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Original Paper

EWS-FLI1 Fusion Transcripts Identified in Patients with Typical Neuroblastoma

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The t(11.22)(q24.q12) results in expression of a chimeric RNA product, EWS-FLI1. This RNA product is expressed in over 85% of tumours belonging to the Ewing's family, and is increasingly used as a definitive characteristic of these tumours. In this study, we evaluated reverse transcriptase-polymerase chain (RT-PCR) for EWS-FLI1 fusion transcripts in 18 neurally derived small round cell tumours. These included six Ewing's family tumours and 12 neuroblastomas. EWS-FLI1 fusion transcripts were identified in all six Ewing's tumours, but also in two of the 12 neuroblastomas. One neuroblastoma contained the classic type 1 fusion transcript, and the second a type 1 transcript containing a 66 bp (base pair) insert that was not derived from the *EWS* or *FLI1* gene. The presence of EWS-FLI1 fusion products in RNA extracted from primary neuroblastoma suggests the identification of EWS-FLI1 fusion transcripts is not pathognomonic for tumours of the Ewing's family. The clinical significance of these fusion transcripts in neuroblastoma is not known.

Key words: EWS-FLI1 mRNA, RT-PCR, diagnosis, neuroblastoma, Ewing's, pPNET, small round cell tumours

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INTRODUCTION

THE t(11.22)(q24.q12) chromosomal translocation fuses the *EWS* gene from chromosome 22q12 to the *ETS* family member, *FLI1*, on chromosome 11q24. This results in expression of a chimeric RNA product, coding for a protein which contains the amino terminal of *EWS* fused to the *ETS* DNA-binding domain of *FLI1* [1, 2] (Figure 1). The chimeric protein expressed by this fusion gene is capable of transformation and appears to act as an abberant transcription factor [3].

This translocation has been identified by conventional cytogenetic analysis in over 85% of tumours belonging to the family of Ewing's tumours [4], and is increasingly used as a diagnostic indicator of tumours belonging to this family. Following identification of the EWS-FLI1 fusion transcript, a reverse transcriptase polymerase chain reaction (RT-PCR) product has formed the basis for a sensitive diagnostic test for tumours containing this translocation [5], and this has been used to define tumours of the Ewing's family [6].

In this study, we have used RT-PCR for the EWS-FLI1 fusion product to characterise a group of 18 neurally derived small round cell tumours, and report the identification of EWS-FLI1 fusion transcripts in tumours from 2 patients diagnosed with neuroblastoma.

MATERIALS AND METHODS

Total cellular RNA was extracted from all 18 characterised tumour samples as previously described [7]. RNA was extrac-ted from the tumour samples in a designated room. The amount and purity of recovered RNA was measured by optical density (OD) at 260 and 280 nm, and its quality confirmed by agarose gel electrophoresis and RT-PCR for the house-keeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

The tumours included six Ewing's and 12 neuroblastomas, diagnosed by conventional criteria including localisation of tumour, age of patient, immunohistochemistry and cytogenetics.

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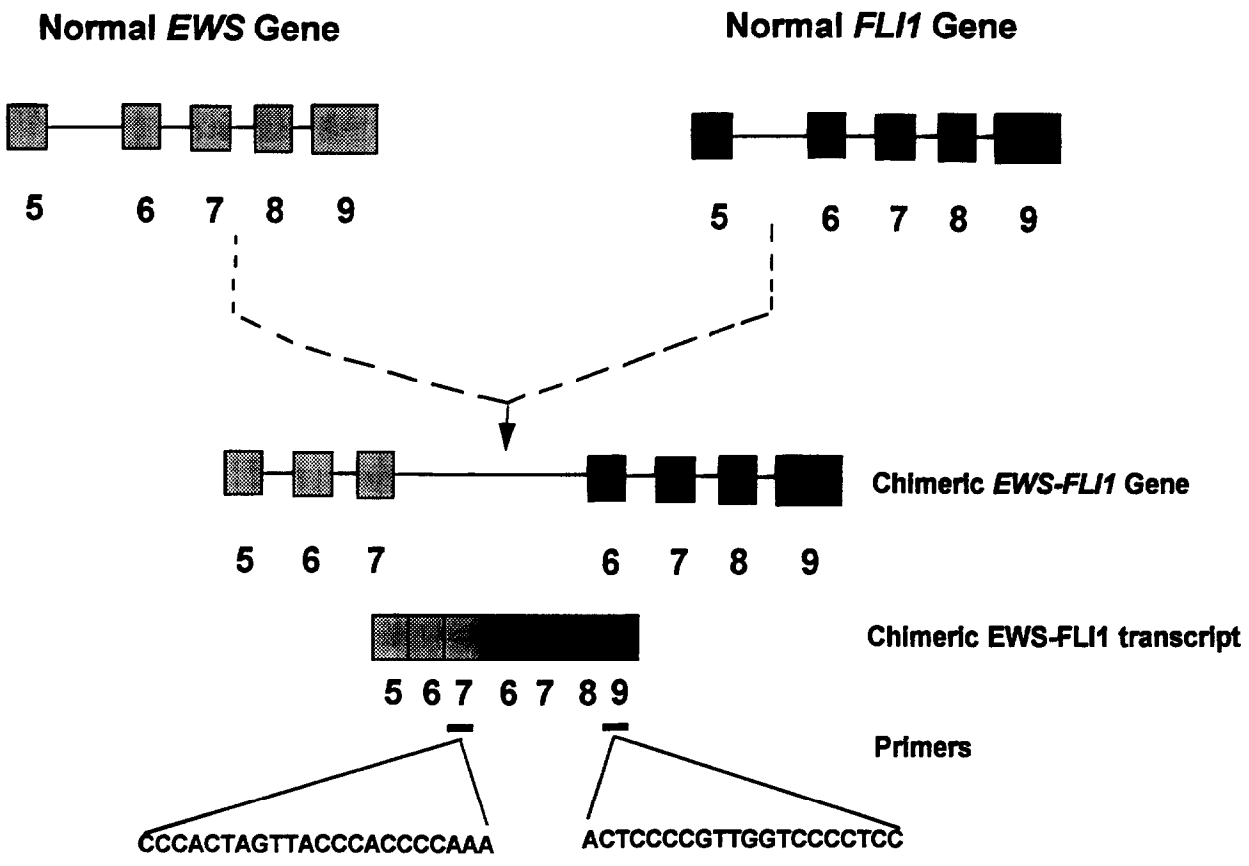

Figure 1. ***EWS-FLI1*** **gene fusion. The t(11.22)(q24.q12) fuses the** ***EWS*** **gene from chromosome 22q12 to the** ***ETS*** **family member,** ***FLI1*****. This results in expression of a chimeric RNA product, EWS-FLI1, which has been used to diagnose tumours belonging to the Ewing's family.**

REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION

The method of RT-PCR for EWS-FLI1 mRNA was based on that previously described for tyrosine hydroxylase [7]. RNA samples (1 μg) were denatured by heating to 95°C for 5 min prior to producing cDNA from extracted RNA, using reverse transcriptase enzyme and a random hexamer primer. Samples were PCR amplified for 40 cycles of 94°C × 1 min, 60°C × 1 min and 72°C × 1 min, followed by 10 min extension at 72°C. Samples were PCR amplified for EWS-FLI1 fusion products using selected primers [6] (Figure 1). For each sample, reverse transcriptase negative controls (RT-ve), in which reverse transcriptase enzyme was omitted, were included. Water negative controls (W) contained all components for the RT-PCR reaction but no target RNA. RNA extracted from cells cultured from a pPNET (peripheral primitive neuroectodermal tumour) (TC-32) known to contain the classic t(11.22)(q24.q12) was included as a positive control. RT-PCR reactions are set up in a designated room and products analysed in isolation from target RNA, RT or PCR reactions.

Amplified products were separated in a 1% agarose gel and stained with ethidium bromide. The identity of RT-PCR products was confirmed by sequence analysis (dideoxy chain-termination method). RNA samples were extracted from a minimum of two tumour samples from each patient, and each sample analysed by RT-PCR four times.

RESULTS

RNA was successfully extracted from all tumour samples, and RT-PCR for the house-keeping gene, GAPDH, generated a single 433 bp (base pair) band in all 18 (Table 1).

RT-PCR demonstrated the classic type 1 EWS-FLI1 fusion product in six of six tumours belonging to the Ewing's family (Table 1), and conventional cytogenetic analysis confirmed the presence of the t(11.22)(q24.q12) in three of these six tumours. Amplification of RNA extracted from the pPNET derived cell line TC-32, with a classic t(11.22)(q24.q12), generated a 310 bp band consistent with the type 1 EWS-FLI1 fusion product (Figure 2). RT-PCR amplified products were not seen in RT-ve or water negative controls.

RT-PCR analysis of RNA extracted from 12 neuroblastomas identified EWS-FLI1 fusion transcripts in two of the 12 (Figure 2; Table 1). The RT-PCR product amplified in

Table 1. *Summary of RT-PCR analysis for EWS-FLI1 fusion transcripts*

	EWS-FLI1 fusion transcript Positive	EWS-FLI1 fusion transcript Negative	GAPDH mRNA Positive
Neuroblastoma (n = 12)	2	10	12
Ewing's/pPNET's (n = 6)	6	0	6

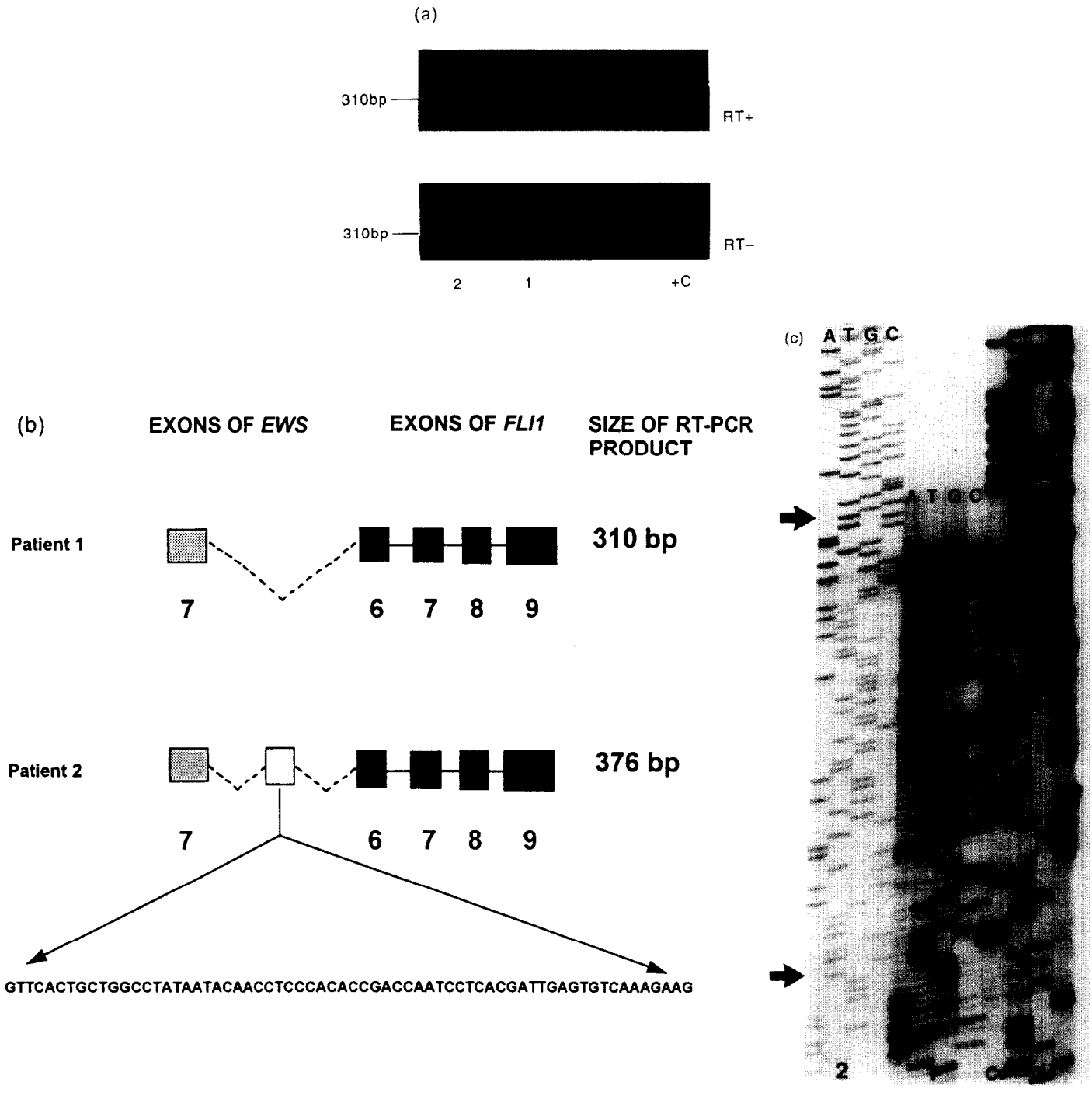

Figure 2. EWS-FLI1 fusion products in RNA from neuroblastomas. (a) RT-PCR products identified in RNA from two neuroblastomas (lanes 1, 2). (b) The 310 bp product was a type 1 fusion product, the 376 bp product a type 1 product containing a 66 bp insert (insert sequence shown is sense). (c) Sequence analysis in the region of the 66 bp insert (antisense sequence was read between the two arrows). C, positive control; RT, negative control.

RNA from the neuroblastoma of patient 1 was 310 bp and of patient 2, 376 bp (Figure 2a). Tumour material from patient 1 was primary tumour removed after six courses of therapy and primary tumour from patient 2 at the time of diagnosis before therapy.

Sequence analysis confirmed the identity of the 310 bp product to be the previously described type 1 EWS-FLI1 transcript. In sample 1, the cDNA EWS sequence was interrupted between the first and second bases of codon 265 (A/GT), and in FLI1 cDNA a break between the first and second base of codon 219 (G/AC) was found (Figure 2a). In the second patient sample, with a product size of 376 bp, the breakpoints in EWS and FLI1 cDNA were identical to those in the sample from patient 1. The 66 bp insert found between these two breakpoints did not match to *EWS* or *FLI1* sequences, but did have a high

degree of sequence similarity with human ERGB transcription factor (98%, determined by comparative sequence analysis using FastA).

Clinical details of the 2 patients with neuroblastoma containing EWS-FLI1 fusion transcripts are given below.

Patient 1 was a 3-year-old boy who presented with a 3 month history of fever, general malaise, intermittent vomiting, bone pain, jaundice and bruising. He developed a unilateral proptosis a few weeks prior to presentation. Clinical examination revealed a very ill child with pallor, proptosis and an adrenal mass. A radiological assessment with CT scan demonstrated a 3×2 cm left adrenal mass without obvious calcification. There were a number of opacities seen in both lung fields and there was a soft tissue mass in the right orbit.

There was marked elevation of urinary catecholamines (HMMA (hydroxymethylmandelic acid) 130 μmol/mmol creatinine, HVA (homovanillic acid) 72 μmol/mmol creatinine and dopamine 3.9 μmol/mmol creatinine) above the normal range for children 3–14 years of age (HMMA 10–15 μmol/mmol creatinine, HVA 12–16 μmol/mmol creatinine, dopamine 0.5–0.8 μmol/mmol creatinine) [8]. Serum ferritin was markedly elevated and trephines demonstrated involvement with clumps of malignant blast cells. An [^{123}I]MIBG scan demonstrated uptake in the primary tumour and all known sites of metastatic disease. Isotope bone scan demonstrated patchy uptake in the skull vault and orbit. Bone marrow chromosomes were apparently normal, and there was no evidence of N-myc amplification by Southern blot or FISH (fluorescent *in situ* hybridisation) analysis.

He was treated according to the European Neuroblastoma Study Group Stage 4 Protocol, with a combination of chemotherapy including vincristine, carboplatin, cisplatin, etoposide and cyclophosphamide (OPEC/OJEC). He had a partial response to treatment and after six courses of therapy had surgical removal of his primary adrenal tumour, which, on pathological examination, contained viable neuroblastoma with focal ganglioneuromatous differentiation. Immunohistochemical analysis demonstrated this tumour was positive for tyrosine hydroxylase* (TH), neurone-specific enolase* (NSE), protein gene product 9.5* (PGP 9.5) and NB84* but negative for MIC-2*, consistent with a diagnosis of neuroblastoma [9]. He resumed chemotherapy, but just as he was due to complete his final course of therapy, his disease recurred in the bone and bone marrow. Urinary catecholamines were once again elevated. After discussion with his parents, he was treated with palliative care alone and he died within 2 months of relapse.

Patient 2 was a $5\frac{1}{2}$-year-old boy, who presented with a short history of vomiting, diarrhoea, increasing abdominal pain and, on examination, had a large adrenal mass. Radiological examination of his abdomen with plain X-ray and CT scan demonstrated a large calcified left adrenal mass with a large para-aortic nodal mass crossing the mid line, displacing the kidney downwards and encircling the aorta.

Urinary catecholamines were elevated (HMMA 22 μmol/mmol creatinine, HVA 42 μm/mmol creatinine, dopamine 0.94 μm/mmol creatinine) above the normal range. Isotope bone scan and bilateral bone marrow aspirations and trephines were negative for evidence of tumour involvements but a diagnostic [^{123}I]MIBG scan showed marked uptake within the primary tumour and abdominal nodal disease, consistent with stage 3 disease. Histology of the primary tumour at the time of diagnosis showed poorly differentiated neuroblastoma consisting of neuroblasts with numerous mitoses in a loose fibrillary neurophil stroma, with occasional rosettes. The cells were positive for TH, NSE, PGP 9.5 and NB84 but did not express MIC-2, consistent with a diagnosis of neuroblastoma [9]. Focal expression of choline acetyltransferase* was recorded. Cytogenetic analysis demonstrated a complex hyperdiploid karyotype with a chromosome count of between 50 and 52. Abnormalities appeared to be mostly numerical, including trisomy, with a structural change in the long arm of chromosome 1. No evidence of double minutes were seen and there was no evidence of N-myc amplification on Southern blotting. Cytogenetic characterisation was incomplete due to poor quality of the chromosomes.

He • was treated according to the European Neuroblastoma Study Group recommendations for patients with Stage 3 Neuroblastoma, with OPEC/OJEC. His disease responded to treatment with marked tumour shrinkage. However, just prior to excision of the treated adrenal tumour, he developed localised and systemic disease, and relapsed with an increase in urinary catecholamines (HMMA 59 μm/mmol creatinine, HVA 40 μmol/mmol creatinine, dopamine 0.6 μm/mmol creatinine). Despite attempts at rescue chemotherapy, his disease progressed rapidly and he died within 2 months of relapse.

Staging of disease was according to International Neuroblastoma Staging System [10].

DISCUSSION

Molecular analysis of tumours for EWS-FLI1 fusion products was simple and rapid to perform, and informative results were obtained in all tumour samples analysed. In all six Ewing's tumours analysed, the RT-PCR product was consistent with the type 1 fusion product [6]. Failure to identify different fusion transcripts as previously described [1, 2, 5, 6] most likely reflects the small cohort of tumours analysed. In three pPNET patient tumour samples, RT-PCR identified EWS-FLI1 fusion transcripts where conventional cytogenetics had failed to identify the characteristic t(11.22)(q24.q12). EWS-FLI1 transcription products have been consistently identified in RNA extracted from tumour material taken from 2 patients diagnosed with neuroblastoma. The fusion product from patient 1 would result from an inframe junction between *EWS* on chromosome 22 and *FLI1* on chromosome 11. This product was identified as a type 1 EWS-FLI1 fusion product [6]. It is not known if the fusion product protein is expressed in this tumour. The RT-PCR products from both patients would suggest the breakpoints in *EWS* and *FLI1* were identical in both patients, although RT-PCR of tumour from patient 2 generated a type 1 transcript that contained 66 bp of additional ma-

*Antibodies to NSE and MIC-2 were from DAKO Ltd, Bucks, U.K.; PGP 9.5 from Biogenesis Ltd, Poole, U.K.; TH and choline acetyltransferase from Chemicon, Quadratech, Surrey, U.K.; NB84 from Nova Castra, Vector Laboratories, Peterborough, U.K.

terial. The inclusion of 66 bp at the identified breakpoint would result in the insertion of 22 additional amino acids. This 66 bp sequence did not match the known *EWS* or *FLI1* sequences, but had a high degree of homology with the human ERGB transcription factor. This might suggest a complex translocation between chromosomes 11, 22 and 14, since ERGB transcription factor is localised on chromosome 14 [11]. This could not be confirmed by conventional cytogenetics. Alternatively, the inserted sequence may code for an unidentified protein with a high degree of homology to ERGB. The sequence amplified in tumour tissue from patient 2 showed 97% homology to the previously described sequence reported in the Ewing's derived IARC-EW11 cell line [11]. What role this translocation might have in the biology of this and related tumours, and whether a protein product is transcribed is not known.

EWS-FLI1 fusion transcripts have been reported in 2/4 undifferentiated, non-catecholamine secreting neuroblastomas [6]. Re-evaluation of these two sarcomas demonstrated MIC-2 expression and the tumours were reclassified as Ewing's sarcoma [6]. In this same study, 20 catecholamine producing neuroblastomas were negative for EWS-FLI1 fusion transcripts [6]. Our study is the first report of EWS-FLI1 fusion transcripts in catecholamine producing MIC-2 negative neuroblastomas. Previous studies have identified the t(11.22)(q24.q12) in tumours with evidence of rhabdomyogenic differentiation [12]. These observations suggest EWS-FLI1 fusion transcripts are not pathognomonic for tumours belonging to the Ewing's family as previously described [1, 2, 6, 7]. We are currently analysing the neuroblastomas by FISH and *in situ* RT-PCR for t(11.22)(q24.q12) and EWS-FLI1 fusion transcripts, respectively, to confirm our findings. *In situ* RT-PCR will be particularly informative, allowing localisation of fusion transcripts to specific cells and identification of the proportion of tumour cells containing the EWS-FLI1 mRNA.

The identification of EWS-FLI1 chimeric products in neuroblastomas suggests there may be a subset of patients with tumours that are of mixed phenotype. Identification of mixed phenotype soft tissue sarcomas in children has been previously described using immunohistochemical and cytogenetic analysis [12]. These observations imply a developmental relationship, and intimate a pluripotent neural crest precursor capable of differentiating along multiple lineages [13, 14]. The significance of mixed phenotype on disease progression and patient outcome is not known, although both patients described in this report had aggressive malignant disease and have subsequently died. We are currently examining the wider significance of these findings in a larger patient group and evaluating the relative value of molecular and histological characterisation of small round cell tumours in predicting clinical behaviour and patient outcome.

In summary, EWS-FLI1 fusion transcripts have been consistently identified in neuroblastomas from 2 patients. This observation suggests the presence of EWS-FLI1 fusion transcripts is not pathognonomic of Ewing's sarcoma or pPNETs. Analysis of small round cell tumours for EWS-FLI1 fusion transcripts by RT-PCR is a useful aid to the characterisation of these tumours, but interpretation of the results should always be made in conjunction with additional pathological and biological data. The implications of these observations for classification and clinical management of patients remains to be seen.

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