Research Paper Management

The Molecular Pathology of Ewings Family of Tumors: Its Relevance to the Diagnosis and Prognosis in the Pediatric Age Group

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ABSTRACT

Background: Ewing's sarcoma family of tumours share the common genetic chromosomal translocation. Though the family of tumours vary according to their location one common fusion protein product of the translocation has been detected since last decade which is constantly associated with the Ewing's family of tumors. This aberrant expression of the genetic product alters the cell proliferation and differentiation which results in Ewing's sarcoma. In today's scenario the detection of this novel genetic specific translocation between chromosome 11 and chromosome 22 is useful in primary diagnosis and prognostic assessment of the patient. Therefore we conducted the present study to assess the patient's outcome after the diagnosis and survival.

Material & methods: Cases of solid tumors in the pediatric age group were included in the study. After relevant clinical details and radiological investigations, fine needle aspiration cytology smears and histopathology sections were evaluated. Special stains and immunohistochemistry were done in the required fields. Molecular analysis was done in selected cases of Ewing's sarcoma.

Results: A total number of 161 cases were studied .Out of 21 cases of Ewing's sarcoma, RT-PCR study was done in 10 cases to find out the molecular biological details. 90% cases showed presence of fusion gene. The patients were followed-up for a period of 2 years to assess their survival.

Conclusion:The diagnostic problem of distinguishing Ewing's sarcoma from other small blue round ell tumours is resolved due to RT-PCR. Also presence of EWS and FLI1 can relate to prognoss and therapeutic response.

KEYWORDS Ewing's tumor, molecular pathology, paediatric age, survival.

Introduction:

Ewing's family of tumors include Ewing's sarcoma (ES), Peripheral primitive neuroectodermal tumor (PNET) and Askin tumors. These are well known as 'Small blue round cell tumours', the most undifferentiated form still the most challenging for the pathologists [1,2]. The sites of affection are both in the bone and soft tissue. As it is most common in the paediatric age group and young adults, it should be differentiated from other small round cell tumours (SRCT) like neuroblastoma, rhabdomyosarcoma, lymphoma and germ cell tumours. Accurate diagnosis can be aided by special stains, immuonohistochemistry(IHC), and identification of specific genetic changes by reverse transcriptase polymerase chain reaction (RTPCR) and fluorescent in situ hybridisation (FISH).

Ewing's sarcoma, a well known highly aggressive osteolytic neoplasm of bone has a marked propensity for haematological dissemination. Hence rapid and accurate diagnosis is essential for early clinical intervention and management. The presence of large amount of cytoplasmic glycogen is not a diagnostic criterion since up to 35% of Ewing's sarcoma cases lack this finding[3]. Another surface marker CD99 or MIC2, a glycoprotein is also positive in other tumours which prove its limitations[4-6]. At molecular level the determination of balanced translocation involving t(11;22)(q24;12) is observed as a novel genetic marker in 83% of cases of Ewings sarcoma [4,7].

Phenotypically, all the tumors of Ewings family exhibit high level of MIC2 antigen expression and consistently bear the t(11;12)(q24;12). The nomenclature of these tumours is decided by depending upon their location and extent of neural differentiation: PNET, Askin tumour, peripheral neuroepithelioma or adult neuroblastoma[8-10]. Till date, though a number of publications are made in the western countries regarding the molecular diagnosis, in our country like India only few studies had been done.

MATERIAL AND METHOD:

During a period of 3 years from 2010 to 2013, we have collected total number of 161 solid tumor cases in the age group from 0-18 years. In every case, clinical details like size of the tumour, site of involvement, necessary additional investigations as ultrasonography, computed tomography, magnetic resonance imaging were carried out. Then from the approachable sites fine needle aspiration cytology and biopsies were done. The morphological diagnoses were made with the help

of ancillary studies like special stain and immunohistochemistry (CD99, Vimentin, desmin, S-100). In small round cell tumours, core biopsy and fine needle aspiration biopsy were done and preserved in RNAlater fluid immediately to preserve the RNA of the live tissue. It was stored in a temperature of -85°c. For the molecular analysis, we have sent the samples (n=10) preserved in RNA later fluid surrounded by frozen ice cubes to Adyar cancer institute, Chennai. The temperature was maintained at -85° c.

Total RNA was isolated from snap frozen tissues using Trizol reagent. The RNA was transcribed to cDNA by reverse transcriptase enzyme. Polymerase chain reaction (PCR) was carried out using suitable probes (forward primer- 5' GGCCAGTAGCATCTGACTTG-3') and (reverse primer-5'-ATGGTACCAGGAGTGTTTCTCC-3'), 0.2 U Taq and 200µM of dNTP mixture. 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 products were transferred to the Nylon membrane with the help of blot apparatus. The Nylon membrane was then hybridised to FLI 1 probe. In 2% agarose gel, these PCR products were run and sequence analysis was done.

RESULT: Biopsy samples of 161 cases revealed 131 malignant ones with a ratio of benign to malignant (1: 4.3). Bone and soft tissue tumours came as the second most common tumours ((30%)) after central nervous system tumours ((52%).) The bone tumors presented clinically as swelling in 82.7% cases (n=27) followed by pain in the joints (n=15, 48.3%) and 2(6.4%) cases with fracture. The most common site of involvement was seen in lower extremity (62.06%) followed by axial skeleton (37.9%)-Fig 1A.

Ewing's sarcoma cases are seen in maximum number (21 cases, 72.4%). In 6 to 10 years age group, 31.03% tumours are seen followed by equal frequencies (17.24%) in 11 to 15 yrs and more than 15 years age group. In all tumours an overall male predominance is noted.Bone scan was done in cases where metastasis was suspected (Fig 1B). Cytology done as the priliminary pathological investigation and revealed monotonous looking small round cells with inconspicuous nucleoli (Fig 1C).Histology revealed sheets of uniform small round cells slightly larger than lymphocytes with little stroma and few mitoses in spite of high cellularity(Fig 2A). The scanty cytoplasm was clear in some cases due to rich glycogen content (PAS +ve-Fig 2C). Homer Wright rosettes characterized by cells arranged around central fibrillary space were seen which indicated neural differentiation. In some cases prominent necrosis and arrangement of tumor cells around the blood vessels were seen. Basing on the criteria, typical and atypical Ewing's sarcoma were classified. Immunohistochemistry CD99 showed strong diffuse membrane positivity in 80% cases(Fig 2D). Bone marrow metastasis was characterized by presence clusters of small round hyperchromatic cells and was found in three cases (Fig 2B) .

Amongst various prognostic factors, the most important ones are the metastasis at the time of presentation and histologic response to chemotherapy. In this study 3 (14.2%) present with metastasis to bone marrow at the time of diagnosis and 6 (28.5%) show > 90% necrosis in response to chemotherapy. 6 (28.5%) patients relapse within 2 years of study and 2 (9.5%) patients had history of relapse. High LDH level is seen in 7 (33.3%) cases. Out of 10 molecularly studied cases typical EWS-FLI1 fusion gene is present in 90% cases(Fig 3).

The molecular study was done in 10 cases of Ewing's sarcoma out of 21. RNA was isolated and RT PCR was done by using ABL primer. The result of EWS-FLI1 product showed two types of fusion depending on the 300 bp product. Type 1 fusion was 330 bp and type 2 fusion was 410 bp(Fig 4). The 330 bp product was observed in 80% cases (8 out of 10) and 1 case showed type 2 fusion. One case didn't show any product.Table 4 shows an overall presentation in Ewings' sarcoma. 90% cases show presence of fusion gene and 85% cases show CD99 positivity.

The amplified PCR products were taken up for sequencing which revealed the fusion of exon 7 of EWS with either exon 6 of FLI 1 (type 1) or with exon 5 (type 2) translocation [fig 1&2]. The presence or absence of fusion was also correlated with clinico-pathological findings. Though it was well known that EWS-FLI1 fusion carries a good prognosis, but in our study,2 cases out of 10, though had EWS–FLI 1 fusion succumb to death within a 3 years follow up period due to extensive metastasis in the whole body.

DISCUSSION: For the purpose of clinical management, accurate diagnosis of pediatric soft tissue tumors is critical. It requires an integration of clinical findings (age, site, pattern of disease spread and radiological characteristics, morphological evaluation, ancillary studies like IHC, cytogenetics and molecular genetics[11,12,13,14]. The cytogenetic demonstration of t(11;22)(q24;q12) translocation in the tumor cells favour the diagnosis of EWS/PNET.

200,000 children develop cancer every year, and 80% of these are from the developing world. In India, with one third of the population under the age of 18, the proportion of childhood cancers is high up to 4.6% [15] as compared to a developed country like England where only 0.5% of all cancers occur in that age group. In this study out of all neoplastic lesions in all age groups (7200), paediatric neoplasms account for 161 cases. The incidence of paediatric tumour is 2.23%. Incidence observed here correlates with the study of Arora RS et al, 2009 where it was in a range of 1.6% - 4.8% in India.

Ewing's sarcoma cases are seen in age groups of all ranges. Incidence peaks from 6 years up to 18 years with a male to female ratio of 1.33:1. These findings also corroborates well with Randall RL et al, 2012[16] who stated that about 25% of cases occur before age 10, while 65% arise between 10 and 20 years old. 10% cases are diagnosed in patients of 20 years old with a male to female ratio of 1.5:1.

Ewing sarcoma is a small round cell tumour, which typically has a clear cytoplasm on H&E stain due to glycogen. This glycogen is demonstrated in this study by PAS stain. CD99 immunostain has significantly highlighted the cell membranes. Though CD99 is also a useful marker in the diagnosis of Ewing sarcoma [17], it also shows positivity in synovial sarcoma, mesenchymal chondrosarcoma, alveolar rhabdomyosarcoma [18]. Study done by Parija, showed CD99 positivity in 78% cases which correlates with present study (85%). These morphologic and immunohistochemical findings are well corroborated with an associated chromosomal translocation. Present study shows fusion gene is present in 90% of Ewing sarcoma cases. Also Parija T et al, 2005[19] studies on 20 biopsies taken from suspected round cell tumours revealed that there is strong concordance between molecular diagnostic methods and standard histopathologic diagnosis in all Ewing sarcoma cases with 90% accuracy.

Ewing's sarcoma is a high grade tumor. Basing on important parameters this study shows 33.3% cases of bad prognosis. On H&E section necrosis >90% is associated with bad prognosis (Table-3). Vukasinovic Z et al, 2007[20] also demonstrated that involvement of distal parts of extremities and axial skeleton are of good prognostic factors, while proximal parts, pelvic girdle, metastatic disease and low index of post-chemotherapeutic necrosis are associated with poor outcome. Jeffrey A et al, 2008[21]stated that staging in Ewing sarcoma should include both local imaging to reveal the full extent of tumour prior to therapy and bilateral bone marrow biopsy, chest CT scaning, radionucleotide total body scanning to evaluate distant metastases.

We have followed up the Ewing sarcoma patients for a period of 2 years. Disease free survival is seen in 57.15 cases where as relapse is noticed in 28.5% patients (Table - 5). Randall et al[16] demonstrated that ESFT is an aggressive cancer with a tendency to recur where it arose (local recurrence) and spread throughout the body (metastasize). Those patients who are

treated with intensive chemotherapy and radiotherapy show a 5 year of survival rate approximately 70-75% (Bacci 2006, Esashvili 2008, Gupta 2010). Unfortunately 15-25% of ESFT patiesnts, when they initially see their doctors will have disease that has metastasized elsewhere in the body. In these patients survival is only 30%.

This study demonstrates that the classic diagnostic problem of distinguishing Ewing's sarcoma from other small blue round ell tumours is resolved due to RT-PCR. Several studies revealed that there is repeated pattern of fusion between N-terminal domain of EWS and Ets domain of FLI 1 or ERG. This unique fusion pattern may lead us to diagnose minimal disease and micro metastasis in the biological samples in future. In our study while doing RT-PCR about 40% of patients showed (4 out of 10) metastatic spread or relapse with positive bone marrow deposits for EWS-FLI 1 transcripts. By quantifying the amount of RT-PCR, we can detect and monitor minimal residual disease which will lead to new therapeutic avenues to improve the patient outcome in recent future[22,23].

Again targeted therapy potentially can be developed based on the molecular pathology of the pediatric soft tissue tumors[24,25,26]. Fusion proteins which are produced act as tumor specific antigen which will be a promising target for immunotherapy[27]. A recent study on synovial sarcoma has revealed that the induction of specific cytotoxic T lymphocytes from normal donor by using in-vitro stimulation with SYT-SSX fusion protein pulsed dendritic cell have the ability to kill the human synovial saroma tumor cells[28]. These findings suggest that a peptide derived from the fusion protein may work as a neoantigen and induce a tumor specific immune response.

Fig 1A-Swelling below knee in the patient of Ewing's sarcoma
Fig 1B-Bone scan revealing multiple skeletal deposits
Fig 1C-Cytsmear showing uniform looking tumor cells, Diff quik stain x 400
Fig 2A-Small round cells in histopathology section of Ewing's sarcoma.H&E stainx400
Fig 2B-Bone marrow metastasis. Leishman's stainx100
Fig 2C-PAS positivity demonstrating presence of glycogen in cytoplasm. PASX400
Fig 2D-CD 99 strongly and diffusely positive. CD99x400
Fig 3-RT-PCR analysis of Ewings' Sarcoma /PNET cases (agarose gel picture showing EWS-FLI1 transcripts)
Fig 4-Sequence electrophoregram

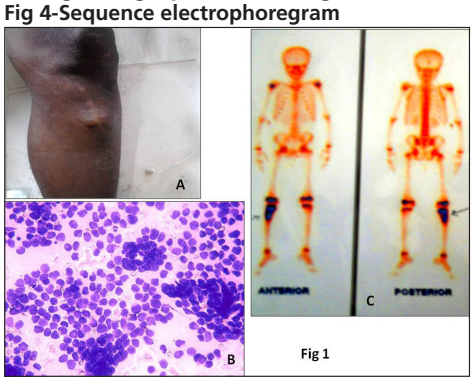

Fig 1

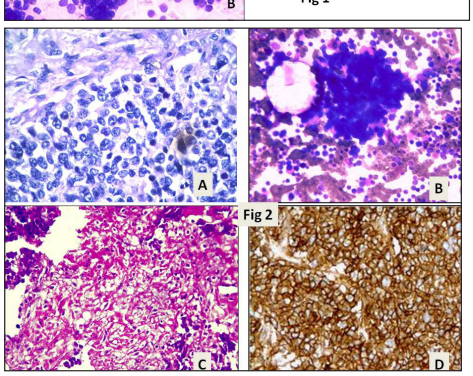

Fig 2

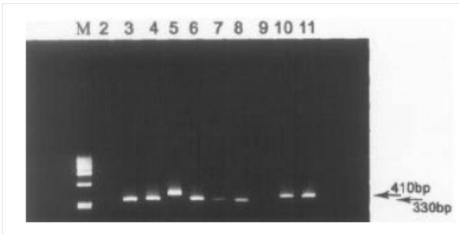

Fig 3

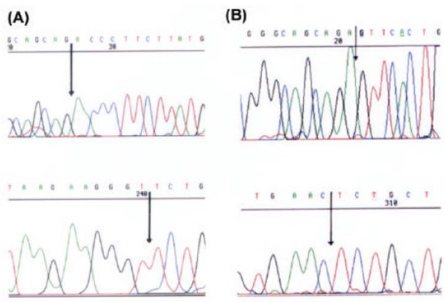

Fig. – 4

Table-1 Frequency of pediatric solid tumors

Tumour types	Frequency	%
CNS tumours	69	52.67
Bone tumours	29	22.13
Soft tissue	10	7.63
Wilm's tumour	5	3.81
Neuroblastoma	5	3.81
Gonadal tumour	5	3.81
Retinoblastoma	4	3.05
Carcinoma and miscellaneous	4	3.05
Total	131	100

p>0.05

Table – 2: Classification of bone tumours.

Age (yr)	Sex	Ewings' sarcoma/ p-PNET	Percentage	Osteosarcoma	Percentage
0 – 5	M	1	6.89	--	—
	F	1		--	
6 – 10	M	5	31.03	--	—
	F	4		--	
11 – 15	M	3	17.24	2	20.68
	F	2		4	
>15	M	3	17.24	1	6.89
	F	2		1	
Total		21	72.41%	8	27.58%

Table –3: Grading and prognostic factors in Ewing's sarcoma.

All the tumors are of high grade. Prognostic factors depend on followings.

Parameters	Good	Cases with good prognosis	Bad	Cases with bad prognosis
Necrosis	< 50 %	16	> 90 %	5
Age of on set	Pediatric	21	Adult	0
Location of tumor	Extremity	15	Central (pelvic)	6
Relapse	> 2 years	2	< 2 years	6
Metastasis	No mets	18	Mets to lungs,BM	3
LDH	Low	14	High	7
Histologic response to chemo	< 50 % necrosis	15	> 90 % or complete necrosis Sis	6
Molecular n=10	EWS-FLI1 fusion	9	Other fusions	1

Table – 4: Molecular study in Ewings' sarcoma.

Age (Yrs)	Sex	HP	Stage	Site of Tumor	EWS-FLI 1 Trans-Location	CD 99 Marker Status
16	Female	ES	M	Right Clavicle	+ve	Positive
12	Male	ES	NM	Rt. Shaft Femur	+ve	Negative
8	Male	ES	M	Left Upper end Femur	+ve	Positive
12	Male	ES	NM	Right Upper end Femur	+ve	Positive
10	Male	ES	NM	Left Tibia below knee	-	Positive
14	Female	ES	M	T-7 Vertebra	+ve	Positive
13	Male	ES	NM	Mediastinal mass	+ve	Non-specific
13	Male	ES	NM	Left Tibia Shaft	+ve	Positive
11	Male	ES	NM	Left Humerus	+ve	Positive
12	Male	PNET	NM	Abdominal Tumour	+ve	Positive

Table – 5: Disease free survival assessment based on grading & staging of Ewings sarcoma:

	Disease free survival	Relapse	Death
Patients (n=21)	12	6	3
Percentage (%)	57.14	28.57	14.28

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