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"RELEVANCE OF RTPCR IN THE DIAGNOSIS OF EWING'S SARCOMA FAMILY OF TUMORS"

Oncology

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ABSTRACT

Background: Translocations diagnosis by fluorescence-in-situ hybridization (FISH) is an established procedure; however, reverse transcriptase polymerase chain reaction (RT-PCR) identifies specific fusion transcripts and works on both fresh and formalin fixed paraffin embedded (FFPE) block tissues. **Objective:** Detection of EWS-FLI1 and EWS-ERG translocations on FFPE samples by RT-PCR for the diagnosis of extra skeletal Ewing sarcoma (EES). **Methods:** Patients diagnosed with small round cell tumors on morphology with a possibility of EES during one year were included in the study. Immunohistochemistry (IHC) was done with a panel of markers as considered appropriate to site, age and morphology, followed by algorithmic molecular analysis. **Results:** Twenty nine patients, in this study, had median age of 16 years with male: female ratio of 1.6:1. The most common site was lower limb. CD99 was positive in all and FLI1 in 28 samples with focal positivity in one each. Twenty six (89.7%) had RT-PCR interpretable results and the test failed due to poor RNA quality in 3 (10.3%). EWS-FLI1 was positive in 19 (73.1%) and 7 (26.9%) were negative for both EWS-FLI1 & EWS-ERG. Five patients had concomitant FISH studies available, which resolved diagnosis in 2 each of RT-PCR negative and failed samples as AFH (EWS-CREB1) in one and EES in 3. In the remaining 5 RT-PCR negative cases, 2 were resolved as lymphoma, 1 synovial sarcoma and 2 EES on IHC. **Conclusions:** RT-PCR from FFPE tissues can be used as a diagnostic test for EES. Combination of molecular techniques would aid in improving the diagnostic accuracy.

KEYWORDS

Extra skeletal Ewing sarcoma, Reverse transcriptase PCR, Immunohistochemistry

INTRODUCTION

Ewing's sarcoma family of tumors (ESFT) is a group of small round cell tumors occurring in bone or soft tissue. Based on SEER database,31% had extra skeletal Ewing sarcoma (EES),which can occur in a wide variety of sites, including chest wall, lower extremities, paravertebral region, pelvis, hip region, retroperitoneum, upper extremities, and rarely intracranial region and viscera.[1,2] Ewing sarcoma is genetically characterized by binding of EWSR1 or other members of the TET/FET family to members of the ETS family. Approximately 85–90% of the Ewing's sarcomas display the translocation t(11;22)(q24;q12) resulting in the EWS/FLI1 fusion gene, and approximately 5–10% harbor a EWSR1-ERG fusion gene. The remaining cases show rare gene partners, such as ETV1, ETV4, and FEV, and EWSR1 can be substituted by FUS [3].

Immunohistochemistry is the first ancillary technique to confirm the diagnosis of ESFT, and distinguish from other small round cell tumors[4] ESFTs also express various markers of neural, epithelial and mesenchymal differentiation.[5]. However, IHC may be non-specific, may be stimulated by different tumor types, or absent in poorly differentiated tumors. FISH is very useful for the diagnosis of translocations and has the advantage of its applicability on FFPE tissues; however, it does not provide the specific fusion transcript and does not differentiate ESFT from tumors that harbor EWSR1 translocations, or may be unsuccessful in certain instances.[6-10] RT-PCR is a valuable method for detecting specific molecular alterations as it has the advantage of identifying both translocation partners.[11] There are very few studies that used molecular methods for the diagnosis of ESFTs, especially from India, using RT-PCR on FFPE tissues. [12-14] To the best of our knowledge, this is the first study from India using RT-PCR on FFPE tissue exclusively in EES.

MATERIAL AND METHODS

This was both retrospective and prospective observational study conducted during the period May 2016 to December 2017. Patients registered in our institute and diagnosed as small round cell tumors on morphology in extra skeletal site, confirmed on imageology, were included in the study. Patients who had inadequate tissue in the blocks or having bone involvement on imageology were excluded from the study. The study was approved by the institutional ethics committee (EC Reference No: IEC/2017/111).

The demographic and clinical data were obtained from the medical records. The site of lesion and extra skeletal location were confirmed on imageology (computed tomography (CT) / magnetic resonance imaging (MRI). The tumors with small round cell morphology were subjected to IHC with cluster designation molecule 99 (CD99) and friend leukemia integration 1 (FLI1). Depending on the age, site and morphological differentiation, further IHC panel including desmin, leukocyte common antigen (LCA), pan cytokeratin (PCK), B cell lymphoma 2 (BCL2), transducing-like enhancer of split 1 (TLE 1) and others (as appropriate) were performed.

Immunohistochemistry was performed for CD 99 (12E7 DAKO) and FLI-1 (MRQ1 cell mark), as per manufacturers instructions. Tumors positive for either or both CD99 and FLI1 were subjected to RT-PCR.

Molecular testing using RT-PCR

To extract RNA, 8-10 sections of 5-8µm thickness were cut from representative paraffin blocks, deparaffinized using xylene (100%) and ethanol (100%) washes. Special attention was paid for cutting and cleaning blades with RNase ZAP solution to avoid cross contamination. Total RNA was isolated from FFPE tissue using Recover All Total Nucleic Acid Isolation kit (Invitrogen by Thermo Scientific), then 1µg RNA was reverse transcribed to cDNA using TRUPCR High Retrotranscritase starter kit (3B Black Biotech India Ltd) as per manufacturers protocol.

PCR

RNA quality was checked using ABL primers as described previously by Parija et al., 2005.[12] Amplification was performed in 20µl master mix consisting of 2µl of 10x PCR buffer, 10 picomoles each of both EWS (FP) and FLI1 (RP), 1U of Amplicon Taq gold, 2.5mM of deoxynucleotide trisphosphate with 2µl of cDNA template, following 35 cycles of 94°C for 30s, 65°C for 1min and 72°C for 1min. Amplicons were checked on 2% agarose gel. EWS FLI1 was subtyped

as Type 1, EWS (exon 7) with FLI1 (exon 6) of 330bp and Type II, EWS (exon 7) and FLI1 (exon 5) of 390bp. EWS-ERG fusion transcripts detection was done as described by Mangham et al, 2006.[15] To exclude false positive results owing to non-specific amplification, all the cases PCR products were sent to Bioserve for sanger sequencing on 3500Dx. Genetic Analyzer. Bioedit software program (http://www.mbio.ncsu.edu/Bioedit/bioedit.html) was used to identify sequences.

RESULTS

There were 29 patients in this study as per the inclusion criteria with 18 males and 11 females with median age of 16 (range 5-36) years. The most common site of involvement was lower limb (10) followed by chest wall (5), para vertebral (4), upper limb (3), head & neck (2), retroperitoneum (1), cheek (1) and visceral (3) (cervix, one and mass adherent & infiltrating lung in two).

Immunohistochemistry:

CD 99 was positive in all 29 (as per inclusion criteria) with focal positivity in one. FLI 1 was positive in 28 with focal positivity in one. The other IHC markers done as per differential diagnosis considered on morphology showed variable positivity and included LCA (2/20), PCK (1/6), desmin (0/9), TLE-1 (1/4), BCL2 (1/2), ERG (0/2), CD31 (0/1), CD34 (0/1) and calponin (1/1).

Molecular analysis:

RT-PCR was done in all 29 samples and FISH in 5 samples only. Of the 29 samples done by RT PCR 26 (89.7%) samples had interpretable results and 3 (10.3%) failed due to low quality of RNA. Of 26 samples, 19 (73.1%) were positive and 7 (26.9%) were negative (Table I). Chimeric EWS-FLI1 Type I fusion was observed in 14 (14/19: 73.7%) and type II fusion in 5 (5/19: 26.3%) samples. All 7 negative EWS FLI1 samples, further tested for EWS-ERG were also negative.

Table I-: Type and number of cases tested for translocations with RT-PCR

RT PCR	N =29	%	
WORKED	26	89.7	
FAILED	3	10.3	
EWS-FLI1 t(11;22)	N=26		
POSITIVE	19	73.1	
Exon 7/6 (Type 1)	14		73.7
Exon7/5 (Type 2)	5		26.3
NEGATIVE	7	26.9	

EWSR1 rearrangement was detected in 3 patients with FISH. These included two samples where RT-PCR failed due to suboptimal RNA extraction. One sample (Table II: S. No. 20), which was positive for EWSR1 by FISH showed EWS-CREB by RT-PCR(done in SRL diagnostics). The diagnosis in this patient was given as angiomatoid fibrous histiocytoma (AFH) correlating with morphology and IHC.

Statistical Analysis:

The diagnostic accuracy of both CD 99 and FLI1 for the diagnosis of ESFT was100% in our cases; however, CD 99 was focal positive and FLI1 was negative in AFH and FLI1 was focal positive in synovial sarcoma. The sensitivity of RT-PCR for the diagnosis of ESFT was 86.36% and specificity of 100%. Due to small sample size, statistical analysis could not be done.

DISCUSSION:

In this study, in addition to morphology and IHC, RT-PCR from FFPE tissues was applied for the diagnosis of extra skeletal Ewing sarcoma. Ewing sarcoma family of tumors are diagnosed based on clinical data, imaging, morphology, IHC and molecular features.

In the present study of 29 small round cell tumors in extra skeletal site with positivity for CD99 and/or FLI1, the diagnosis of ESFT was made by RT-PCR in (26/29: 89.65%) tumors (excluding 3 cases where RNA was suboptimal).

Table III ESFT: Demographic features, site involved, results of IHC, FISH, RT-PCR and final diagnosis

S No	Age	Gender	Site	CD 99	FLI 1	Others	FISH	RT-PCR		Final Diagnosis
							EWSR1	EWS-FLI1	EWS-ERG	
1	32	F	Leftt cheek	FP	P	Desmi, TLE: N	ND	P	ND	ESFT
2	32	M	Right arm	P	P	ERG: N	ND	P	ND	ESFT
3	34	F	Cervix	P	P	PCK: P	P	P	ND	ESFT
4	36	F	Right lumbar	P	P	-	ND	P	ND	ESFT
5	11	M	Left thigh	P	P	PCK: N	ND	P	ND	ESFT
6	33	M	Left lumbar	P	P	LCA: N	ND	P	ND	ESFT
7	16	M	Paravertebral	P	P	PCK, LCA:	ND	P	ND	ESFT
8	10	M	Right leg	P	P	Desmin: N	ND	P	ND	ESFT
9	9	F	Right shoulder	P	P	-	ND	P	ND	ESFT
10	8	M	Right thigh	P	P	LCA: N	ND	P	ND	ESFT
11	13	F	Forearm	P	P	LCA: N	ND	P	ND	ESFT
12	19	F	Hip	P	P	LCA: N	ND	P	ND	ESFT
13	5	M	Rightt chest wall	P	P	LCA, PCK: N	ND	P	ND	ESFT
14	13	M	Right thigh	P	P	LCA, TLE, Desmin: N	ND	P	ND	ESFT
15	11	M	Leg mass	P	P	LCA: N	ND	P	ND	ESFT
16	12	F	Right thigh	P	P	LCA, Desmin,	ND	P	ND	ESFT
17	8	F	Chest wall	P	P	LCA: N	ND	P	ND	ESFT
18	12	F	Scalp	P	P	LCA, Desmin: N	ND	P	ND	ESFT
19	21	F	Lumbar	P	P	LCA: N	ND	P	ND	ESFT
20	25	M	Left leg mass		N	Desmin, CD31, CD34: N Calponin:P	P	N	N	AFH (EWS-CREB1+)
21	30	M	Retroperitoneal mass	P	P	Bcl2:P	P	N	N	ESFT
22	9	M	Right chest wall	P	P	LCA, Desmin: N	ND	N	N	ESFT
23	8	M	Chest wall	P	P	LCA: N	ND	N	N	ESFT
24	14	M	Right leg mass	P	P	LCA: P PCK: N	ND	N	N	Lymphoma
25	25	F	Mass adherent to lung	P	P	LCA:P	ND	N	N	Lymphoma
26	25	M	Chest wall	P	P	LCA: N TLE1:P	P	N	N	Synovial sarcoma (SYT-SSX)
27	29	M	Right thigh	P	P	LCA, Desmin: N	P	failed	ND	ESFT
28	22	M	Left supra clavicular region	P	P	LCA: N	ND	Failed	ND	ESFT
29	33	M	Mass adherent to lung	P	P	PCK, Desmin, Bcl 2, ERG: N	P	Failed	ND	ESFT

Abbreviations: M-Male, F-Female, ESFT-Ewing sarcoma family of tumors, P-Positive, FP- Focal Positive, N-Negative, ND-Not done

Demographic features and sites of involvement:

Patients with EES were reported to have a higher mean age, less likely to be male and had predilection for axial involvement, when compared to skeletal ESFT.[1] In the present study, the median age was 16 years with male predominance and lower extremities were the most common site of involvement, followed by chest wall, similar to other studies.[16, 14] Though soft tissues in the extremities, chest wall, head & neck and paravertebral region were common sites of involvement, deep soft tissues in thorax, retroperitoneum and visceral involvement was noted in the present study.

Morphology and immunohistochemistry:

The morphology of ESFT may be varied but the most common pattern is small round cell morphology as seen in the present study.[16] One tumor, which showed marked pleomorphism, on further evaluation was diagnosed as AFH in the present study. The diagnosis of ESFT is based on diffuse membranous positivity of CD99, along with nuclear positivity of FLI1 and negativity for LCA.[6,9,14] CD99 is sensitive and FLI1 more specific. The combination of both was reported to increase the specificity of IHC in diagnosis of ESFT and hence used in the present study. [4,5,16,17] The diagnostic accuracy was 100% for both

CD99 and FLI1 for ESFT in the present study; however, the sensitivity for the diagnosis in small round cell tumors was 100% and 95% respectively for CD99 and FLI1. These observations were in agreement with others.[16,14,18,19] LCA was negative in all ESFT and also helped to diagnose lymphoma in the present study. PCK and BCL2 were positive one each in ESFT in the present study and similar observations were made earlier.[16,5,20]

Molecular analysis:

In majority of cases, the molecular alterations involve the EWS gene (EWSR1), with members of ETS family of transcription factors, EWS is a promiscuous gene and can fuse to non-ETS genes, forming chimeras.[5] Fusion of EWS to ETS family of genes is unique to ESFT. The tumor phenotype is not altered by variant translocations but fusion of EWS to non-ETS genes occurs in other tumors like DSRCT, AFH, clear cell sarcoma and others.[10,9] Hence, identifying the partner gene by RT-PCR is important as it specifies the tumor type. In the present study, RT-PCR was done for FLI1 and ERG genes.

Molecular alterations for the diagnosis of ESFT were found in 19/26(73.1%) cases and none for EWS-ERG using RT-PCR similar to Parija et al.[12] on FFPE tissues in ESS. The sensitivity of RT-PCR for the diagnosis of ESFT was 100%.[21] EWS-FLI1 was the most common gene fusion reported in ESFT, as was seen in the present study.[9] In the present study, EWS-FLI1 fusions included type 1 fusion (exons 1-6 of EWS fuse to FLI1 exons 6-9) in 14 (73.7%) and type 2 (exons 1-7 of EWS fuse to FLI1 exons 5-9) in 5 cases (26.3%). No correlation between morphology and molecular alteration was observed in the present study.

In the present study, 7 cases were negative for RT-PCR. A negative result does not necessarily rule out the possibility of ESFT as both partner genes may have variant translocations.[5] The final diagnosis was resolved by FISH as ESFT in 1 case, and specific non-ESFT in 2 cases in the present study. Of which 1) one case, on further testing showed positivity for TLE on IHC and SYT fusion transcript on FISH, confirming the diagnosis of synovial sarcoma. 2) Second case was positive for EWS-CREB fusion transcript (done in SRL laboratory, a referral laboratory). Correlating with morphology and IHC, a final diagnosis of AFH was given. Additional IHC markers as deemed appropriate on morphology helped in ruling out ESFT and make specific diagnosis like lymphoma in 2 cases. The diagnosis remained probable ESFT in 2 without molecular confirmation, based on clinical, radiological, morphological and IHC features. Similar observations were made earlier.[6,9]

Three (10.34%) cases were non-informative by RT-PCR due to lack of RNA, of which 2 biopsy samples were processed in other diagnostic centres before referral and hence the pre analytical factors were not under control. Of the 3, EWSR1 by FISH was confirmed in 2 cases. Gamberi et al[6] reported a failure rate of 15.32% (both fresh and FFPE tissues) whereas Noujaim et al reported[10] 18% failure rate (FFPE tissues) by RT-PCR. FFPE tissue is reported to have limited sensitivity when compared to fresh tissue; however, fresh tissue may not be available in routine diagnostic surgical pathology, especially in small biopsies and referral cases.[9] A combination of FISH and RT-PCR was recommended to increase the sensitivity and accuracy of diagnosis of Ewing sarcoma.[8] Table III–compares the results of the present study with similar studies using RT-PCR for the diagnosis of ESFT.

Table IV-: Comparison of present study with other similar studies using RT-PCR on FFPE for the diagnosis of ESFT

Author and Year (Reference)	Morphology & IHC	RT-PCR			FISH Positive	Final diagnosis	
	N	Positive	Negative	Failed			
Bridge et al 2006	43	8 (excluding EWS-FLI1 fusion transcript in focal round cell component in one each of fibrosarcoma and malignant teratoma)	12	7	9	ESFT	17
						DSRCT	2
						Fibrosarcoma	2
						Malignant teratoma	1
						Unresolved round cell tumor	11
Qian X et al 2005	18	15 EWS-FLI1 -13 (Type 1- 10; Type 2- 3) EWS-ERG 2	-	3	15	ESFT	18
Gamberi et al 2011	222	121 EWS-FLI1-108 (Type 1: 73;Type 2: 28;others: 7) EWS-ERG - 12 EWS-FEV - 1	67	34	23	ESFT	144
						ESFT (IHC)	12
						Undifferentiated small round cell tumor	4
						Non-ESFT	28
Machado et al 2016	200	148 EWSR1-FLI1 121 EWSR1-ERG 11 EWSR1-FEV 1 SYT-SSX 3 EWSR1-WT1 1 CIC-DUX4 6	19	33	-	ESFT	133
						Synovial sarcoma	3
						DSRCT	1
						Ewing-like tumors	11
						Undifferentiated small round cell tumors	19
Noujaim et al 2017	86	50	19	17	72	ESFT	76
						Possible ESFT	4
						Non-ESFT	6
Parija et al 2005	20 SK	18 EWS-FLI1 18 (Type 1: 11 Type 2: 7)	2	-	-	ESFT	18
						DSRCT	1
						SRCT	1
Jambhekar et al 2006	32 SK & ESK	29 FFPE 23 FNAC 6	3	-	10 FISH+1 0/11	ESFT	29
						DSRCT	1
						Undifferentiated sarcoma	1
Gautam et al 2010	44 FNAC	24 EWS-FLI1- 20 (Type 1: 11 ;Type 2 : 5 ;Type 4: 4) EWS-ERG 4 PAX3/7-FKHR 2 EWS-WT1 2	1	-	-	ESFT	25
						Neuroblastoma	13
			2			Alveolar Rhabdomysarcoma	4
						DSRCT	2
Rekhi et al 2014	58 SK & ESK	56 EWS-FLI1 55 EWS-ERG 1	2	-	12	ESFT	56
						Others	2
Present study	29 ESK	19 EWS-FLI1 - 19 Type 1: 17; Type 2: 2	6	3	4	ESFT	22
						Synovial sarcoma	1
						Lymphoma	2
						AFH	1
						Possible ESFT	3

Abbreviations: ESFT- Ewing sarcoma family of tumors , DSRCT - Desmoplastic small round cell tumor , SRCT - small round cell tumors, SK - Skeletal, ESK - Extra skeleltal, FFPE- Formalin fixed paraffin embedded tissue

CONCLUSION:

The present study confirms that optimal use of ancillary techniques like IHC, RT-PCR and FISH are necessary in addition to morphology for the diagnosis of ESFT, especially in extra skeletal sites including visceral organs. EWS-FLI1 was the most common translocation partner with type 1 fusion being the commonest. RT-PCR has proven to be sensitive and cost effective as a diagnostic test for EES, ensuring proper fixation and processing. The limitation of the study is that we could not assess those cases that were morphologically ES but failed to show the translocation by RT PCR. Larger studies are therefore warranted covering all the translocations of Ewing's sarcoma and ES like tumors.

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