

Type 1 (11; 22)(q24: q12) translocation is common in Ewing's sarcoma/peripheral neuroectodermal tumour in south Indian patients

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The Ewing's sarcoma family can present diagnostic difficulties. In the past the basis of diagnosis has been an exclusion. Identification of a specific translocation especially t(11; 22) (EWS-FLI 1 fusion gene), which is seen in nearly 85% of Ewing's sarcoma cases can help in precise diagnosis. We have carried out a study on twenty patient samples diagnosed to have Ewing's sarcoma/peripheral neuroectodermal tumour (PNET)/small round cell malignant tumour. The study involved RT-PCR analysis for the fusion transcript, followed by sequencing to identify the specific type of fusion. Ninety percent (18/20) of the samples tested were found to be t(11; 22) translocations involving EWS-FLI 1 genes. Sixty-one percent (11/18) were found to be type 1 fusion and seven were type 2 (39%). This is the first study in India with quantitative information about the types of EWS-FLI 1 translocations present in Ewing's family of tumours in south Indian patients.

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1. Introduction

The term small round cell malignant tumour (SRCMT) traditionally describes a group of undifferentiated pediatric sarcomas that include Ewing's sarcoma, neuroblastoma, lymphoma, desmoplastic small round cell tumour and rhabdomyosarcoma, which represent about 15% of all childhood cancers. Cytogenetic studies have revealed specific chromosomal translocations in these SRCMTs. The translocations break specific genes in the involved chromosomes and create novel chimeric genes that encode a fusion protein with a different and unique function (Aurias *et al* 1983; Zucman *et al* 1993; Sorensen *et al* 1994).

Ewing's sarcoma is a small round cell tumour that shows limited neural differentiation typically arising within bones in children and adolescents. It is closely related pathologically to peripheral neuroectodermal tumour (PNET) that shows more definite neural features (de Alava *et al* 1998). Ewing's sarcoma accounts for approximately 6%

to 10% of malignant bone tumours and is the second most common malignant bone tumour of children and young adults (Delattre *et al* 1994). Approximately 85% of Ewing's sarcoma/PNET harbour the translocation t(11; 22) (q24: q 12) (Dowing *et al* 1993; Zucman *et al* 1993). At the molecular genetic level the chromosome 22q12 breakpoints are clustered within a single gene designated EWS and chromosome 11q24 breakpoints are within a gene called FLI 1. In this rearrangement, the 3' portion of FLI 1 oncogene is juxtaposed to the 5' portion of the EWS, thereby creating a functional EWS-FLI 1 fusion gene. As a consequence of this rearrangement EWS/FLI 1 transcript is expressed from the EWS gene promoter. In a small subset of Ewing's sarcoma (about 10%), the EWS gene is joined to the ERG gene that is closely related to FLI 1 but is located on chromosome 21 (Sorenson *et al* 1994). There are also other translocations, which result in EWS-ETV1 {t(7; 22)}, EWS-E1AF {t(17; 22)} and EWS-FEV {t(2; 22)} fusion transcripts but they are rare (de Alava and Gerald 2000). Zucman *et al* (1993) de-

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scribed different types of in-frame and out-of-frame EWS-FLI 1 chimeric transcripts found in Ewing's sarcoma/PNET cases. The two most common fusions join EWS exon 7 in frame with either exon 6 (type 1) or exon 5 (type 2) of FLI 1 gene. Apart from aiding in diagnosis, the RT-PCR based approach can help in better staging (Peter *et al* 1995; Pfliederer *et al* 1995) and can provide prognostic information (Zoubek *et al* 1996; de Alava *et al* 1998). In the present study we analysed the presence of t(11; 22) translocation and the type of fusion in south Indian patients diagnosed to have Ewing's sarcoma/PNET/SRCMT.

2. Materials and methods

2.1 Sample collection

The current study included 20 biopsy samples from cases suspected to have SRCMTs. The initial histological diagnosis made by the pathologists revealed that out of the 20 cases, 15 were Ewing's sarcoma, 3 were PNET, one was a desmoplastic SRCMT and one was SRCMT for which a definite diagnosis could not be made. One case of spindle cell sarcoma was also included in this study as a negative control for each experiment.

2.2 Oligonucleotide primers

Primers and probes used are listed in table 1.

2.3 RNA Isolation and RT-PCR

Total RNA was isolated from snap frozen tumour tissues using Trizol reagent (GIBCO BRL, Germany) according to manufacturer's recommendations. One microgram of total RNA was reverse transcribed into cDNA using random hexamers at 37°C for 90 min followed by 95°C for 5 min. Two µl from the reaction was PCR amplified using Abl primers (forward primer-5'-GGCCAGTAGCATCTG ACTTTG-3'; reverse primer-5'-ATGGTACCAGGAGTG TTTCTCC-3') in a 20 µl reaction volume containing 10 pmol each of the forward and reverse primer 200 µM of dNTP mix, 0.2 U of Taq (Amersham Biosciences UK),

2 µl 10 × PCR buffer, to check for the quality of the RNA isolated from each sample. Subsequently, 40 cycles of RT-PCR reactions were performed with each specific primer pairs (EWS 22.3 and FLI 11.3; EWS 22.8 and ERG 11). PCR conditions were as follows: 94°C for 30 s, 65°C for 1 min and 72°C for 1 min. Amplified PCR products were checked in 2% agarose gel. One positive control t(11; 22) sample, one spindle cell sarcoma as negative control and one water only (no cDNA) negative control were included in each run.

2.4 Slot blot analysis

PCR products were transferred to the nylon membrane with the help of slot blot apparatus (Hoefer). The nylon membrane was hybridized to biotinylated FLI 1 probe at 55°C overnight. ABC complex/HRP Kit (Dako, Denmark) was used to detect the hybridization of probe (Levy and Herrington 1995).

2.5 Sequencing of PCR products

PCR products were run and extracted from 2% agarose gel and purified using gel extraction kit (Qiagen, GmbH, Germany). Purified products were re-amplified and direct sequencing was done by cycle sequencing using Big Dye Terminator Kit V 3.0 (Applied Biosystems, Foster City, CA, USA) with both forward and reverse primers and then loaded onto ABI 310 Genetic Analyser. Sequencing analysis was done to check the break points of each fragment.

2.6 CD 99 immunohistochemistry

This was done using the CD 99 monoclonal antibody (Dako, Denmark) using a three layered ABC technique (Vijayalakshmi *et al* 2002).

3. Results

Biopsy samples were collected from patients ($n = 20$) whose clinical and radiological data were compatible

Table 1. Primer and probe sequences.

Primer/probe	Sequences
EWS 22.3	5' TCC TAC AGC CAA GCT CCA AGT C 3' (Peter <i>et al</i> 1995)
FLI 11.3	5' ACT CCC CGT TGG TCC CCT CC 3' (Naito <i>et al</i> 2000)
EWS 22.8	5' CCC ACT AGT TAC CCA CCC CAA A 3' (Peter <i>et al</i> 1995)
ERG 11	5' TGT TGG GTT TGC TCT TCC GCT C 3' (Peter <i>et al</i> 1995)
FLI 1 probe	5' TGC CAC AGC TGGATCTG 3' (Pfliederer <i>et al</i> 1995)

with a diagnosis of Ewing's sarcoma/PNET or SRCMT. Pathological examination revealed fifteen Ewing's sarcoma, three PNET, one desmoplastic SRCMT and one SRCMT for which further classification was difficult to make. RNA was isolated and RT-PCR was performed with ABL oligonucleotide primer to check for the quality of the RNA isolated from each sample, which amplified a 300 bp product. The EWS-FLI 1 product was either 330 bp (type 1 fusion) or 410 bp (type 2 fusion). Eighteen of the twenty samples tested were found to be positive for EWS-FLI 1 translocation (90%); of the 18 cases that

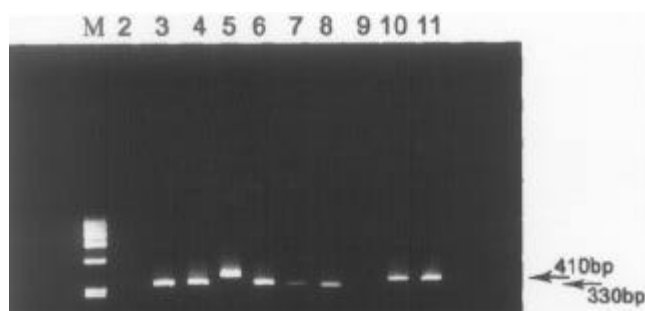


Figure 1. RT-PCR analysis of Ewing's sarcoma/PNET cases. Lane 1, marker; lane 2, negative control; lanes 3, 4, 6, 7 and 8, type 1 EWS-FLI 1 transcript; lanes 5, 10 and 11, type 2 EWS-FLI 1 transcript; lane 9, absence of EWS-FLI 1 transcript.

were histopathologically diagnosed as ES/PNET, 15 were found to have the EWS-FLI 1 translocation (83%). The case diagnosed as desmoplastic SRCMT and the SRCMT for which no further opinion was possible, both showed EWS-FLI 1 translocation (figure 1). None of the cases showed EWS-ERG fusion transcript in the present study (data not shown). Amplified PCR products were hybridized with FLI 1 specific probe for further confirmation and all positive cases were found to hybridize to the FLI 1 probe (figure 3). PCR products were taken up for direct sequencing.

Sequencing of amplified product revealed two different EWS-FLI 1 in-frame junctions, which results in the fusion of exon 7 of EWS with either exon 5 (type 2) or exon 6 of FLI 1 (type 1). In our study 61% (11/18) cases showed type 1 translocation and 39% (7/18) showed type 2 translocation (figure 2). The presence or absence of fusion transcript was also correlated with clinico-pathological findings (table 2). Interestingly, type 1 fusion was found to be associated with non-metastatic disease (8/10) (80%) and type 2 fusion more commonly with metastatic disease (5/8) (63%). However, this did not reach statistical significance, possibly due to the smaller numbers of cases.

CD 99 was found to be positive in 14/18 (78%) cases; the four cases that were negative included the desmoplastic SRCMT and the case diagnosed as SRCMT for which no further opinion was possible.

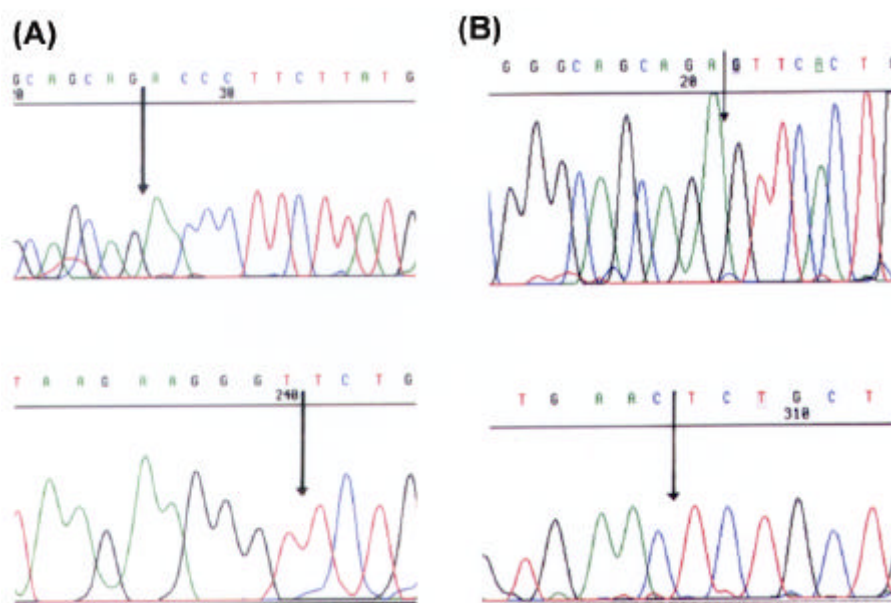


Figure 2. Sequence electropherogram of type 1 EWS-FLI 1 fusion transcript (A) and type 2 EWS-FLI 1 fusion transcript (B) showing the break point region (arrow). The top panel is the forward reaction sequence and the lower panel is the reverse reaction sequence.

4. Discussion

The precise classification of small round cell malignant tumours occasionally is very difficult, especially in case of unusual location, recurrences or poorly differentiated cases. Presence of fusion genes resulting from a chromo-

somal translocation is a typical event in small round cell tumours. In addition to providing important insight into our understanding of molecular mechanism of tumour development, the identification of such genetic alterations has practical value in tumour management.

In the current study, we explore the use of RT-PCR assay for detecting the fusion transcript and the types in Ewing's sarcoma/PNET. The results demonstrated a strong concordance between molecular diagnostic methods and standard histopathologic diagnosis in all Ewing's sarcoma cases. Two cases showed discordance with a histopathologic diagnosis of Ewing's sarcoma but a negative RT-PCR based result. It is possible that these tumours might contain variant translocations, such as t(17; 22) or t(7; 22) etc, that could not be detected by the primer combinations we used. Alternatively, there might have been a sampling error in the small biopsy material provided resulting in unrepresentative material being processed (Naito *et al* 2000). Another discordant case was the identification of EWS-FLI 1 translocation in the tumour classified as desmoplastic small round cell sarcoma. This tumour has been described to have t(11; 22) involving EWS and WT 1 gene (Ladanyi and Gerald 1994). However, one report by Katz *et al* (1997) described EWS-FLI 1 gene fusion, in a tumour with mixed desmoplastic SRCMT and Ewing's sarcoma. Our result could suggest a common histogenetic origin for these tumours.

Several lines of experimental evidence suggest that the EWS-FLI 1 fusion initiates and maintain tumorigenesis

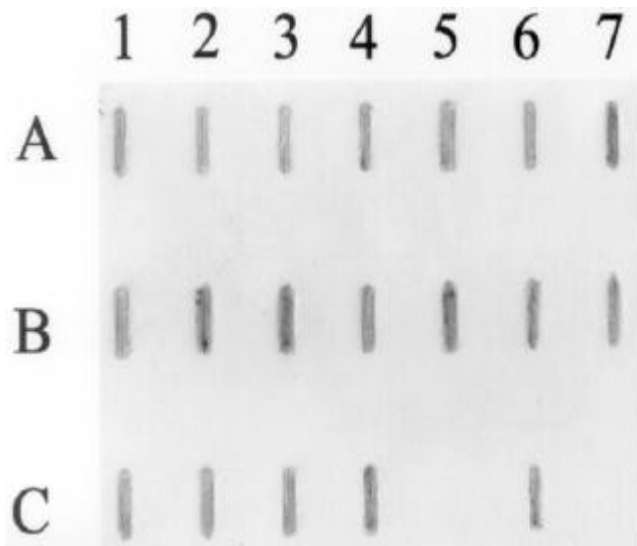


Figure 3. Slot blot analysis of Ewing's sarcoma with FLI 1 specific probe. Fifth and sixth sample in row 'C' are the PCR negative and positive control, respectively and seventh sample in the same row shows the absence of EWS-FLI 1 translocation.

Table 2. Clinico-pathologic characteristics of patients studied.

Age in years	Sex	HPE	Stage	Site	EWS-FLI 1 translocations present	CD 99 marker status
7	F	ES	NM	Radius	Type 1	-
16	M	ES	NM	Femur	Type 1	-
10	M	ES	NM	Humerus	Type 1	Not done
14	F	ES	NM	Mandible	Type 2	+
18	F	Desmoplastic SRCMT	NM	Retro-peritoneum	Type 2	-
10	F	ES	NM	Maxillary antrum	Type 1	+
3	M	ES	NM	Fibula	Type 1	+
55	M	PNET	NM	Sternum	Type 1	+
20	F	ES	NM	Fibula	-	+
8	F	PNET	NM	Mandible	Type 1	+
2	M	PNET	NM	Foot	Type 1	+
3	M	ES	NM	Chest wall	-	+
19	F	ES	Me	Femur	Type 2	+
36	M	ES/PNET	Me	Fibula	Type 2	Not done
14	M	ES	Me	Humerus	Type 2	+
22	M	SRCMT	Me	Femur	Type 1	-
13	M	ES	Me	Tibia	Type 2	+
14	F	ES	Me	Femur	Type 1	+
17	M	ES	Me	Fibula	Type 2	+
14	M	ES	Me	Femur	Type 1	+

HPE, Histopathology examination; M, male; F, female; ES, Ewing's sarcoma; NM, non-metastatic; Me, metastatic.

in ES/PNET (de Alava *et al* 1998). It is the most heterogeneous gene fusion in cancer. At the molecular level, there are several types of in-frame EWS-FLI 1 chimeric transcript, most of which have been observed *in vivo* and represent different combinations of exons from EWS and FLI 1 gene. The two most common fusion joins EWS exon 7 in frame with either exon 6 (type 1) or exon 5 (type 2) of FLI 1 gene (Zucman *et al* 1993). Type 1 fusion is more frequent (60–70%) than type 2, which is seen in 30% of cases. In our study also 62% of cases showed fusion of exon 7 of EWS with exon 5 of FLI 1 (type 1) and 38% showed fusion of exon 7 of EWS with exon 6 of FLI 1 (type 2). The analysis of EWS-FLI 1 chimeric transcript in Ewing's sarcoma/PNET is not only of molecular diagnostic interest but can also provide powerful independent prognostic information (de Alava *et al* 1998). Zoubek *et al* (1996) reported a possible association of the type 1 EWS-FLI 1 fusion with longer relapse free survival in a subgroup of 55 patients with localized Ewing's sarcoma/PNET. In our series, the type 1 fusion were more commonly detected in non-metastatic Ewing's sarcoma, whereas type 2 fusion were more common in the metastatic disease. This could possibly indicate the more aggressive behaviour of the tumours with the type 2 fusion. However, since the numbers are still few and since nearly 50% of non-metastatic Ewing's sarcoma patients studied are still on treatment, it is premature to comment on their prognostic significance.

Although cytogenetic analysis can detect a wide variety of chromosomal translocation, it is time consuming and the success rate is quite variable and is dependent on the success of short-term cultures. Only 50% of time, conventional cytogenetics can provide the answer. According to Barr *et al* (2002) and McNicol and Farquharson (1997), RT-PCR assays are more sensitive and have a higher technical success rate than standard cytogenetic and are comparable to fluorescence *in situ* hybridization (FISH). In our series only 4 of the 20 cases had a cytogenetic positivity, primarily due to difficulty in getting metaphase spreads.

CD99 is also a useful marker in the diagnosis of SRCMT (Stevenson *et al* 1994). Apart from being positive in Ewing's sarcoma/PNET family of tumours, CD 99 can also be positive in synovial sarcoma, mesenchymal chondrosarcoma, alveolar rhabdomyosarcoma (Granter *et al* 1996). In our series 78% (14/18) of the tumours were positive for CD99. The two cases that did not reveal a t(11; 22), were CD99 positive. The immunohistochemical evaluation for CD99 is a useful adjunct in the work up of a tumour diagnosed as a SRCMT.

This is the first study in India that gives information about the different types of EWS-FLI 1 translocations present in Ewing's sarcoma/PNET in south Indian patients. The data we presented in this report suggests analysis of

EWS-FLI 1 translocation will improve the reliability of diagnosis among small round cell tumour of patients and might provide prognostic information. A larger series will need to be studied to confirm the association of the different fusion types (type 1 versus type 2) with stage of disease.

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