Variability of *EWS* chimaeric transcripts in Ewing tumours: a comparison of clinical and molecular data

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Summary

Ewing tumours (ET), including Ewing’s sarcoma and peripheral primitive neuroectodermal tumour, are well characterised at the molecular level by a unique chromosomal rearrangement which fuses the *EWS* gene to one of two closely related *ETS* proto-oncogenes, FLI-1 or ERG. Expression of the resulting chimaeric transcripts can be readily detected by reverse transcriptase polymerase chain reaction (RT–PCR). This approach led to the identification of a number of different exon combinations at the junction site of coding sequences. The physiological consequences of the observed variability in the hinge region of *EWS* chimaeric proteins are not known. We have analysed tumour-derived material from 30 ET patients with well-documented clinical course (18 with localised and 12 with metastatic disease at diagnosis) for the presence of *EWS/FLI-1* or *EWS/ERG* RNAs. Karyotypes were obtained in 21 cases. A chromosome 22 rearrangement was demonstrated in 18 cases (67%). In contrast, RT–PCR revealed the presence of chimaeric transcripts in 28 tumours (93%), with fusions of *EWS* exon 7 to *FLI-1* exons 6 (19/28), 5 (4/28) and 7 (1/28). In addition, *EWS/FLI-1* exon combinations 10/5 and 9/4 were observed in one case each. In the last tumour, the presence of at least four additional splicing variants corresponding to fusion of *EWS* exon 7 to *FLI-1* exons 4, 6, 8 and 9 was demonstrated. Two tumours expressed *EWS/ERG* fusion transcripts involving *EWS* exon 7 and *ERG* exon 6 in this study, *EWS/FLI-1* exon combinations 7/6 (type I) predominated over 7/5 (type II) in localised ET (14 versus 1) and were more abundant in tumours affecting the long bones (9 versus 0), whereas in central axis tumours and metastatic disease there was only little difference in the frequency of the two types. So far, no correlations between different chimaeric *EWS* transcripts and any other clinical parameters have been identified.

Among cytogenetic aberrations in Ewing’s sarcoma (ES) and peripheral primitive neuroectodermal tumours (pPNET), recently referred to as ‘Ewing tumours’ (ET), the reciprocal translocation between the long arms of chromosomes 11 and 22, t(11;22)(q24;q12) occurs in 83% of cases. In an additional 9% complex translocations involving chromosome 22 have been identified (Aurias et al., 1983; Turc-Carel et al., 1983; Whang-Peng et al., 1984). Moreover, tumour cells of ES and pPNET are well characterised by the extraordinary high expression of the pseudoautosomal *MIC* gene (Kovar et al., 1990), which represents a highly selective feature of these cells, making an assignment of these small round-cell tumours as one entity even more likely (Ambros et al., 1991).

The recent cloning of the chromosome breakpoints of the ET-specific t(11;22) translocation revealed that the sites of rearrangement were localised within two regions, EWSR1 on chromosome 22 and 11 respectively (Zucman et al., 1992). On chromosome 22, EWSR1 is nested within a novel gene of unknown function, *EWS* (Plougastel et al., 1993). The gene involved in the translocation on chromosome 11 was revealed to encode the human homologue of murine FLI-1, a member of the ETS family of transcription factors. In mice, FLI-1 resides at the insertion site of Friend murine leukaemia virus in induced erythroleukaemias. Proviral integration causes FLI-1 activation and consequently neoplastic transformation of erythroid progenitor cells. As a result of the gene rearrangement in human ET cells, the C-terminal FLI-1 portion is constitutively expressed from the *EWS* gene promoter as part of a chimaeric protein. In addition to FLI-1 rearrangements, fusion of *EWS* to the closely related ETS proto-oncogene *ERG*, located on chromosome 21q22, was revealed in t(11;22)(q24;q12)-negative ETs (Zucman et al., 1993; Sorenson et al., 1994). The positions of the chromosome translocation breakpoints were shown to be restricted to introns 7–10 of the *EWS* gene and widely dispersed within introns 3–9 of the ETS-related genes. Among the rearrangements that result in in-frame exon fusion, joining of exon 7 of *EWS* to either exon 6 or 5 of *FLI-1* has been observed most frequently (81%) (Zucman et al., 1993). These rearrangements correspond to type I and II fusion transcripts originally reported by Delattre et al. (1992). Regarding the *EWS/ERG* rearrangements, four different chimaeric transcripts have been reported so far (Zucman et al., 1993). The minimal coding sequences present in all ET-specific fusion transcripts is composed of *EWS* exons 1–7 and by the *FLI-1* or *ERG* region downstream of the exon 8/9 boundary. These RNAs encode chimaeric proteins with a C-terminal glutamine-rich *EWS* regulatory domain (*EWS-ERD*) fused via a variable hinge region to a unique DNA-binding domain that is characteristic of all *ETS* transcription factors. The activity of a second transactivating domain, which resides in the C-terminus of *FLI-1* or *ERG*, is dependent on the presence of *EWS-ERD* (Ohno et al., 1993). However, nothing is known about the function and the physiological consequences of the variant region lying in between *EWS-ERD* and the DNA-binding domain, except that type I and a chimaeric transcript 66 bp shorter than type I transform NIH-3T3 cells equally well (May et al., 1993). We now report on the analysis of different gene fusions and accessory splicing variants in 30 ETs by means of RT–PCR. The molecular data are compared with all relevant clinical data available in order to identify any correlations of specific *EWS* chimaeric variants with the course of the disease in ET patients.

Materials and methods

Patients and tumours

The tumour-derived material analysed in this study originated from 30 ET patients, 14 females and 16 males with a median age of 13 years (range 3–25) (Table I). Eight
primary tumours were located in the lower extremities, eight in the thoracopulmonary region, five in the pelvis, four in the upper extremities, one in the mandible and one in the spine; one patient had multifocal bone disease. Two patients had tumours not involving the bone (extraosseous ET) evolving from the iliopsoas muscle and the chest wall (patients 22 and 23 respectively). Moreover, two patients suffered from ETs as second malignancies after acute lymphoblastic leukaemia and large-cell anaplastic lymphoma (patients 23 and 28). At diagnosis, 18 patients presented with localised disease, whereas in 12 patients metastatic disease was revealed. Metastases to the lungs could be found in seven patients, to the bones in seven patients, to the bone marrow investigated by light microscopy in four patients and to the liver in one patient.

Twenty-nine patients were treated according to the Cooperative Ewing's Sarcoma Study (CESS) protocols (Jürgens et al., 1988). One patient received the CWS 86 protocol for soft-tissue sarcoma because of an extraosseous ET (Koscielniak et al., 1991). Six patients with metastases at diagnosis and two patients after relapse received autologous or allogeneic bone marrow transplantation with a conditioning regimen consisting of melphalan, etoposide and carboplatin (Emminger et al., 1991). Follow-up information was available for 28 patients.

**Collection of tumour samples**

At least two surgical samples of tumour were collected from every patient at diagnosis. After cryostat examination one piece was formalin fixed and paraffin embedded for immunohistochemical studies. The second part of the tumour was dissected and placed into RPMI-1640 (Gibco, Paisley, UK) for the derivation of cell lines and routine cytogenetic analysis. Another part was immediately snap frozen in liquid nitrogen and transferred to the laboratory on dry ice for RNA preparation.

As negative controls for the RT–PCR analysis, RNA from different solid tumours with a diagnosis other than ET were used. They included three rhabdomyosarcomas, one lymphoma, one neuroblastoma, one osteosarcoma and one malignant melanoma.

**Immunohistochemistry**

For diagnosis of small round cell tumours a panel of antibodies was used directed towards MIC-2\(^{22.3}\) antigen (12E7, R. Levy, Stanford University, USA; HBA71, G. Hamilton, University of Vienna, Austria), vimentin, desmin (Biomarker, Rehovot, Israel; D-1033, Sigma), common leucocyte antigen, anti-muscle-specific actin (HHF-35, Enzo, New York, NY, USA), HNK-1/Leu 7 (Becton Dickinson, Sunnyvale, CA, USA), neuron-specific-enolase (Bioscience, Emmenbrücke, Switzerland) and S-100 protein (Dakopatts, Glostrup, Denmark). All tumours were reviewed by the Vienna Bone Tumour Registry (M. Salzer-Kuntschik) and classified as ETs according to the CESS criteria (Jürgens et al., 1988; Ambros et al., 1991).

**Nucleic acid isolation**

Total RNAs from frozen tumours or cell lines were isolated using the TRIzol extraction kit (Gibco BRL, Life Technologies, Gaithersburg, MD, USA) based on the acid guanidinium–phenol–chloroform method (Chomczynski & Sacchi, 1987).

**Reverse transcription and PCR amplification**

Aliquots of 1–4 μg of total RNA were reverse transcribed with either random hexamers, olio-DT or with the FLI-1 specific primer 11A (Table II) using Moloney murine leukaemia virus reverse transcriptase (Gibco BRL, Life Technologies).

The resulting cDNAs were PCR amplified using primers 11.3 and 22.3 (Delattre et al., 1992) or primers 22.3 and ERG 3 (Table II). Internal control for specific amplification was provided by nested PCR using primers 11.4 and 22.4 or primers 22.4 and ERG 4. In order to evaluate the quality of

### Table 1 Patients' characteristics and clinical data

| Patient no. | Age (years) | Localisation | Metastases at diagnosis | RNA | Chromosome 22 rearrangement |
|-------------|-------------|--------------|-------------------------|-----|---------------------------|
| 1           | 13F         | Humerus      | None                    | EWS ex 7/FLI-1 ex 6 | t(11;22) |
| 2           | 16M         | Fibula       | None                    | EWS ex 9/FLI-1 ex 4 | del 22q |
| 3           | 10F         | Femur        | None                    | EWS ex 7/FLI-1 ex 6 | t(11;22) |
| 4           | 3F          | Chest wall   | None                    | EWS ex 7/FLI-1 ex 6 | t(11;22) |
| 5           | 14M         | Femur        | Bone marrow             | Negative                  | neg        |
| 6           | 9M          | Fibula       | Bone marrow             | EWS ex 7/FLI-1 ex 6 | t(11;22) |
| 7           | 4F          | Chest wall   | None                    | EWS ex 7/FLI-1 ex 5 | t(11;22) |
| 8           | 16F         | Pelvis       | Lungs, bone             | EWS ex 7/FLI-1 ex 5 | t(11;22) |
| 9           | 16F         | Pelvis       | Lungs                   | EWS ex 7/FLI-1 ex 6 | t(11;22) |
| 10          | 10F         | Chest wall   | Liver, lungs            | Negative                  | t(2;22) |
| 11          | 13M         | Scapula      | None                    | EWS ex 7/FLI-1 ex 6 | t(11;22) |
| 12          | 16F         | Chest wall   | Bone                    | EWS ex 10/FLI-1 ex 5 | t(6;11;22) |
| 13          | 17M         | Multifocal   | Bone, bone marrow       | EWS ex 7/FLI-1 ex 5 | t(11;22) |
| 14          | 10F         | Chest wall   | Bone                    | EWS ex 7/FLI-1 ex 5 | t(11;22) |
| 15          | 12F         | Fibula       | None                    | EWS ex 7/FLI-1 ex 6 | ND |
| 16          | 17F         | Mandibula    | Lungs, bone             | EWS ex 7/FLI-1 ex 6 | ND |
| 17          | 17F         | Metatarsus   | None                    | EWS ex 7/FLI-1 ex 6 | ND |
| 18          | 4F          | Chest wall   | None                    | EWS ex 7/FLI-1 ex 6 | ND |
| 19          | 25M         | Pelvis       | Lungs, bone, bone marrow| EWS ex 7/FLI-1 ex 5 | ND |
| 20          | 6M          | Thumb        | None                    | EWS ex 7/FLI-1 ex 6 | ND |
| 21          | 15M         | Humerus      | Lungs, bone             | EWS ex 7/ERG ex 6 | t(2;22) |
| 22          | 11F         | Chest wall   | None                    | EWS ex 7/ERG ex 6 | Negative |
| 23          | 7F          | Mus. iliopsoas | None              | EWS ex 7/FLI-1 ex 6 | t(11;22) |
| 24          | 11M         | Fibula       | None                    | EWS ex 7/FLI-1 ex 6 | t(11;22) |
| 25          | 14M         | Pelvis       | None                    | EWS ex 7/FLI-1 ex 6 | ND |
| 26          | 8F          | Spine        | None                    | EWS ex 7/FLI-1 ex 6 | ND |
| 27          | 15M         | Tibia        | None                    | EWS ex 7/FLI-1 ex 6 | ND |
| 28          | 20F         | Pelvis       | Lungs                   | EWS ex 7/FLI-1 ex 6 | t(11;22) |
| 29          | 20M         | Chest wall   | None                    | EWS ex 7/FLI-1 ex 6 | ND |
| 30          | 14M         | Chest wall   | None                    | EWS ex 7/FLI-1 ex 6 | t(11;22) |

*Extraosseous Ewing tumour. F, female; M, male; ND, not determined because of non-growth or insufficient amount of material.*
RNA preparations, RT-PCR was performed in parallel using MIC2\(^{20-22}\) primers 1 and 2 under identical conditions (Table II). MIC2\(^{20-22}\) transcripts are expressed ubiquitously in human cells but are highly abundant in ETs (Kovar et al., 1990).

In RT-PCR experiments, strict precautions were taken to avoid cross-contamination or product carry-over that would result in false positives. Pre- and post-amplification steps were separated from each other. Negative controls were included at every step of sample preparation, RNA extraction, reverse transcription and PCR. First-round PCR included 35 cycles of denaturation at 94°C for 30 s, annealing at 65°C for 1 min and elongation at 72°C for 1 min. PCR was preceded by a 10 min incubation at 94°C. Nested PCR was performed with 30 cycles under the same conditions as first-round PCR. Since primers ERG 3 and ERG 4 matched the corresponding region of FLI-1 at 18 out of 20 and at 16 out of 18 nucleotides, respectively, the annealing temperature was increased to 68°C for more stringent conditions. Amplified products were analysed on 1.5% agarose gels.

**DNA sequence analysis**

Sequencing was performed by the dideoxy chain-termination method modified for fluorescent-based DNA sequencing using an Applied Biosystems 373 DNA sequencer.

Direct sequencing of double-stranded PCR products was carried out using the Femtomole-Sequencing System (fmol, Promega). Reaction products were analysed in 8% polyacrylamide gels.

**Hybridisation analysis**

PCR products were routinely hybridised to EWS-, FLI-1- or ERG-specific probes either in gel or on filters. For in-gel hybridisation PCR products were electrophoresed through a 2% agarose gel which was dried for 1 h at room temperature followed by 3 h at 60°C. Dried gels were swollen in water and incubated successively in 0.5 M sodium hydroxide, 0.15 M sodium chloride, and in 0.5 M Tris pH 8.0. 0.15 M sodium chloride, for 30 min, each. Gels were equilibrated in 6 x SSC (1 x SSC = 0.15 M sodium chloride, 0.15 M sodium citrate, pH 7.0) for 15 min and hybridised to radiolabelled FLI-1-specific oligonucleotide 11.6 (Table II) for 2–3 h at 60°C in 5 x SSPE (1 x SSPE = 0.15 M sodium chloride, 0.010 M sodium hydrogen phosphate, 0.001 M EDTA), 5 x Denhardt's solution (1 x Denhardt = 0.2 g l\(^{-1}\) Ficoll, polyvinylpyrrolidone and bovine serum albumin) and 0.1% SDS followed by 2–3 washes with 6 x SSC.

For filter hybridisations gels were blotted onto a nylon membrane (Hybond/Amersham), which was subsequently prehybridised and hybridised to EWS-, FLI-1- or ERG-specific probes according to standard procedures (Maniatis et al., 1982). Hybridised gels and filters were exposed to Kodak XAR-5 film for autoradiography.

**Results**

Reverse-transcribed RNA from tumour-derived material, either cell line or primary tumour tissue, was analysed by PCR using primers 22.3 and 11.3 or ERG 3 respectively. The specificity of the amplification reactions was confirmed by nested PCR with primers 22.4 and 11.4 or ERG 4. PCR products were routinely hybridised to EWS-, FLI-1- or ERG-specific probes (Figure 1). Amplification products with sizes other than those expected for the well-defined type I and type II EWS/FLI-1 RNA as well as EWS/ERG products were subjected to direct sequence analysis.

EWS chimaeric RNA was detected in 28 of 30 ETs. The processed transcripts displayed fusion of EWS exon 7 to FLI-1 exon 6 (19/28) or 5 (4/28). In this study, therefore, the two most common EWS/FLI-1 variants, type I and type II, were detected in 68% and 14% respectively. One tumour expressed a chimaeric transcript combining EWS exon 7 with FLI-1 exon 7, which has not been observed before. One
tumour displayed a fusion of EWS exon 10 to FLI-1 exon 5. RT–PCR analysis of a cell line established from a metastasis from patient 2 resulted in multiple amplification products. In order to discriminate between specific and non-specific bands all fragments were excised from the gel, reamplified using primers 11.4 and 22.4 and subjected to direct sequence analysis (Figure 2). The most prominent product obtained corresponded to EWS FLI-1 exon fusion 9.4, which is in concordance with the observation of a single DNA break.

In contrast, routine cytogentic revealed chromosome 22 rearrangements in only 18 out of 27 tumours (67%), partly because of failure to obtain mitotic figures (5.9) and partly because of insufficient tumour-derived material (4.9). Fifteen tumours revealed a t(11;22)(q24;q12) and one tumour a del22q. In all of these cases an EWS/FLI-1 fusion was demonstrated by RT–PCR. In one tumour (patient 10) displaying a t(2;22)(q25;q12), no fusion transcript could be detected although control amplifications of MIC2p-32 confirmed satisfactory quality of the RNA samples. This tumour was a typical MIC2p-32-positive chest wall tumour evolving from the right ninth rib (Askin tumour) with pleural effusion and metastases to the lungs and to the liver. In contrast, another patient (no. 21) with t(2;22)(q35;q12) expressed an EWS/ERG chimaeric RNA.

One patient (no. 5), who repeatedly failed to demonstrate the presence of chimaeric tumour transcripts in RT–PCR using either FLI-1 or ERG-specific primers, had a tumour from the right femur reaching to the gluteal region with bone marrow involvement as investigated by light microscopy. The tumour stained positive for the MIC2p-32 antigen but was negative for desmine and several neuroendocrine markers (i.e. neuron-specific enolase, S100, ganglioside GD2, chromogranin). The tumour cell morphology in light microscopy was compatible with a diagnosis of Ewing's sarcoma.

The tumour karyotype was 48,XY.del(1)(p34), +6, +12 with cytogenetically normal pairs of chromosome 22, 21 and 11. In addition, Northern blot analysis did not reveal aberrant EWS transcripts or expression of rearranged FLI-1 RNA.

Finally, RNA extracted from all tumours with a diagnosis other than ET (i.e. three rhabdomyosarcomas, one lymphoma, one neuroblastoma, one osteosarcoma and one malignant melanoma) did not reveal any EWS chimaeric transcript in RT–PCR.

A comparison of the distribution of primary tumour sites with the different exon combinations revealed the following (Table III). The relative frequency of the most common EWS FLI-1 exon combination 7/6 – type I – (19 tumours) was higher in tumours of the extremities (six legs, three arms) than in central axis tumours (four chest wall, three pelvis) or in tumours in other locations (three cases). In contrast EWS exon 7/FLI-1 exon 5 transcripts – type II – (4 tumours) were present in all cases (two cases each) only. Studies of the extent of disease showed that 78% (14/18) of patients with localised disease expressed type I RNA. In contrast, type II transcripts predominated in patients with disseminated disease at diagnosis (three cases) as compared with localised tumours (one case). This patient (no. 7) with a thoracopulmonary ET achieved a partial remission after two courses of chemotherapy, but shortly afterwards showed a tumour progression with lung and bone metastases. This tumour displayed a p53 mutation, which is very rare in patients with ET (Kovar et al., 1993), and this may have been responsible for the aggressive tumour growth.

In this study, EWS chimaeric transcripts were also detected in two patients with metastatic ET (nos. 22 and 23). One tumour, evolving from the chest wall with secondary infiltration of the scapula, showed a fusion of EWS and ERG (no. 22). In the other case (patient 23), ET developed as a secondary malignancy from the ilio-psoas muscle after acute lymphoblastic leukaemia and expressed a chimaeric transcript with EWS exon 7 fused to FLI-1 exon 7.

Figure 2 a. Nested RT–PCR analysis of a cell line derived from a metastasis from patient no. 2 (right lane). Arrows point to amplification products that turned out to be derived from EWS FLI-1 chimaeric RNAs after nucleotide sequencing. EWS FLI-1 exon combinations are indicated. All other bands resulted from amplification of either non-specific sequences, as is often observed in nested PCR, or and heteroduplex DNA formed between the specific alternative products. Left lane, size marker b. Results from sequence analysis of excised PCR products resulting from alternative splicing.
**Fusion transcripts and clinical outcome**

Response data to chemotherapy were available for all patients positive for EWS rearrangements. The clinical outcome could not be evaluated in two patients because they were lost for follow-up. Ten patients showed no evidence of disease for the median duration of 11 months. Six of these ten patients had tumours expressing EWS exon 7/FLI-1 exon 6. Eight patients died as a result of disease progression. In this subset of patients, no preference for any exon combination has been observed. Two patients in complete remission died because of septic complications after allogeneic bone marrow transplantation. Furthermore, six patients are still on therapy and so far were not analysed for disease survival. In view of the relatively short follow-up in most patients, these results have to be considered preliminary.

**Discussion**

Owing to the lack of differentiation, cytologically distinguishing ETs from other small round-cell tumours of childhood and adolescence is often difficult. Among immunological markers only MIC2-30-32 is consistently highly expressed on ET cells, but it is not limited to this group of malignancies. Because of the high incidence of chromosome 22q12 aberrations, cytogenetic demonstration of these rearrangements should be pivotal to the unambiguous diagnosis of ET. However, routine cytogenetic analysis is often hampered by non-growth of tumour cells in vitro. Moreover, complex rearrangements with and without involvement of chromosome 11 have been identified at the chromosomal level in approximately 9% of cytogenetically analysable ET cases (Turc-Carel et al., 1988). This observation might be partially explained by the orientations of the genes involved in the translocations: EWS and FLI-1 are orientated from the centromere to the telomere, whereas ERG is orientated from the telomere to the centromere (Crete et al., 1993). Presumably, in-frame fusion of EWS and ERG can only be accomplished by either interstitial or complex translocations involving other chromosomes. This hypothesis might explain the lack of cytogenetic evidence for chromosome 21 rearrangements in ETs and the 17% translocation negatives observed in earlier studies (Turc-Carel et al., 1988). In our cohort patients 21 and 22 expressing EWS/ERG fit into this category. The molecular demonstration of chimaeric EWS RNA expression by RT–PCR as exemplified in this study appears to be more reliable than classical cytogenetics for ET diagnosis. Chimaeric EWS transcripts were identified in 93% of all cases investigated. By contrast, routine cytogenetics displayed a chromosome 22 rearrangement in only 67% of all tumours analysed. Only in 2 out of 30 tumours (7%) could no EWS chimaeric transcript be revealed by RT–PCR and Northern blotting (data not shown). In one of these cases an EWS rearrangement was demonstrated by molecular cytogenetics, suggesting the involvement of another so far unidentified ETS-related gene (data not shown). The other tumour (patient no. 5) was negative for any chromosome 22q12 rearrangement. Because of the clinical and histopathological appearance this tumour was classified as 'atypical Ewing's sarcoma'. However, this term is poorly defined. Tumours assigned to this group should therefore be reclassified on the basis of EWS/FLI-1 or EWS/ERG expression. Thus, it cannot be excluded that this patient suffered from some neoplasm other than ET. The data presented in this report confirm the high degree of variability of EWS chimaeric transcripts and resultant PCR products in ETs reported earlier.

In this study, the percentage of tumours expressing either type I or type II transcripts was 82% (23/28), which is similar to the proportion (38/47) recently observed by Zucman et al. (1993). The data collected in our study from a relatively small number of cases suggest that the EWS/FLI-1 exon combination 7/6 predominates in fusion transcripts of localised tumours of the extremities, whereas the combination EWS exon 7/FLI-1 exon 5 was more frequent in metastatic disease and tumours of the central axis. However, the significance of these findings has to be carefully evaluated in much larger cohorts of patients. Moreover, we could demonstrate the presence of a t(1;12)(q24;q12) and a t(21;22)(q24;q12) in two cases of malignancies of soft tissues at the molecular level, thus confirming the diagnosis of extraosseous ET. One of these tumours, which occurred as a second malignancy after treatment of acute lymphoblastic leukaemia, expressed an EWS/FLI-1 exon 7/7 fusion transcript which has not been reported before and therefore appears to be rare. In another unusual case with a single defined gene rearrangement at the DNA level (data not shown), a large EWS/FLI-1 transcript including EWS exons 1–9 as well as FLI-1 exons 4–9 was coexpressed with a number of deduced splicing variants, all lacking EWS exons 8 and 9 and fused to FLI-1 exons 4, 6, 8 or 9. In the protein encoded by the smallest product (EWS/FLI-1 exons 7/9) the EWS regulatory region is directly joined to the FLI-1 DNA-binding domain. This product has been predicted to occur as a parallel to the smallest EWS/ERG fusion observed by Zucman et al. (1993). The coexistence of alternative splicing variants seems to be common in cells carrying chromosomal translocations and has been demonstrated to occur in BCR-ABL-, PML/RAR- and PBX1/E2A-positive leukaemias (Shivelman et al., 1986; Solomons et al., 1991; Izraeli et al., 1992). The possible presence of multiple differentially processed fusion transcripts in one tumour makes the application of additional methods for specification mandatory. We performed nested PCR as well as hybridisation to FLI-1 and ERG-specific probes and sequence analysis in order to distinguish specific amplification products from non-specific ones. Moreover, these very sensitive methods will be of particular help in the analysis of minimal metastatic and residual disease. The protocol applied in this report allowed detection of one tumour cell in 10⁶ nucleated cells by nested PCR in multiple mixing experiments (C. Pfleiderer et al., manuscript in preparation).

In conclusion, we think that comparison of molecular and clinical data as initiated in this report should be extended to much larger cohorts of ET patients since the results presented do not exclude a biological influence of the variable hinge region of EWS chimaeric oncoproteins on the clinical course of the disease in ET.

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**Table III Different exon combinations, tumour localisation and extent of disease**

| Chest wall | Pelvis | Upper extremities | Lower extremities | Others | Local disease Metastases |
|------------|--------|------------------|-------------------|--------|--------------------------|
| EWS ex 7/FLI-1 ex 6 | 4 | 3 | 3 | 6 | 3 | 14 | 5 |
| EWS ex 7/FLI-1 ex 5 | 2 | 2 | 0 | 0 | 0 | 1 | 3 |
| Others | 1 | 0 | 0 | 1 | 1 | 2 | 1 |
| EWS/ERG | 1 | 0 | 1 | 0 | 0 | 1 | 1 |
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