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A second Ewing's sarcoma translocation, t(21;22), fuses the EWS gene to another ETS-family transcription factor, ERG

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The t(11;22)(q24;q12), present in 85% of Ewing's sarcoma and related tumours, fuses the EWS gene from chromosome 22q12 and the *ETS* family member, *FLI-1*. This results in the expression of a chimaeric protein containing the amino-terminal portion of EWS fused to the ETS DNA-binding domain of FLI-1. We have identified a second Ewing's sarcoma translocation, t(21;22)(q22;q12), that fuses *EWS* to a different *ETS* family member, the ERG gene located on band 21q22. Identical *EWS* nucleotide sequences found in the *EWS/FLI-1* fusion transcripts are fused to portions of *ERG* encoding an ETS DNA-binding domain resulting in expression of a hybrid EWS/ERG protein. These findings suggest that fusion of *EWS* to different members of the *ETS* family of transcription factor genes may result in the expression of similar disease phenotypes.

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Specific chromosomal translocations are associated with a large number of human malignancies. The pathophysiologic effects of these rearrangements are mediated by transcriptional deregulation or structural alteration of proto oncogenes. Transcription factors have been frequent targets of tumour-associated chromosomal rearrangement (reviewed in ref. 1).

Ewing's sarcoma and primitive neuroectodermal tumour (PNET) are related solid tumours of childhood that, in 85% of cases, show cytogenetic evidence of a characteristic translocation between chromosomes 11 and 22 (refs 2–4). As a consequence of this translocation, the 5' portion of the EWS gene from band 22q12 is fused to the 3' portion of the FLI-1 gene from band 11q24 (refs 5,6). This results in the formation of chimaeric *EWS/FLI-1* mRNAs and proteins[5,6]. Detection of these chimaeric transcripts has formed the basis of a sensitive and specific diagnostic assay for these tumours[7].

EWS is a ubiquitously expressed gene of unknown function. *FLI-1* (refs 8–10) is a member of the ETS gene family of transcription factors which have now been found in many species (reviewed in ref. 11). ETS proteins have structurally related sequence-specific DNA binding domains (ETS domains) that are typically located in their carboxy termini. Their amino terminal regions are thought to interact with other proteins to form complexes involved in transcriptional activation of target genes. The chimaeric EWS/FLI-1 molecule consists of the glutamine-rich amino terminal portion of EWS fused to the carboxy terminus of FLI-1 (refs 5,6). The EWS/FLI-1 fusion protein efficiently transforms NIH3T3 cells. This transformation ability requires both the EWS domain and the DNA binding domain of FLI-1 (ref. 6). EWS/FLI-1 appears to act as an aberrant transcription factor that is functionally distinct from normal FLI-1 (ref. 12).

These data suggest that the formation of the *EWS/FLI-1* chimaera is important in the oncogenesis of Ewing's sarcoma. Approximately 15% of Ewing's sarcoma and PNET fail to show cytogenetic evidence of simple or complex translocations involving bands 11q24 and 22q12 (ref. 4). Of this 15% approximately half show variant translocations or other alterations involving band 22q11–12. This observation has led to the suggestion that in these cases, *EWS* is fused to transcription factors other than *FLI-1*. In the present study, we provide evidence for this phenomenon in two Ewing's sarcoma cases with 21;22 chromosomal translocations. We demonstrate that as a result of this rearrangement, *EWS* is fused to another *ETS* family member, *ERG*, located on band 21q22. We show that chimaeric *EWS/ERG* transcripts and proteins are expressed in cell lines containing this variant translocation.

Results

21;22 translocation in Ewing's sarcoma

Two cell lines, TTC-466 and TTC-633, were derived from primary tumour tissue from two paediatric patients with Ewing's sarcoma. Pathologic diagnosis in both cases was made according to established morphologic, immunocytochemical and ultrastructural criteria for Ewing's sarcoma (see Methodology); each case showed pathological features typical for this class of tumours.

Because Ewing's sarcoma cells frequently carry characteristic karyotypic abnormalities, cytogenetic analyses were performed on both tumour derived cell lines. Surprisingly, neither TTC-466 nor TTC-633 contained an 11;22 chromosomal translocation typical of

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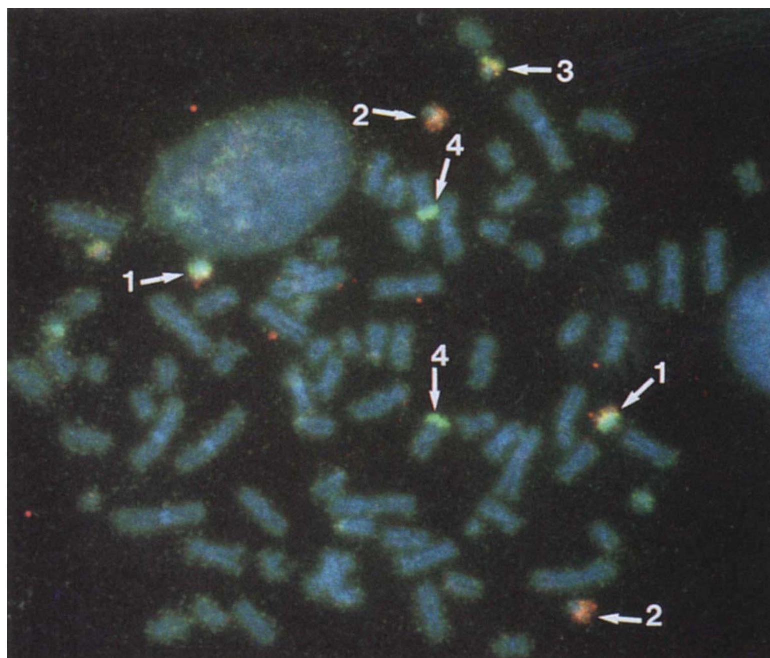

Fig. 1 Dual colour fluorescence *in situ* hybridization (FISH) analysis demonstrating fusion between chromosomes 21 and 22 in TTC-466 cells. Metaphase spreads from TTC-466 were prepared and analysed by FISH using chromosome 21 (green) and 22 (red) painting probes. Chromosomal species are labelled as follows: 1, derivative 21/22 chromosomes; 2, normal chromosomes 22; 3, normal chromosome 21; 4, derivative ?/21 chromosome. Painting with chromosome 11 probe revealed no rearrangement with chromosome 22 (data not shown).

Ewing's sarcoma. Cytogenetic examination of 38 complete metaphases from TTC-466 revealed an apparent reciprocal t(21;22) involving bands 21q22 and 22q12 in virtually all cells examined (D.L-T. *et al.*, manuscript in preparation). Structural abnormalities of chromosome 11 were also observed in some TTC-466 clones, but not involving band 11q24. Similary, cytogenetic examination of TTC-633 cells revealed a t(21;22)(q22;q12) rearrangement but no apparent abnormalities of chromosome 11.

To better delineate the structures of the chromosomal translocations present in TTC-466 and TTC-633 cells, fluorescence *in situ* hybridization (FISH) studies were performed. Two colour FISH experiments were performed by hybridizing TTC-466 metaphase chromosomes simultaneously to chromosome 21 and 22 painting probes (Fig. 1). These analyses demonstrated derivative chromosomes with chromosome 21 sequences juxtaposed to chromosome 22. Similar results were obtained for TTC-633 cells using single colour FISH with chromosome 21 and 22 probes (data not shown). However, FISH experiments using a chromosome 11 probe failed to detect any abnormalities in either cell line indicative of a 11;22 translocation (data not shown).

RT-PCR detection of fusion transcripts

The Ewing's sarcoma 11;22 translocation fuses the *EWS* to *FLI-1*. We have shown previously, by Southern blot analysis, that the EWS gene is rearranged within the same 2 kb genomic fragment in both TTC-466 and TTC-633 cells[7]. This work suggested that these cell lines contained a translocation involving *EWS* and a gene on chromosome 21. *ERG* , also a member of the ETS-family of transcription factors, is very similar to *FLI-1* (68% amino acid identity) and has been mapped to the same band on chromosome 21 that is involved in the above 21;22 translocation[13]. This suggested that *EWS* and *ERG* might be fused in TTC-466 and TTC-633 cells as a result of the 21;22 rearrangement.

To detect possible *EWS/ERG* hybrid transcripts, reverse transcription (RT)-PCR experiments were performed. Oligonucleotide primer pairs were used that could detect *EWS/FLI-1* and *EWS/ERG* fusions. The 5' oligonucleotide (ESBP-1) of each pair was specific for the EWS gene. Different 3' oligonucleotides were used that hybridized to *FLI-1* alone (ESBP-2) or to both *FLI-1* and *ERG* (ERG1). Initial RT-PCR reactions were performed with the ESBP-1/ESBP-2 primer pair that should only amplify *EWS/FLI-1* fusion templates (Fig. 2, lanes a–f). A single 329 basepair (bp) amplfication product was observed for TC-71, a Ewing's sarcoma cell line containing the t(11;22) (ref. 7). No amplification products were detected using RNA from TTC-466 or TTC-633 cells, indicating that these cell

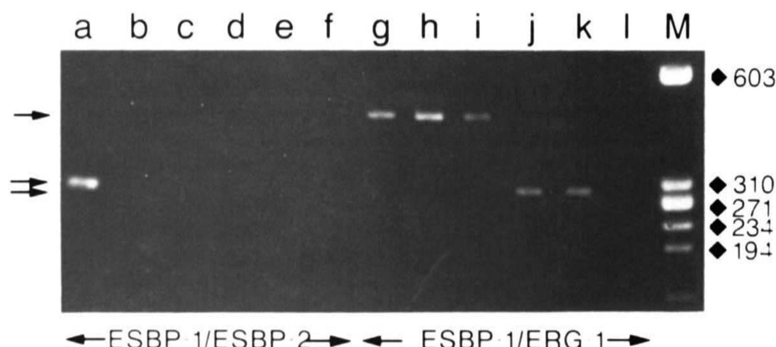

Fig. 2 RT-PCR analysis of TTC-466 and TTC-633 cell lines. Total RNA extracted from cell lines was analysed by RT-PCR using the *EWS*-specific primer ESBP-1 in combination with the *FLI-1*-specific primer ESBP-2 (lanes a–f) and the *ERG* primer ERG-1 (lanes g–l). Arrows indicate the positions of amplified products electrophoresed using 2% agarose gels and stained with ethidium bromide. Lanes a and g, TC-71; lanes b and h, TTC-466 cell line; lanes c and i, TTC-466 primary tumour tissue; lanes d and j, TTC-633 cell line; lanes e and k, TTC-633 primary tumour tissue; and lanes f and l, the rhabdomyosarcoma cell line A204. M, Size markers in bp.

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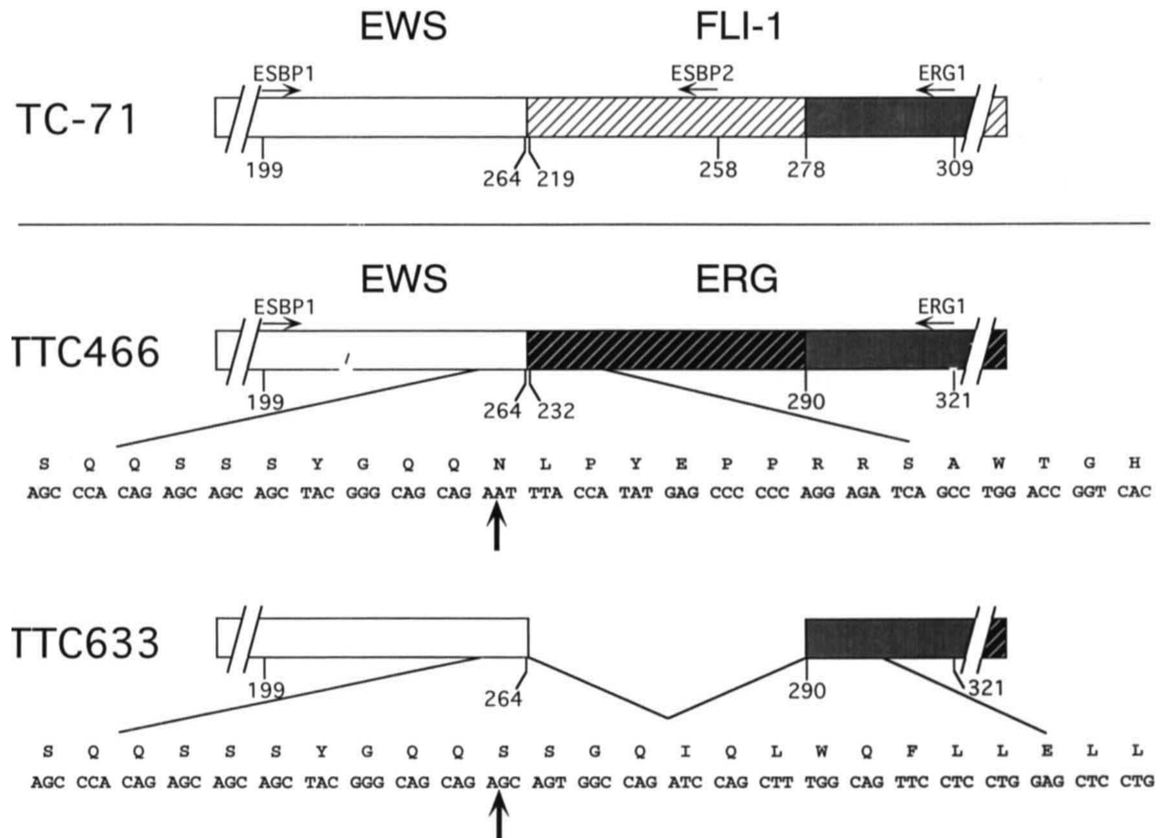

Fig. 3 Schematic showing EWS/FLI-1 and EWS/ERG fusion genes in different Ewing's sarcoma cell lines. RT-PCR using ESBP1/ERG1 primers was used to amplify chimaeric junctions involving *EWS* in tumour cell lines. TC-71 contains a 11;22 translocation which results in a type 1 fusion between *EWS* and *FLI-1* (sequence data not shown). TTC-466 and TTC-633 contain *EWS/ERG* chimaeric fusions that are joined at the same location in *EWS* as in TC-71 but at different sites in *ERG*. Sequence analysis depicted under each *EWS/ERG* schematic demonstrates that in both TTC-466 and TTC-633, fusions are in frame. Vertical arrows indicate *EWS/ERG* fusion points. Amino acid locations of fusion points and PCR primers are shown in schematics. Clear, *EWS*; Light stripe, *FLI-1*; Dark stripe, *ERG*; and Grey, *FLI-1/ERG* ETS common domain.

lines do not contain *EWS/FLI-1* fusion transcripts.

The ESBP-1/ERG1 primer pair was then used to detect possible *EWS/ERG* fusion species. Since the ERG1 primer matched at 18 out of 20 nucleotides of the corresponding region of *FLI-1*, an amplification product was observed with the TC-71 cell line (Fig. 3, lane g). A 465 bp RT-PCR product was detected for TTC-466 and a 315 bp product for TTC-633 (Fig. 2, lanes h–k). Identical results were obtained using RNA isolated from either original tumour tissue or tumour-derived cell lines, indicating that these transcripts were present in primary tumours and were not artefacts of tissue culture. No amplification products were observed using RNA from A204, a rhabdomyosarcoma cell line that has neither a t(11;22) nor a t(21;22) (Fig. 2, lanes f, l). Identical results were obtained when PCR products were blotted onto nylon filters and probed with an *EWS*-specific ^{32}P-labelled oligonucleotide probe.

Different *EWS/ERG* fusion species

To better delineate the structures of possible *EWS/ERG* fusion transcripts in TTC-466 and TTC-633 cells, RT-PCR amplified products using ESBP-1/ERG1 primers were subcloned and sequenced. Nucleotide sequence analyses demonstrated in frame fusions between *EWS* and *ERG* in both Ewing's sarcoma cell lines (Fig. 3). The *EWS* fusion points were identical for both TTC-466 and TTC-633. This is concordant with the observation that the *EWS* breakpoints from both cell lines localized to the same 2 kb genomic region (see above). The *ERG* fusion points differed between TTC-466 and TTC-633. Analysis of the TTC-466 RT-PCR product demonstrated the 5' juxtaposition of *EWS* sequences to 270 bp showing 100% sequence identity with *ERG* codons 232–321 (ref. 13). In contrast, the TTC-633 product contained *ERG* sequences commencing at *ERG* codon 290. This corresponds to the first amino acid of the ETS-common domain of *ERG*, known to be required for sequence specific DNA binding.

Expression of *EWS/ERG* fusion gene

Our RT-PCR data detected a fusion transcript between *EWS* and *ERG* in Ewing's sarcoma cell lines containing a 21;22 translocation. To determine whether these transcripts were functional, northern and immunoprecipitation analyses were performed. Northern blots containing poly A$^+$ RNA from TTC-466 and the TC-32 PNET cell line containing the t(11;22) were first hybridized with an *EWS* cDNA probe. In addition to a 2.2 kb germline *EWS* band, a 3.1 kb species was detected in TTC-466 that comigrated with an *EWS/FLI-1* fusion band present in TC-32 cells (Fig. 4*a*). Only the 3.1 kb species was detected in TTC-466 when this blot was hybridized

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with ERG-specific probes isolated from coding or untranslated *ERG* sequences 3' to the EWS/ERG fusion point (Fig. 4*a*). Hybridization with a *FLI-1* cDNA fragment detected the 3.1 kb band only in TC-32 cells (data not shown). These data identify the *EWS/ERG* fusion mRNA and demonstrate that it is specific to cells containing a t(21;22).

To detect EWS/ERG protein products, immunoprecipitation experiments were performed using antisera raised to an EWS/FLI-1 polypeptide fragment. Included in this fragment was a portion of FLI-1 that was very similar to ERG (111/114 amino acids). This antisera, as well as recognizing both EWS and FLI-1 epitopes, could also be used to detect ERG protein products. Immunoprecipitation from metabolically labelled TTC-466 cells revealed a 68 kD band corresponding to an EWS/ERG fusion protein (Fig. 4*b*). This band was not detected in lysates from HeLa cells that do not contain a 21;22 translocation. The size of this protein product is concordant with the size predicted from *EWS/ERG* nucleotide sequence data. The EWS/ERG protein was virtually identical in size to a previously described EWS/FLI-1 fusion protein[6]. A 90 kD species was present in both TTC-466 and HeLa cells which corresponds to germline EWS.

Discussion

These studies document the formation of *EWS/ERG* fusion genes in two separate Ewing's sarcoma cell lines harbouring a 21;22 chromosomal translocation. This rearrangement is distinct from translocations involving chromosomes 11 and 22 that are found in 85% of Ewing's sarcoma karyotypes[4]. The frequency of the 21;22 translocation is unclear although it is likely to represent a major proportion of the 7% of Ewing's sarcomas that have 22q12 chromosomal abnormalities in the absence of 11q24 alterations. We have detected the t(21;22) in two (16%) of 12 Ewing's sarcoma cell lines. The PCR primer pairs described here can distinguish between *EWS/ERG* and *EWS/FLI-1* fusions, and will be useful in determining the incidence of the former in Ewing's sarcoma and related tumours.

ERG and *FLI-1* have similar structures and are likely to play similar roles in chimaeric fusions with *EWS*. *ERG* shares an overall 68% amino acid identity with *FLI-1* (ref. 9). The ETS-common domain required for sequence specific DNA binding shows 98% identity (83/85 amino acids). This domain is required for transformation by *EWS/FLI-1* (ref. 6). The fact that this domain is preserved in both TTC-466 and TTC-633 fusions suggests that DNA-binding is also important in EWS/ERG function. The carboxy terminal 87 amino acids immediately 3' to the ETS-common domain are 72% identical between ERG and FLI-1. The function of this region is unknown. It is possible that it may modulate DNA binding which would suggest that the natural binding sites of ERG and FLI-1 while being very similar, may not be identical.

Formation of EWS/ERG chimaeras occurs in the absence of EWS/FLI-1 fusion in TTC-466 and TTC-633 cells. Cytogenetic and FISH analyses as well as PCR and northern data indicate that FLI-1 is neither rearranged nor expressed in these tumours. This suggests that EWS/ERG is able to replace EWS/FLI-1 in the oncogenic pathway to Ewing's sarcoma. Recent evidence suggests that EWS/FLI-1 is an aberrant transcription factor where EWS acts as a transcriptional activation domain linked to the DNA-binding domain of FLI-1 (ref. 12). Considering the structural similarities of EWS/ERG and EWS/FLI-1, it is likely that both chimaeras act by this same mechanism to activate a very similar repertoire of target genes.

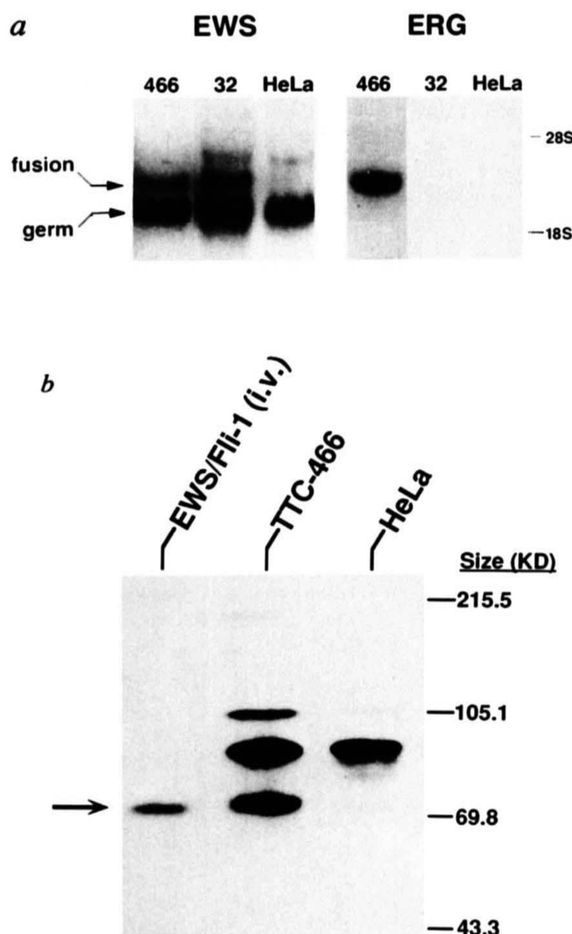

Fig. 4 *a*, Northern analyses showing *EWS/ERG* fusion transcript in TTC-466 cells. Identical northern blots containing Ewing's sarcoma RNAs were hybridized to *EWS* and *ERG* cDNA probes. The *EWS* probe detected a 2.2 kb germline band present in all cell lines. An additional 3.1 kb band was detected in TTC-466 (lane 466) that was not present in HeLa cells. This band was approximately the same size as the *EWS/FLI-1* fusion transcript found in TC-32 cells (lane 32) containing a t(11;22). Hybridization with an *ERG* cDNA probe (codons 232–300) detected the same 3.1 kb band only in TTC-466 cells, consistent with this band being an *EWS/ERG* fusion. *b*, Immunoprecipitation of EWS/ERG fusion protein from TTC-466 cells. Antisera generated from an EWS/FLI-1 fusion polypeptide containing a region of FLI-1 very similar to ERG (see text) was used to immunoprecipitate ^{35}S-labeled TTC-466 and HeLa cell extracts. TTC-466 displayed a unique band consistent with the predicted size of an EWS/ERG fusion protein (arrow). This fusion protein was nearly identical in size to *in vitro* translated EWS/FLI-1 protein (EWS/FLI-1 (i.v.)). The additional band at 90 kD, that is present in both TTC-466 and HeLa cells, likely represents germline EWS protein.

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We have shown that two distinct tumour-associated chromosomal abnormalities occur in the same tumour type. It is possible that this reflects genomic regions prone to rearrangement in the Ewing's sarcoma cell of origin. It has been hypothesized that c-*ETS-1* and *FLI-1*, which are closely linked on band 11q24, arose by gene duplication and that gene dispersion later led to the formation of the closely linked c-ETS-2 and ERG genes on band 21q22 (refs 8,14,15). This suggests that intronic and chromatin structures involved in rearrangement with *EWS* might also be similar in *ERG* and *FLI-1*. Alternatively, the 11;22 and 21;22 translocations might reflect biological selection. Though ETS proteins are known to bind an overlapping set of DNA sequences *in vitro*, they may be more discriminating *in vivo*. In this regard, ERG may be one of the few transcription factors that, when fused to EWS, can function in the same oncogenic pathways as EWS/FLI-1. It has recently been shown that EWS is fused to the transcription factor ATF as a result of a 12;22 translocation found in malignant melanoma of soft parts[16]. Thus EWS when fused to different classes of transcription proteins may promote formation of distinct tumour phenotypes.

Variant translocations involving a common gene and a set of distinct chromosomal loci have been observed in other human malignancies. In some instances they suggest that different oncogenic pathways can lead to the same tumour phenotype. The 1;19 and 17;19 translocations that occur in a subset of acute lymphocytic leukemia (ALL) result in formation of structurally disparate chimaeric genes: in the t(1;19) the transcription activation domain of the E2A gene is fused to the DNA-binding domains of PBX, a homeobox gene[17,18], while in the t(17;19) E2A is fused to HLF, a leucine zipper transcription factor[19]. It seems likely that these chimaeric products are participating in different oncogenic pathways in phenotypically similar tumours. In other instances, it appears that different genomic rearrangements may initiate the same oncogenic pathway. In Burkitt's lymphoma, the t(8;14) juxtaposes *c-MYC* to the immunoglobulin heavy chain locus and t(2;8) and t(8;22) rearrangements have been described which fuse *c-MYC* to an immunoglobulin light chain locus (reviewed in ref. 20). In each case the biologic effect is the same, leading to transcriptional deregulation of *c-MYC*. Similarly, chromosome 11q23 translocations in certain subtypes of childhood acute leukaemia fuse the human homologue of the Drosophila trithorax gene with a variety of distinct but structurally similar genes[21–25]. Our findings, together with the occurrence of variant rearrangements in other human tumours, suggest that there may be redundancy in human oncogenesis at both the genetic as well as the biological level.

Methodology

Case material and cell lines. Original tumour tissue from the first case was obtained from a surgically excised pulmonary metastasis of a cervical paraspinal soft tissue tumour in a 4 1/2 year old female admitted to the Childrens Hospital of Los Angeles. The primary tumour and pulmonary metastasis showed identical pathological features. Light microscopy revealed a diffuse growth of undifferentiated small round cells with areas of necrosis. Immunostains were positive for neuron-specific enolase, neurofilament protein and the MIC-2 antigen, and ultrastructural analysis showed primitive cells with intracytoplasmic glycogen accumulation, neuritic processes and atypical neurosecretory granules. A diagnosis of extraosseous Ewing's sarcoma was made. The second case was a bone lesion from the right ischium of a 7 1/2 year old female. Light microscopy was similar to that of the first case. Immunostains were also positive for neuron-specific enolase, neurofilament protein and the MIC-2 antigen. Electron microscopy showed small round cells with frequent neurosecretory granules and primitive neurites. A diagnosis of Ewing's sarcoma with peripheral neuroepithelioma phenotype was therefore made. The cell lines TTC-466 (case 1) and TTC-633 (case 2) were established by standard methods using fresh tumour tissue and are maintained in RPMI 1640 with 20% fetal bovine serum.

Cytogenetic analysis and fluorescence *in situ* hybridization. Metaphase spreads for cytogenetic analysis were prepared using conventional methods[26]. Karyotypes were designated according to the International Society for Cytogenetic Nomenclature (ISCN), 1991. The procedure for fluorescence *in situ* hybridization (FISH) was based on published methods[27], and was performed overnight at 37 °C under low stringency conditions (50% formamide and 2× sodium saline citrate buffer). Probes, labelled with biotin, included the chromosome 11, 21 and 22 specific painting probes as well as the chromosome 13/21 (D13Z1/D21Z1) satellite probe (all from Oncor) and were detected using fluorescein-labelled avidin (Oncor). Chromosomes were counterstained with propidium iodide and visualized using an Olympus BH2 microscope equipped with Olympus BP495 filters. For dual colour studies, chromosome 21 and 22 painting probes were labelled with biotin and digoxigenin respectively. Signals were detected using fluorescein-labelled avidin and rhodamine-labelled anti-digoxigenin, respectively. Chromosomes were counterstained with DAPI.

RT-PCR. RT-PCR detection of chimaeric transcripts involving *EWS* was performed as previously described, using total RNA as the starting material[7]. Oligonucleotide primers used for PCR included *EWS*-specific ESBP-1: 5'–CGACTAGTTATGATCAGAGCAGT–3' (ref. 6), *FLI-1*-specific ESBP-2: 5'–CCGTTGCTCTGTATTCTT-ACTGA–3' (ref. 6), and *ERG*-specific ERG-1: 5'–ACTCCCCG-TTGGTGCCTTCC–3'. Thirty five cycles of PCR were performed, consisting of denaturation at 94 °C for 30 s, annealing at 65 °C for 60 s and extension at 72 °C for 120 s. Products of amplification were identified by gel electrophoresis using 2% agarose gels followed by ethidium bromide staining. PCR products were also transferred to Nytran filters (Schleicher and Schuell), by standard protocols, and probed with a ^{32}P-end labelled oligonucleotide probe (5'–AGCCAACAGAGCAGCAGCTACGGGCAGCAGA–3') derived from *EWS* cDNA sequence data[7].

Cloning and sequencing of PCR products. PCR products were isolated from 2% low melting point agarose gel slices using β-agarase (New England Biolabs). Purified products were then cloned into pCRII plasmids using the Invitrogen TA Cloning System (Version 1.3, Invitrogen), with minor modifications of the protocols described by the manufacturer. Direct sequencing of purified recombinant plasmid DNA was then carried out using an Applied Biosystems 373A DNA Sequencer. Sequencing primers included Sp6, T7, m13 reverse and m13 forward (–20) primers (Invitrogen). Sequences were analysed using MacVector Version 4.0 Sequence Analysis software.

RNA analysis. Total or Poly (A)$^+$ RNA was isolated from cell lines[28] or frozen primary tumour tissue[7] as described. Northern analysis was performed according to standard methods[28]. Blots were hybridized with the following cDNA probes: *EWS*, 700 bp *Pst*I/*Bam*HI fragment encompassing codons 12–245; *FLI*, 210 bp *Pvu*II/*Pst*I fragment within the ETS-common domain; *ERG* (3' coding) 203 bp *Nde*I/*Sac*I fragment from codon 232–300 and (3' untranslated) 700 bp *Eco*RI/*Ava*I (courtesy of Craig Thompson). After hybridization, blots were washed under stringent conditions (0.1× SSC, 50 °C, 20 min) and autoradiographed.

Immunoprecipitation analysis. Antisera was raised to a glutathione-S-transferase-EWS/FLI-1 fusion polypeptide consisting of EWS amino acids 245–64 (ref. 5) fused to FLI-1 amino acids 240–369 (ref. 9). A 450 bp *Bam*HI/*Pst*I fragment, from a EWS/FLI-1 fusion cDNA isolated from a tumour cell line containing an 11;22 translocation[7], was subcloned into the expression plasmid pGEX-2T (Pharmacia). This construct was expressed in *E. coli*, fusion protein was purified using glutathione-agarose[29] and protein was

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injected subcutaneously into rabbits. This antisera recognized epitopes from both EWS and FLI-1 (S.L.L. and C.T.D., unpublished observations). TTC-466 cells were metabolically labeled with ^{35}S-methionine and whole cell lysates were subjected to two cycle immuno-precipitation using EWS/FLI-1 antisera. Immune complexes were precipitated with protein A-sepharose (Pharmacia), fractionated by SDS/PAGE and autoradiographed.

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