chest wall (EO)	EWING	EWS exon7–E1AF
10	12y/F	Paravertebra (EO)	EWING	EWS exon7–FLI-1 exon6
11	13y/F	Vertebra (O)	EWING	EWS exon7–FLI-1 exon5
12	13y/M	Pelvis (EO)	EWING	EWS exon7–FLI-1 exon5
13	14y/F	Iliac (O)	EWING	EWS exon7–FLI-1 exon6
14	14y/M	Rib (O)	EWING	Not detected
15	14y/M	Foot (O)	EWING	Not detected
16	15y/M	Pelvic cavity (EO)	EWING	EWS exon7–ERG exon9
17	16y/F	Tibia (O)	EWING	EWS exon7–FLI-1 exon5
18	16y/M	Abdomen (EO)	EWING	EWS exon7–ERG exon7
19	17y/M	Mediastinum (EO)	EWING	EWS exon7–ERG exon7
20	18y/M	?	EWING	EWS exon10–FLI-1 exon6
21	22y/F	Retroperitoneum (EO)	EWING	EWS exon7–FLI-1 exon6
22	27y/M	Retroperitoneum (EO)	PNET	EWS exon7–FLI-1 exon5
23	30y/M	Thigh (EO)	PNET	EWS exon7–FLI-1 exon5
24	34y/M	Retroperitoneum (EO)	EWING	EWS exon7–FLI-1 exon6
25	34y/M	Chest wall (EO)	EWING	EWS exon7–FLI-1 exon6
26	35y/M	Peritoneum (meta)	EWING	EWS exon7–FLI-1 exon6
27	F	Lung (meta)	EWING	Not detected
28	M	Scapula (O)	EWING	EWS exon7–FLI-1 exon6
29	M	?	EWING	Not detected
30	?	?	EWING	EWS exon7–FLI-1 exon5

NCR-EW2 and SCCH196 have a typical chromosomal abnomality, t(11;22)(q24;q12).
O, osseous; EO, extra osseous.

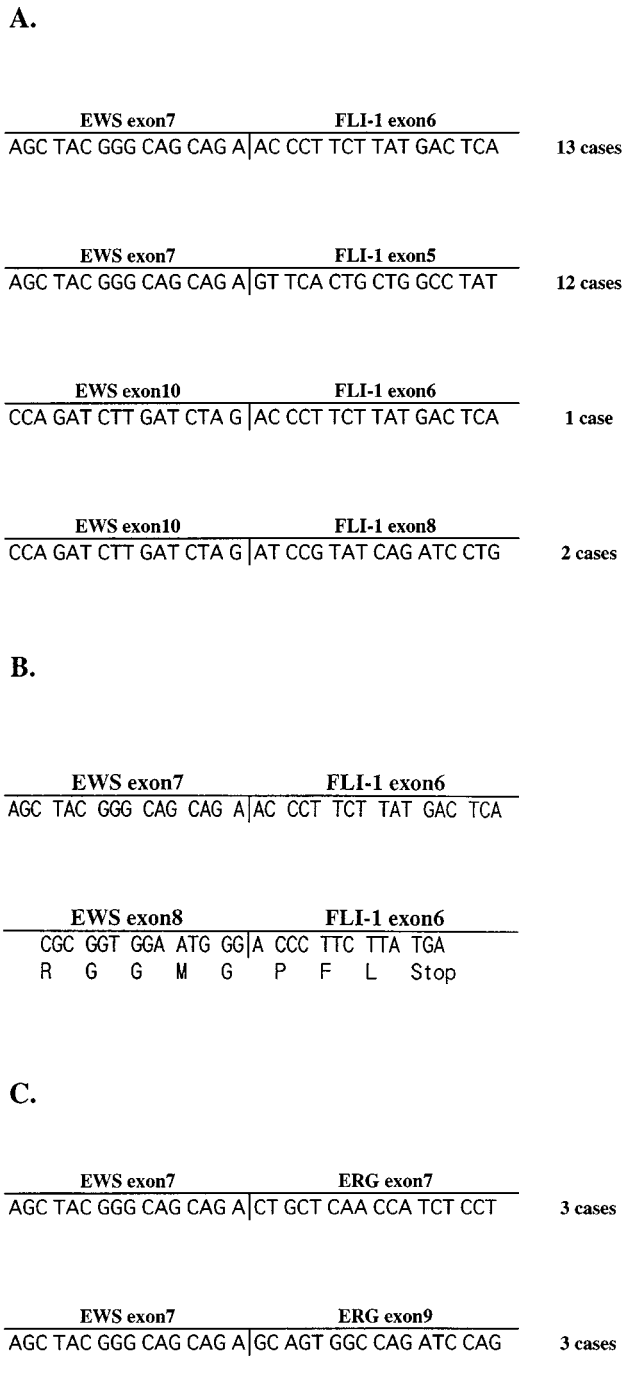

Fig. 1. Sequences of the *EWS-FLI-1* and *EWS-ERG* junctions. Vertical lines indicate the nucleotide position of the junctions of the two genes. The number of cases which demonstrated each type of junction is shown on the right side. A. Four different *EWS-FLI-1* in-frame fusion transcripts are shown. B. Two different *EWS-FLI-1* fusion transcripts in the same tumor (SCCH196). The position of the stop codon (stop) in the out-of-frame fusion is indicated. C. *EWS-ERG* in-frame fusion transcripts are shown.

Table III. Fusion Transcripts in Ewing Family of Tumors (Cell Lines)

	Diagnosis	Fusion transcripts
NCR-EW1	EWING	Not detected
NCR-EW2	EWING	EWS exon7–FLI-1exon5
NCR-EW3	EWING	EWS exon7–E1AF
NCR-EW4	EWING	Not detected
K-EW1	EWING	EWS exon7–ERG exon9
SK-ES1	EWING	EWS exon7–FLI-1exon5
SYM-1	EWING	EWS exon7–FLI-1exon5
RD-ES	EWING	EWS exon7–FLI-1exon5
SCCH196	EWING	EWS exon7–FLI-1 exon6
W-ES	EWING	EWS exon7–ERG exon9
EES-1	EWING	EWS exon7–FLI-1exon6
EW93	EWING	EWS exon7–FLI-1exon6
PN-1	PNET	Not detected
MURAOKA	PNET	EWS exon10–FLI-1 exon8
KU-9	PNET	EWS exon7–FLI-1exon5
KK-2	PNET	EWS exon7–FLI-1 exon6

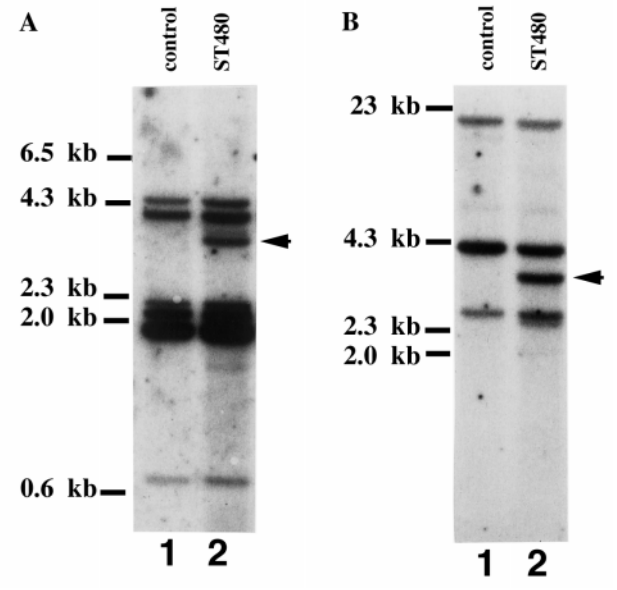

Fig. 2. Detection of *EWS* (A) and *E1AF* (B) rearranged fragment in a case of Ewing's sarcoma (ST480). Genome DNAs were isolated from lymphocytes of a normal person (lane 1) and a surgical sample of Ewing's sarcoma (lane 2), and digested with *Pst*I. A. The blot was hybridized with *EWS* probe (exon 6-12). The tumor-specific rearranged fragment is indicated by an arrow. B. The same filter was hybridized with *E1AF* probe and the rearranged band was detected at the same position as with the *EWS* probe (arrow).

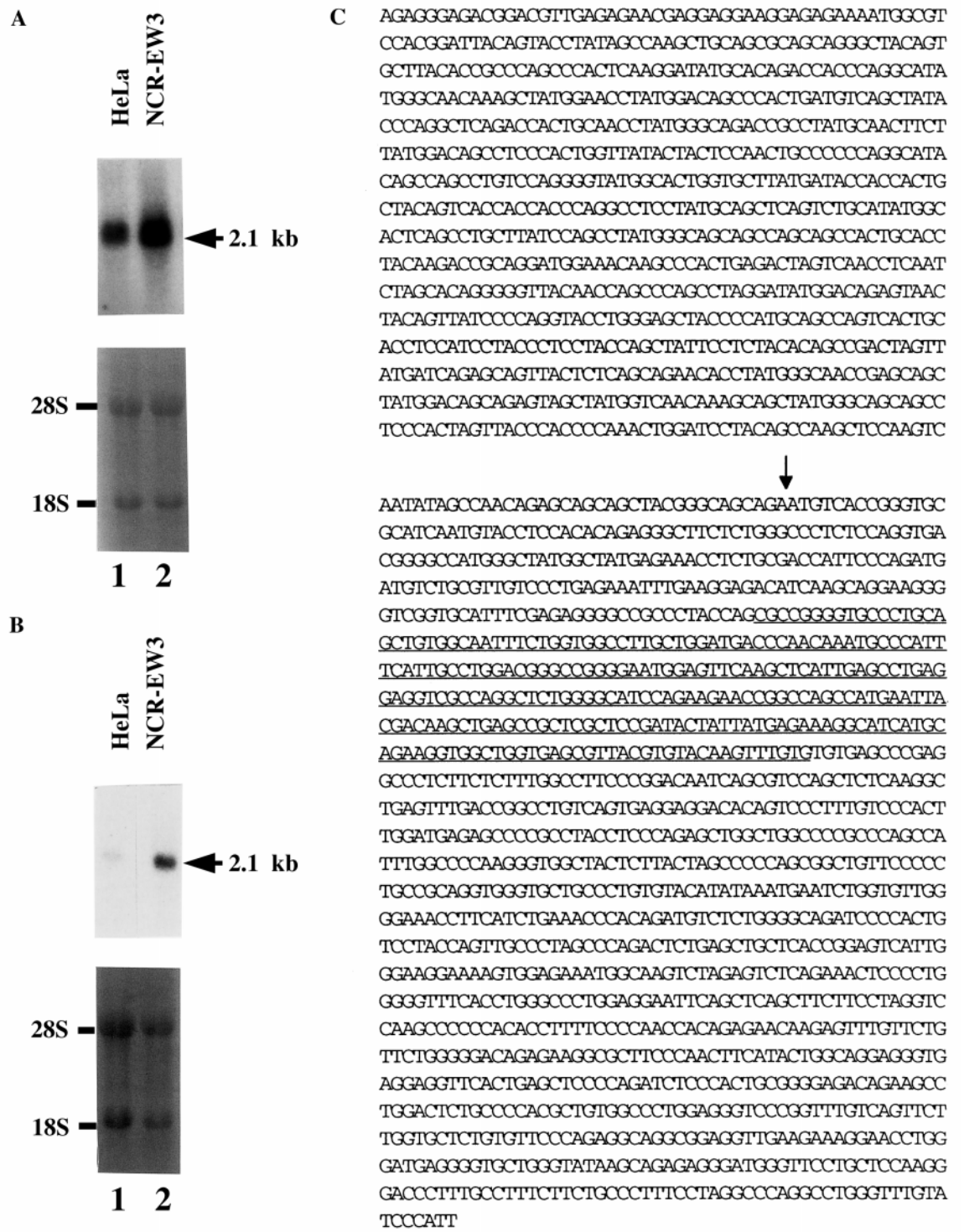

Fig. 3. Expression of the tumor-specific chimera gene in a Ewing's sarcoma cell line, NCR-EW3. A and B. RNA blot analyses showing the *EWS-E1AF* fusion transcript in NCR-EW3 cells. Identical RNA blots were hybridized with *EWS* (A) and *E1AF* (B) probes. The *EWS* probe detected a 2.1-kb fusion transcript band in NCR-EW3 cells. This band was approximately the same size as the germline *EWS* transcript found in HeLa cells. Hybridization with the *E1AF* probe detected the same 2.1-kb fusion transcript band only in NCR-EW3 cells. C. Sequence of the *EWS-E1AF* chimera cDNA. The arrow shows the breakpoint. The sequence of the Ets DNA binding domain is underlined. The nucleotide sequence of the *EWS-E1AF* chimera cDNA has been deposited in the GenBank database (Accession No. U35622).

not. In this case, the in-frame fusion transcript is generated by splicing out of *EWS* exon 8 (Fig. 1B). *EWS-ERG* fusion transcripts had two types of junctions; *EWS* exon 7 to *ERG* exon 7 and *EWS* exon 7 to *ERG* exon 9 (Fig. 1C). The same fusion transcripts were detected from the original surgical samples and from cell lines which were established from the surgical material. In one case, surgical samples were taken four times from the same patient at the age of 17 to 20 years old (Table II, case 19) and the same fusion transcript was detected from all the samples. This indicates that the same fusion transcripts are maintained even after relapse, metastasis or progression, and generation of a cell line. The maintenance of the fusion genes is considered to be necessary for these processes, since the tumor cells could not survive *in vivo* and *in vitro* once the chimera gene is lost. We did not detect these fusion genes from any other pediatric solid tumors examined, including neuroblastoma, rhabdomyosarcoma, osteosarcoma, malignant lymphoma and Wilms' tumor.

***EWS* rearrangement by Southern blot analysis and chromosomal analysis** In some cases, we could not detect the known fusion genes, *EWS-FLI-1*, *EWS-ERG* and *EWS-ETV1*. To determine if there were new fusion genes in these cases, we performed Southern blot analysis with the *EWS* cDNA probe on surgical tumor samples from the patients. In a case of Ewing's sarcoma (Table II, case 9), the bands of genome *EWS* showed a different pattern from that of the normal germ line (Fig. 2A); an extra-band was detected at 3.5 kb. Identical results were obtained with the cell line NCR-EW3, which was established from that sample. The same membrane was hybridized with 3′ *E1AF* cDNA probe. The abnormal band was detected at the same position (Fig. 2B). This indicates that the 3.5 kb fragment contained the breakpoint between *EWS* and *E1AF*.

Expression and whole cDNA sequence of the *EWS-E1AF* fusion transcript To determine the expression of the *EWS-E1AF* fusion gene, we performed RNA blot analysis with total RNA from the cell line NCR-EW3 (Fig. 3, A and B). A discrete signal of 2.1 kb fusion transcript was detected with both *EWS* and *E1AF* probes. This indicates that the *EWS-E1AF* fusion is highly expressed in NCR-EW3 and the transcript does not have large deletions in the *EWS* 5′ part and *E1AF* 3′ part.

To determine if the whole cDNA of the *EWS-E1AF* gene has a deletion or mutation, we sequenced two different PCR products; one obtained with primers EU-17 specific to *EWS* and EU-11 specific to *E1AF*; the other with a primer specific to *EWS*, EU-6, and a non-specific poly-T primer (Table I). The whole sequence revealed no deletion or mutation in the *EWS* part or *E1AF* part of the fusion gene (Fig. 3C). As in the cases of other fusion genes, the *E1AF* part of the fusion gene contains the DNA binding domain of the Ets gene family.

Correlation of histopathological examination and genetic alterations of the tumor samples To determine histopathological characteristics of the tumor, we basically employed conventional morphological analysis of hematoxylin and eosin-stained tumor sections under a light microscope. Electron microscopic analysis and immunohistochemical analysis were also performed. We think it essential to differentiate the Ewing's sarcoma and PNET, because Ewing's sarcoma has an undifferentiated neural pattern of neurofilament expression with differential usage of polyadenylation sites, which is different from the differentiated neural pattern of neurofilaments of PNET.[19] To differentiate the Ewing's sarcoma and PNET, we examined the presence of neurosecretory granules by electron microscopy in all cases. To clarify whether the type of fusion gene determines or correlates with the morphology and phenotypes of the tumor, we re-examined the histopathology of the tumors by microscopic analysis, ultrastructural analysis, and immunohistochemical analysis. We did not find any significant correlation between the morphological diagnostic criteria (neurosecretory granules, periodic acid-Schiff reactivity, and rosette-like formation) and the results of genetic analysis (type of chimera genes and positions of the breakpoints).

DISCUSSION

In this study, we extensively investigated a new fusion gene, joining *EWS* and *E1AF* through t(17;22)(q21;q12), in Ewing's sarcoma and concluded that *EWS-E1AF* is another fusion gene available for the diagnosis of Ewing's sarcoma. The *EWS-E1AF* chimeric gene is expressed at a high level in a Ewing's sarcoma cell line, NCR-EW3, indicating that the preservation of the chimera gene during and even after generation of the cell line is necessary for cell survival. At the genome level, we identified *EWS* and *E1AF* genes in a 3.5 kb genome fragment, suggesting that both genes are proximately located in the tumor; a recent analysis revealed that the sequence at the breakpoint in the NCR-EW3 has a high homology (86%) with the sequence of the breakpoint between bcr-abl in chronic myeloid leukemia.[20] *EWS-E1AF* contains the Ets DNA binding domain, like *EWS-FLI-1*, *EWS-ERG* and *EWS-ETV1*. This domain is reported to be required for transformation by *EWS-FLI-1*.[18, 21–23] Hence, sequence-specific DNA binding is considered to be functionally important for *EWS-E1AF* activity. The lack of mutation or deletion in both the *EWS* and *E1AF* parts is consistent with the lack of mutation in other types of fusion genes in Ewing's sarcoma. In contrast, many mutations and deletions have been detected in the translocated c-myc of the Burkitt lymphoma.[24]

We also conducted genetic analysis of Ewing's sarcoma and PNET, in addition to histopathological analysis. It is

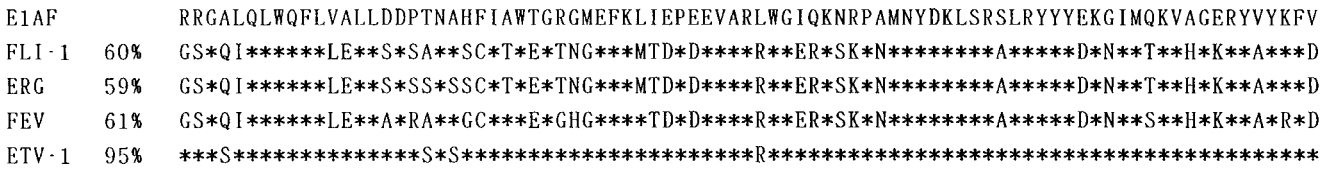

Fig. 4. Comparison of the amino acid sequence of the Ets DNA binding domain of *E1AF* with those of *FLI-1*, *ERG*, *FEV* and *ETV1*. *, identical amino acid. Percentage amino acid identity is shown on the left side.

sometimes very difficult to diagnose the tumor on the basis of the clinical features and morphology. Immunohistochemical analysis by monoclonal antibodies and ultrastructural analysis are helpful, but not specific. In contrast, detection of fusion genes with RT-PCR is reliable, convenient and quick. Although RT-PCR could theoretically be performed from a single cell, a sample of 1 mm^3 provides sufficient RNAs to afford good experimental reliability or to allow examination with a series of primer sets. Thus, punch biopsy samples are sufficient. In cases of bone tumor and soft-part sarcoma, small amounts of samples should be stored in guanidine isothiocyanate solution for possible future analysis.

Sequencing of the PCR products is necessary for certain sets of primers to detect the chimera genes. The primers ESBP-1 and ERG-1, designed for the *EWS* and *ERG* genes, respectively, amplified the *EWS-FLI-1* fusion gene, which has a breakpoint involving *EWS* exon 10 and *FLI-1* exon 8 (Table I, Table II, case 7 and Table III, MURAOKA). This is probably because of the high sequence homology in the DNA binding domain. To determine the precise fusion genes in the tumors, sequencing of PCR products is necessary.

We could not detect the known fusion genes in some cases which were diagnosed as Ewing's sarcoma and PNET. There are three possible explanations for this: limitation of RT-PCR, limitation of diagnosis with conventional morphological analysis, and the presence of new fusion genes in Ewing's sarcoma and PNET. It is very difficult to eliminate the limitation of RT-PCR completely, since many factors, such as annealing temperature, positions of primers, length of PCR products, quality of RNA, amount of fusion transcripts, number of cycles and so on, may influence the outcome. The second possibility is that the diagnosis of the tumor on the basis of morphological criteria was not precise. The criteria for morphological diagnosis of small-round-cell tumor are controversial. Thus, the detection of the fusion genes as well as ultrastructure and immunohistochemistry would all contribute to the diagnosis. When the fusion transcripts can not be detected, we should reevaluate the diagnosis, since more than 90% of Ewing's sarcoma is considered to contain a chimera gene, e.g. *EWS-FLI-1*, *EWS-ERG*, *EWS-ETV1*, and *EWS-E1AF* presented in this manuscript.[10–14, 25] The PNET cell line, NCR-PN1, established from a sciatic nerve tumor of a 2-month-old girl, does not contain a fusion gene (Table III) and NCR-PN1 cells have neuronal character. NCR-PN1 is an exceptional type of PNET, in terms of both the age of the patient and the clear neuronal character. NCR-PN1 may have had a different origin from common PNET tumors. Furthermore, we could not eliminate the third possibility of a new fusion gene. We isolated the new fusion gene, *EWS-E1AF*, from a tumor sample (Table I, case 9) in which no known fusion gene was detected. Recently, an *EWS-FEV* fusion was also isolated.[26] Thus, the presence of other new fusion genes is still possible.

Pathologists tend to classify these tumors into Ewing's sarcoma without neural differentiation and PNET with neural differentiation from the point of view of diagnostic pathology. However, the neurogenic potential of Ewing's sarcoma and the results of genetic analysis, including our data suggest that Ewing family tumors include both Ewing's sarcoma and PNET in the spectrum of a single biologic entity.

The target genes for the *EWS-E1AF* remains to be elucidated. *E1AF* is most closely related to a distinct subfamily of Ets gene, *ETV1* which has previously been implicated in Ewing's sarcoma. The Ets DNA binding domain of the *E1AF* part shows 95% amino acid identity with that of *ETV1* (Fig. 4). In contrast, *E1AF* has only 60% amino acid identity with the *FLI-1* and *ERG* Ets domains. The consensus sequence of *E1AF* recognition sites is not known yet, while those of ETS1 and ETS2 have been reported.[27–31] For the chimera genes including *EWS-E1AF*, matrix metalloproteinase (MMP) genes might be targets, since MMP genes have recognition sites for Ets family genes in their regulatory element.[32] MMP genes have been reported to determine cell characteristics related to high metastatic capability. However, the fusion genes are thought to be responsible for oncogenesis. Thus, the most important thing is to isolate the target gene which functions in cell growth and proliferation *in vivo*. The Ewing's sarcoma cell line NCR-EW3 generated in our laboratory, with the *EWS-E1AF* fusion gene, should be a suitable material for identification of the target genes *in vivo*.

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