EWS/FLI-1 Rearrangement in Small Round Cell Sarcomas of Bone and Soft Tissue Detected by Reverse Transcriptase Polymerase Chain Reaction Amplification

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Recent cloning of the t(11;22) region has led to the detection of a number of sequences involved in the breakpoints by substituting a sequence which encodes a putative RNA binding domain for that of the DNA binding domain of the human homologue of murine FLI-1. Several tumours display consistent translocation at t(11;22) (q24;q12), a finding that suggests these fusion transcripts could be expressed and detected by reverse transcriptase polymerase chain reaction amplification. To date, only a small number of Ewing's sarcomas (Es) and peripheral neuroectodermal tumours (pPNET) of bone have been tested with this novel molecular biology approach. In this study, we confirmed the presence of the three putative chimaeric transcripts on 7 cases of Es and pPNET sarcomas of bone and soft tissue, providing 100% positivity for the tested tumours. For comparative purposes, a number of other neuroectodermal tumours were analysed with negative results: esthesioneuroblastoma, retinoblastoma, Schwannoma. A primitive soft tissue sarcoma (ectomesenchymoma) with a 22 chromosome rearrangement did not express any transcript, nor did a number of non-neuroectodermal small round cell sarcomas of soft tissue (rhabdomyosarcomas) and bone (microcellular osteosarcoma), conventional bone sarcomas, leiomyosarcomas, malignant fibrous histiocytomas and synovial sarcomas. These results reinforce the value of molecular biology techniques for the correct assessment of histology difficult evaluable neoplasms, such as the group of small round cell tumours within the Es family.
Key words: RT-PCR, Ewing's sarcoma, pPNET, EWS/FLI-1
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INTRODUCTION

WITHIN THE category of small round cell tumours, several highly malignant neoplasms of childhood are considered in which Ewing's sarcoma (Es) of bone and soft tissue constitutes the most representative category; the histological diagnosis has always been a challenge for the pathologist, and their identification, facing a number of similar but biologically distinct neoplasms for purposes of treatment and prognosis, is a demanding necessity [1–5].

The former category of Es has been to a large extent replaced by the concept of peripheral primitive neuroectodermal tumours or pPNET (also known as PN or PNET), and introduces a histogenetic concept within the morphology: all these neoplasms occurring outside the central nervous system in children, young adults and, exceptionally, in more advanced ages, seem to express a neuroectodermal phenotype, but with a biology different to those well-known neuroblastomas (NB) of the sympathetic system [6–8]. The latter comprise genetically, biologically and even morphologically, a different family of malignant neoplasms somewhat related to but distinct from pPNET [9, 10].

The histologies of Es and pPNET are closely related and only minor differences have been demonstrated with conventional techniques [11, 12]. Atypical variants of Es, superimposed with pPNET plus the factor of subjectivity, may influence the diagnosis at routine histology [13]. The introduction of ancillary techniques, such as electron microscopy and immunohistochemistry, has been essential for their more exact categorisation [1, 3, 7, 14].

A further aid for the accuracy of this diagnosis has been the demonstration of a reciprocal translocation on Es and pPNET, t(11;22)(q24;q12), first described by Aurias and colleagues [15–17]. This finding has provided further support to the categorisation of this group of malignant small round cell sarcomas of childhood.

Based upon this finding, recent cloning of the t(11;22) region has been possible by molecular genetic approaches, leading to the detection of a number of sequences involved in the breakpoint. The translocation alters the open reading frame of a gene on chromosome 22, by substituting a sequence which encodes a human homologue of the murine FLI-1 gene, on chromosome 11q24, with a second unknown gene (EWS) on chromosome 22q12. The cloning and sequencing of complementary DNA

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from these fusion transcripts facilitates the use of molecular genetics in the identification of the t(11;22) in this family of neoplasms. As a direct consequence, the identification of fusion transcripts involved in the translocation has led to the detection of at least three types of chimaeric transcripts. These products differ from EWS protein by substitution of the RNA binding domain by the ETS-homologous DNA binding domain of human FLI-1 [18].

A limited number of cases of these neoplasms (Es and pPNET) has already been examined with this methodology, and the presence of such breakpoints has been confirmed, together with the t(11;22) chromosomal abnormality [18–20], but still the number of cases tested is limited and, therefore, further confirmation is necessary before introducing this novel methodology into routine diagnostic procedures.

Based upon extensive experience in the biology of this family of tumours, we have undertaken such a study comparing conventional Es versus pPNET and other small round cell sarcomas (rhabdomyosarcoma, neuroblastoma, microcellular and conventional osteosarcoma), as well as a number of soft tissue sarcomas (malignant fibrohistiocytomas, leiomyosarcomas and synovial sarcomas).

In the present study, we have performed reverse transcriptase polymerase chain reaction (RT-PCR) using oligonucleotide primers derived from EWS and FLI-1 sequences and total RNA of the putative malignant tumours (primary sarcomas and xenografts), comparing results with a cell line already tested as positive (Tc-71) for the transcript [20].

MATERIALS AND METHODS

For our study, we selected a series of 30 primary tumours and nude mice xenografts (Table 1). Most of these cases have been previously identified and the morphological, biological and immunohistological characteristics described previously [21–26].

The histological diagnosis was supported by electron microscopy in all cases. The following groups were distinguished:

Conventional Es

Three cases belong to this tumour type (cases 1, 2, 6). Histologically, the tumour showed a homogeneous cell pattern with sparce stroma and isolated vessels. The reticular network was non-existent and exclusively limited to perivascular fields. The cells were small, regular in size and homogeneously distributed. Glycogen content was abundant. Immunomarkers showed constant positivity for vimentin, neuron-specific enolase (NSE) or HNK-1 (Leu-7). Furthermore, we tested antibody HBA-71, which recognises epitope P30/32 MIC 2 [27, 28], and it proved positive in all cases. At electron microscopy, no neural differentiation was observed; no neurofilaments, neurotubules, or neurosecretory granules were found.

Atypical Es with neuroectodermal features (pPNET)

Four cases were tested; three were soft tissue tumours (cases 3–5) and the fourth was located in bone (case 7); the latter presented large cells. Microscopical differences compared to the first group were mainly the presence of pseudorosettes of Homer-Wright type, composed of groups of six to 10 cells centrally oriented toward a core, and nuclei with more condensed chromatin, prominent nucleoli and spindle contour. The reticular framework was better developed with a basket-like, lobular distribution. Moreover, a fibrillary background was found

between the cells. At electron microscopy, neural differentiation was confirmed by the presence of neurotubules, neurosecretory granules (membrane covered with a dense core) and synaptic-like figures. Immunohistochemically, all tumours showed vimentin positivity as well as NSE and HNK-1 (Leu 7). The antibody HBA/71 for MIC-2 was positive in all cases [22]. The three soft tissue cases expressed neural differentiation when induced in vitro [23].

Other small round cell tumours

For comparative purposes, we tested a number of other neoplasms with defined morphology.

Two small round cell tumours with rhabdomyogenuous differentiation (RMS): one was a solid variant of alveolar rhabdomyosarcoma with isolated rhabdoblasts and positive expression of desmin and NSE, but failed to express HBA/71. At electron microscopy, striated fibrilles were seen (case 13). The second RMS showed embryonal differentiation (case 14).

Six osteogenic sarcomas with diverse degrees of osteogenesis were also tested. One case (case 21) was a small anaplastic osteosarcoma with Ewing-like fields: vimentin and NSE were positive but HBA/71 was negative. Osteonectin and osteocalcin antibodies were present in isolated cells. By electron microscopy, the presence of osteoide material confirmed the osteogenic potentiality of these cells [24]. Osteogenic maturation was found to varying degrees in the other five neoplasms, which was also confirmed by electron microscopy (cases 18–20, 22–23).

Neural neoplasms

These were also included for comparative purposes: one was an esthesioneuroblastoma with typical Homer-Wright rosettes and focal microcalcifications. Neurosecretion was confirmed by positivity with NSE, and some cells expressed S-100 protein (case 9) [25].

A second case was a retinoblastoma (case 10), also having neural rosettes of Homer-Wright and typical neuroblastic differentiation. Immunohistochemistry showed NSE, HNK-1 positivity but absence of HRA-71. Electron microscopy confirmed the neural nature of this tumour.

An immature teratoma of the testis, displaying neuroectodermal differentiation with clusters of Homer-Wright rosettes and medulloblastoma-like figures, was also included in this analysis (case 11) [26].

Case 12 was a Schwannoma of soft tissue displaying fascicular Schwann cells oriented in bands. S-100 antigen was present, but no NSE, HNK-1 or HBA-71.

A primitive sarcoma with biphasic differentiation of small round cell type, primary of soft tissue, was considered an ectomesenchymoma (case 8). The cells expressed both desmin and myoglobin as well as NSE and HNK-1. Electron microscopy revealed neurosecretory granules, but no rhabdomyofilaments were observed.

A number of soft tissue tumours of histiocytic, fibroblastic and leiomyomatous nature were also tested as controls [21]. None of these cases posed differential diagnostic problems in terms of a pPNET or Es of soft tissue; limited experience with this technology prompted us to study them (cases 15–17, 24–30). For positive control purposes, we used a cell line (Tc-71) which had been previously tested for the presence of the t(11;22) and its breakpoint expression [19, 20].

Total cellular RNA was extracted with the guanidine isothiocyanate-caesium chloride method [29]: cells and tissues were homogenised in 5 volumes of 4.0 M guanidinium thiocyanate,

25 mM sodium citrate, 0.5% sarcosyl and 0.1 M β-mercapto-ethanol in a tissue homogeniser. After clarification, the homogenate was mixed with 1.6 volumes of caesium chloride and the resulting mixtures centrifuged in SW27 polyallomer tubes in a Beckman ultracentrifuge for 35 h, at 25 000 rpm. RNA migrating to the bottom was separated and ethanol precipitated. As described by Delattre and colleagues [18], RNA from the tumours was aliquoted to perform RT-PCR. Oligonucleotide 11A (5'-AGAAGGGTACTTGTACATGG-3') was used as a primer for the reverse transcription of 1 μg of total RNA using Gen Amp RNA PCR kit (Perkin Elmer). The resulting cDNA was subjected to 35 cycles of PCR amplification with primers 11.3 (5'-ACTCCCCGTTGGTCCCCTCC-3') and 22.3 (5'-TCCTACAGCCAAGCTCCAAGTC-3'), each cycle consisting of a denaturing step at 94°C for 30 s, annealing at 65°C for 1 min, and extension at 72°C for 2 min. The amplified fragment was identified by gel electrophoresis and ethidium bromide staining.

RESULTS

Table 1 and Figure 1 show results of the present analysis. The RNA isolated from the 30 tumours was subjected to RT-PCR, using primers derived from EWS and FLI-1 coding sequences flanking the EWS/FLI-1 breakpoint [18, 19]. The products of amplification were visualised by ethidium bromide stained agarose gels, and were seen in all Es cases as well as in the atypical Es with pPNET features. The line Tc-71 tested for

control proved positive. The presence of the three types of chimaeric transcripts were seen in the different cases of Es/pPNET. Case 7 (line 3, Figure 1b) revealed a chimaeric transcript with 329 bp; cases 1–4 and 6 (lines 2 to 6, Figure 1a) and control Tc-71 (line 7, Figure 1b) presented a band of 395 bp, and the 581 bp was seen in case 5 (line 2, Figure 1b). All the other types of neoplasms proved negative, although the ectomesenchymoma of soft tissue presented a variant aberration of chromosome 22.

Therefore a complete correlation was obtained between tumour type and detection of EWS/FLI-1 fusion transcripts. The group of Es/pPNET, located either in bone or soft tissue, showed the t(11;22), with varying degrees of evidence for neuroectodermal immunomarkers and the MIC-2/HBA-71 antibody expression, all presented the fusion transcripts, as did the cell line Tc-71, which served as control. Alternatively, none of the small round cell tumours of various origin (neuroectodermal origin included) presented any fusion transcript, nor did the other bone and soft tissue malignant neoplasms.

The specificity of the technique was effective in 100% of our cases, demonstrating extremely high accuracy for this technique.

DISCUSSION

As in other types of solid neoplasms, including lymphomas, the cloning of chromosomal abnormalities in small round cell sarcomas (Es and pPNET) is providing new insights for the

Table 1. Pathological and cytogenetic characteristics of the tumours

Cases	Diagnosis	22q abnormality	RT-PCR
1 PT	Ewing's sarcoma bone	Not done	+
2 PT	Ewing's sarcoma bone	Not done	+
3 XT	Ewing's sarcoma soft tissue	t(11;22)(q24;q12)	+
4 XT	Ewing's sarcoma soft tissue	t(11;22)(q24;q12)	+
5 XT	Ewing's sarcoma soft tissue	t(9;11;22;13)(p22;q24;q12;q22)	+
6 XT	Ewing's sarcoma bone	Not done	+
7 XT	Ewing's sarcoma bone	t(11;22)(q24;q12)	+
8 XT	Primary soft tissue sarcoma with neural and muscular differentiation	t(3;22;18)(p14-21;q12;q23)	−
9 XT	Esthesioneuroblastoma	None	−
10 XT	Retinoblastoma	None	−
11 PT	Immature teratoma (PNET testis)	None	−
12 PT	Schwannoma	Not done	−
13 XT	Phabdomyosarcoma	del 22(q12)	−
14 XT	Rhabdomyosarcoma	None	−
15 PT	Fibrohistiocytoma	Not done	−
16 PT	Fibrohistiocytoma	Not done	−
17 PT	Fibrohistiocytoma	Not done	−
18 PT	Osteosarcoma	Not done	−
19 XT	Osteosarcoma	t(22;?)(q12;?)	−
20 XT	Osteosarcoma	Not done	−
21 XT	Osteosarcoma	None	−
22 XT	Osteosarcoma	Not done	−
23 XT	Osteosarcoma	t(22;?)(q12;?)	−
24 PT	Leiomyosarcoma	Not done	−
25 PT	Leiomyosarcoma	Not done	−
26 XT	Leiomyosarcoma	Not done	−
27 PT	Synovial sarcoma	None	−
28 XT	Synovial sarcoma	None	−
29 XT	Synovial sarcoma	None	−
30 XT	Synovial sarcoma	None	−

PT, primary human tumour; XT, mouse xenograft; RT-PCR, reverse transcriptase polymerase chain reaction.

(a)

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

395 bp 2 ⟶

1 Marker ladder 100 (BRL)
2 Case 3 Es soft-tissue
3 Case 4 Es soft-tissue
4 Case 1 Es bone
5 Case 2 Es bone
6 Case 6 Es bone
7 Case 8 Primitive sarcoma
8 Case 10 Retinoblastoma
9 Case 13 Rhadomyosarcoma
10 Case 21 Osteosarcoma
11 Case 25 Leiomyosarcoma
12 Case 29 Synovial sarcoma
13 Control normal liver nude mice
14 Control (no RNA)
15 Marker ladder 100 (BRL)

(b)

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

581 bp 3
395 bp 2
329 bp 1

1 Marker ladder 100 (BRL)
2 Case 5 Es soft-tissue
3 Case 7 Es bone
4 Case 9 Esthesioneuroblastoma
5 Case 11 PNET testis
6 Case 12 Schwannoma
7 Control TC-71 Es cell line
8 Case 14 Rhabdomyosarcoma
9 Case 15 Fibrohistocytoma
10 Case 21 Osteosarcoma
11 Case 23 Osteosarcoma
12 Case 24 Leiomyosarcoma
13 Case 28 Synovial sarcoma
14 Control (no RNA)
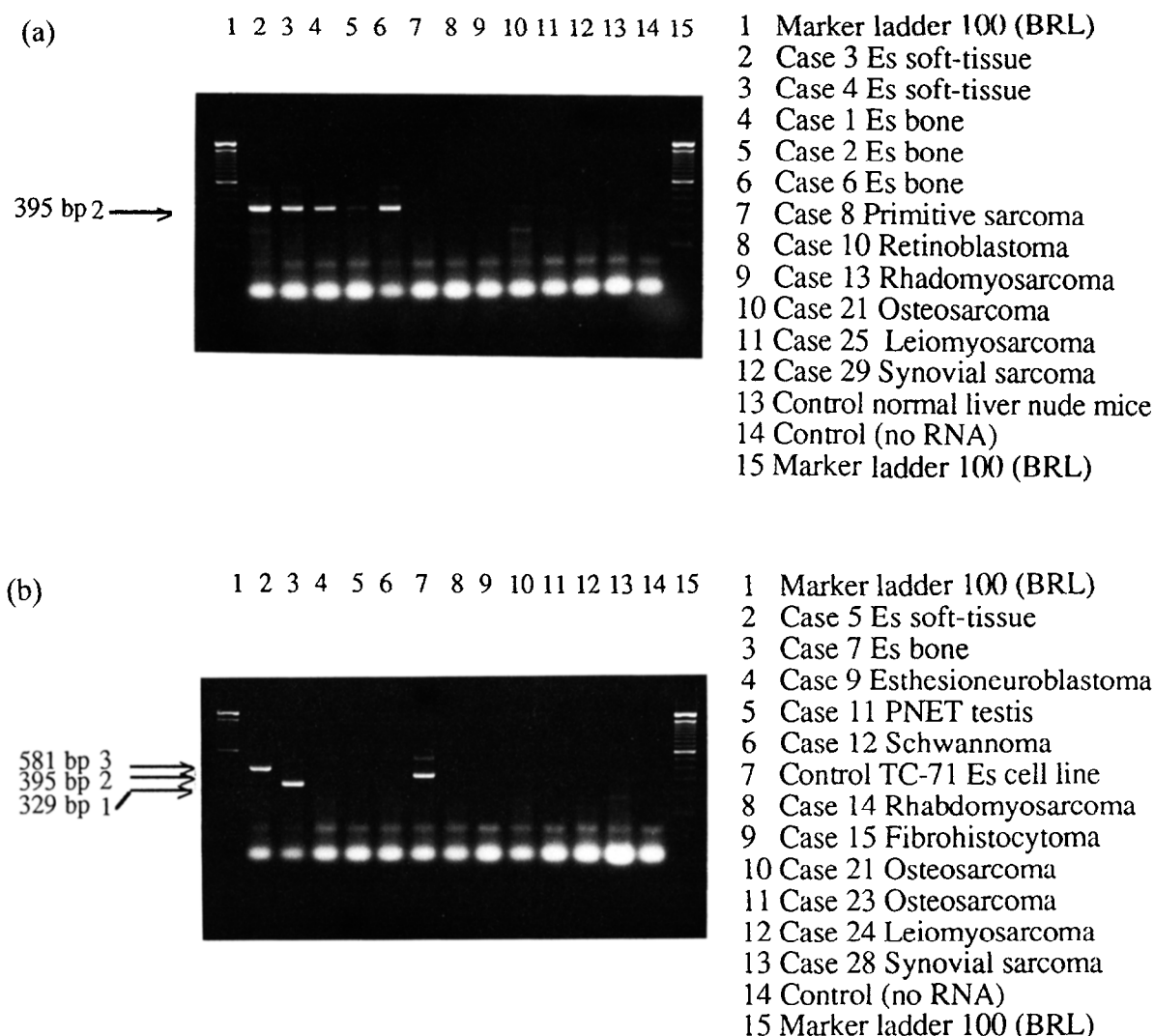15 Marker ladder 100 (BRL)

Figure 1. Identification of amplified transcripts EWS/FLI-1. (a) Reverse transcriptase polymerase chain reaction (RT-PCR) detection of one type of chimaeric transcript (2). (b) RT-PCR detection of three types of chimaeric transcripts (1, 2, 3).

histogenesis, biology and diagnosis of these tumours. Since the first communication of the cloned breakpoints on chromosome t(11;22)(q24;q12) by Delattre and colleagues [18], few new studies have confirmed this observation. Zucman and colleagues [19] demonstrated these breakpoints on 20 Es cases, using genomic probes. A chromosome 22 breakpoint was demonstrated in every tumour tested, whether the karyotype contained typical, complex or variant translocations, and was even detected in a normal karyotype case. Similar findings have been discussed by Sorensen and colleagues [20] on 10 PNET cell lines and 20 primary tumours: eight of the cell lines and 18 of the pPNET solid neoplasms possessed the fusion transcript detected by RT-PCR. Interestingly enough, both cell lines failed simultaneously EWS rearrangement and the Southern analysis. More exciting is the finding of these chimaeric transcripts in conventional Es and in pPNET, but not in any other type of neuroectodermal neoplasm so far tested ([20], Landanyi, personal communication).

The present findings provide further support to the value of this diagnostic test for the presence of EWS/FLI-1 fusion transcripts both in Es and in pPNET, a fact which reinforces the common biological, cytogenetical and morphological characteristics of both types of tumour. In all 7 cases tested (3 Es and 4

pPNET), we were able to confirm the presence of such a genomic abnormality together with the existence of a t(11;22)(q24;q12). One point of further interest is that cases 3–5 were atypical Es with neuroectodermal features of soft tissue location, and that following *in vitro* culture of the three cell lines, clear neuroectodermal markers as well as a neural phenotype were determined by electron microscopy [23]. This confirms the common biological nature of Es/pPNET, not only when located in bone but also in extraskeletal sites. As pointed out by several pathologists [1–3, 25] and by ourselves [1–3, 30–31], the Es family of neoplasms extends morphologically and biologically in a continuum from conventional Es, devoid of any neural traces, to pPNET, expressing a varied number of neural structures. A clear-cut distinction between this spectrum of tumours does not exist, neither genetically not with prognostic or therapeutic significance.

Another interesting finding in this analysis is the negativity for neuroectodermally derived tumours other than Es/pPNET. We could not detect positive results by RT-PCR on an esthesioneuroblastoma, a retinoblastoma and a Schwannoma. These three neoplasms have an accredited neuroectodermal origin but lack t(11;22) rearrangement. This was also the finding of Sorensen and colleagues [20] on a set of analogous neoplasms.

Furthermore, in the present study, a number of Es-like neo-plasms, such as small anaplastic osteogenic sarcoma and solid alveolar rhadomyosarcoma, proved negative, as did four conventional osteosarcomas which showed neither the chimaeric transcripts nor the t(11;22).

Case 8, a primitive soft tissue sarcoma with a t(3;22;18)(p14-21;q12;q23) and other clonic abnormalities, is noteworthy. The tumour did not express the EWS/FLI-1 fusion transcripts. This is somewhat in contrast to another malignant ectomesenchymoma [20] [which had the t(2;13)(q35;q14), a rearrangement typical of alveolar rhabdomyosarcoma, and a t(11;22)(q24;q12), typical of Es/pPNET], which have shown positive results by RT-PCR. The heterogeneity of these tumours [32] requires further classification, and should not be grouped within the Es family of sarcomas.

The availability of probes for the translocated breakpoint in Es/pPNET enhances the diagnostic accuracy for this intriguing group of tumours, complementing the existing positivity for MIC-2 antigen [21, 22], as detected by immunohistochemistry (antibody HBA-71), which shows a specificity in nearly 90% of cases. In this study, all tumours which expressed positivity for HBA-71 were also genetically translocated, involving ESW and FLI-1 genes; the potential diagnostic value because of its high level of specificity in this group of neoplasms is appparent, and represents a new complementary tool for the characterisation of the Es family.

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