

Ewing Sarcoma With Emphasis on Extra-skeletal Ewing Sarcoma: A Decade's Experience From a Single Centre in India

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ABSTRACT

INTRODUCTION: The diagnosis of Ewing sarcoma family of tumours (ESFT) is challenging, especially in adults and in extra-skeletal or visceral location. Several morphologic mimics with varied treatment options and prognosis confer diagnostic dilemmas. Application of ancillary diagnostic modalities in surgical pathology in clinical routine has enabled accurate diagnosis of ESFT in bone, soft tissues, and viscera.

AIM: The study aims to assess the clinicopathological features including molecular test results of ESFT with emphasis on sex, age, and location, especially extra-skeletal soft tissue and visceral location.

MATERIAL AND METHODS: Data of clinicopathological, molecular tests (wherever performed), diagnosis rendered in 302 ESFT over a decade from our centre were reviewed. Statistical comparison of skeletal and extra-skeletal tumours with reference to age and sex was done using SPSS package. The *P* value of <.05 was considered significant.

RESULTS: The cohort included 302 ESFTs with 49% skeletal and 51% extra-skeletal tumours. Thigh was most common site among skeletal tumours; chest wall, paraspinal location, and retroperitoneum among soft tissues (39.4%); and kidney, ovary, and cervix among visceral tumours (11.3%). Fluorescence in situ hybridisation for *EWSR1* gene rearrangement was positive in 54 patients and reverse-transcriptase polymerase chain reaction in 19 patients. Predominance of male sex, younger age and location in extremities among skeletal tumours and lack of gender predilection, higher age and axial location in extra-skeletal tumours were noted, which were statistically significant. Molecular tests were performed more frequently in extra-skeletal tumours, especially in visceral tumours to establish the diagnosis.

CONCLUSIONS: The study showed statistically significant differences in the age, sex, and location between skeletal and extra-skeletal ESFT. The increased percentage of extra-skeletal tumours especially in viscera was attributed to the increased awareness and availability of ancillary techniques.

KEYWORDS: Ewing sarcoma family of tumours, skeletal, extra-skeletal, visceral, fluorescence in situ hybridisation, reverse-transcriptase polymerase chain reaction

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Introduction

The Ewing sarcoma family of tumours (ESFT) encompass all the previous terminologies such as Ewing sarcoma of bone, extra-skeletal Ewing sarcoma, peripheral primitive neuroectodermal tumour, Askin tumour of thoraco-pulmonary region, and atypical Ewing sarcoma based on the presence of a unifying pathognomonic chromosomal translocation.^{1–9} Tumours involve bones, soft tissues, and viscera and typically appear as solid sheets of uniform, small blue round cells with minimal cytoplasm and little extracellular matrix. The disease arises from some mesenchymal or neural crest-derived stem or progenitor cell and hence may show variable neuronal, mesenchymal, and

epithelial differentiation.^{2,6,10} The potential mimics of ESFT with reference to age, site, and morphology are lymphoblastic lymphoma, rhabdomyosarcoma, neuroblastoma, poorly differentiated synovial sarcoma, desmoplastic small round cell tumour, and others.^{2–7,9,11} The treatment and prognosis of these various tumours are very different, necessitating accurate diagnosis. The diagnosis is challenging, especially in adults and in extra-skeletal or visceral location.

Availability of ancillary techniques in diagnostic surgical pathology enables accurate diagnosis of more tumours in the extra-skeletal location.^{12–14} The clinicopathological features and prognosis were reported to be different in skeletal and



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extra-skeletal tumours.¹²⁻²¹ This article aims to study the clinicopathological features and the role of ancillary techniques in the diagnosis of ESFT with particular emphasis on extra-skeletal soft tissue and visceral location.

Materials and Methods

A total of 346 patients suspected clinically or on radiology as ESFT and tumours with small round cell morphology on core biopsies or resected specimens, with a possibility of ESFT during the period September 2009 to December 2019, were included in the study. The study was approved by the institutional ethics committee. The demographic, clinical, and radiology findings with respect to site of tumour were noted from the medical records. The biopsies and resected specimens were fixed in 10% neutral buffered formalin and processed for paraffin sections and stained with hematoxylin and eosin (H&E). The architectural details, cellularity, atypia, mitoses, areas of necrosis, and differentiation, if any, were noted.

Depending on the morphological differential diagnosis considered, immunohistochemistry (IHC) was performed on formalin-fixed paraffin embedded (FFPE) tissues as per the protocols on automated immunostainer Roche Ventana Benchmark XT. Antibodies used were cluster of differentiation 99 (CD99) (12E7 Dako; ready to use [RTU]), Friend leukaemia integration (FLI1) (MRQ1; Cell Marque, Rocklin, CA USA, 1:50), leukocyte common antigen (LCA) (PD7126/162B11; Biocare, CA USA, 1:100), vimentin (V9; Biocare, RTU), Bcl2 (124; Dako, Glostrup, Denmark, RTU), pan cytokeratin (pan CK) (AE1/AE3, RTU), synaptophysin, chromogranin, CD56, transducin-like enhancer of split 1 (TLE1), Wilms tumour gene 1 (WT1). Weak/moderate/strong staining was considered as positive staining and equivocal staining as negative staining; diffuse membranous staining was considered positive for CD99 and nuclear positivity for FLI1; focal staining in the tumour cells was reported as focal positivity, no staining as negative; endothelial cells and lymphocytes were used as internal control.

Fluorescence in situ hybridisation (FISH) test results of *EWSR1* gene rearrangement status in FFPE tissues were reviewed. Locus-specific Vysis dual colour break-apart probe for *EWSR1* gene located at 22q12 (Vysis, Inc., Downers Grove, IL, USA) was used. Hybridisation signals in 50 nonoverlapping nuclei were reported. Break in *EWSR1* gene was considered to be positive for *EWSR1* rearrangement. The cut-off established was 15%.

Reports of reverse-transcriptase polymerase chain reaction (RT-PCR) test, which was done in-house or out-sourced, were reviewed. The procedure involved extraction of total RNA from FFPE tissue, using Recover All Total Nucleic Acid Isolation kit (Invitrogen by Thermo Scientific, Baltics UAB, Lithuania). RNA, 1 µg, was reverse transcribed to cDNA using TRUPCR High Retro Transcriptase Starter kit (3B BlackBiotech India Ltd, Bhopal, India) as per manufacturer's

protocol. Amplicons were checked on 2% agarose gel. *EWS-FLI1* was subtyped as Type 1, *EWS* (exon 7) with *FLI1* (exon 6) of 330bp (base pairs) and Type II, *EWS* (exon 7) and *FLI1* (exon 5) of 390bp. The PCR products were sent to Bio-serve for sequencing on 3500Dx Genetic Analyzer. Bio-edit software programme (<http://www.mbio.ncsu.edu/Bioedit/bioedit.html>) was used to identify sequences.

Final histopathologic diagnosis rendered was based on correlation of clinical, morphologic, IHC (positivity for CD99 and FLI1), and/or positivity for *EWSR1* rearrangement by FISH and/or *EWS-FLI1* by RT-PCR. Tumours other than ESFT, confirmed on IHC, FISH, or RT-PCR, were excluded from the study.

Statistics

Analysis was done using SPSS statistical software version 17.0. Age and sex in skeletal versus extra-skeletal sites were correlated. The *P* value, from which statistical significance was assumed, was considered as significant when *P* < .05.

Results

Of the total of 346 suspected ESFT, 44 were excluded due to lack of clinical details in 17 and diagnosis of sarcomas other than ESFT in 27 patients. The cohort included 302 patients with 149 (49.34%) having tumours originating in skeletal system, 119 (77.7%) in soft tissues, and 34 (11.26%) in viscera. Final diagnosis was made correlating clinical features, imaging, morphology, and IHC. Molecular confirmation by FISH for *EWSR1* was present in 54 patients and RT-PCR in 19 patients (including both in one).

Skeletal ESFT (n = 149): This group included 101 (67.8%) men and 48 (32.2%) women including 64 (42.9%) children (<15 years). Age ranged from 3 to 61 (median 16) years. The sites included long tubular bones in 74 (49.7%) with bones of upper limb (humerus, radius, and ulna) in 24 (32.4%) and bones of lower limb (femur, tibia, fibula, and calcaneum) in 50 (67.57%). Tumours involved flat bones in 75 (50.3%) that included cranial bones (8), mandible (4), maxilla (2), scapula (7), clavicle (6), ribs (20), pelvis (13), and vertebrae (4). Immunohistochemistry for both CD99 and FLI1 was positive in 109 (20 confirmed by FISH for *EWSR1* rearrangement), CD99 and *EWSR1* positivity in 6, only *EWSR1* positivity in 5, only CD99 in 28, and only FLI1 in 1. Pan CK, desmin, and Bcl2 were variably positive and LCA was negative. Neuroendocrine markers (synaptophysin, chromogranin, CD56 and NSE) were present in 5. Fluorescence in situ hybridisation for *EWSR1* gene rearrangement was positive in 31 patients.

Extra-skeletal ESFT (n = 153): There were 79 (51.63%) men and 74 (48.37%) women, including 41 (26.80%) children (<15 years). Age ranged from 7 months to 65 (median 25) years. Soft tissue tumours were noted in 119 (77.7%) patients, which

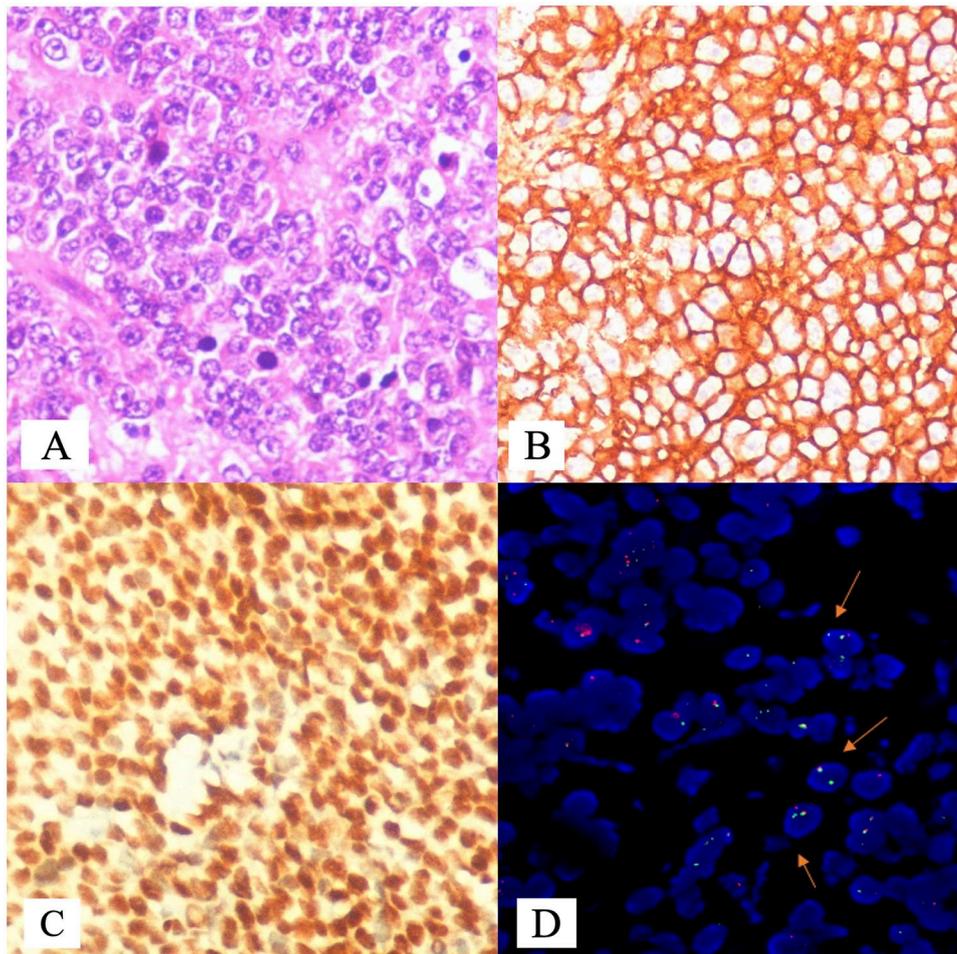


Figure 1. Photomicrographs of ESFT: (A) diffuse sheets of round cells with vesicular nuclei, marginated nucleoli and moderate amounts of cytoplasm (hematoxylin-eosin, original magnification $\times 100$), (B) immunohistochemistry (IHC) for CD 99 demonstrating membrane positivity in tumour cells (CD99 $\times 400$), (C) IHC for FLI1 showing nuclear positivity (FLI1 $\times 400$), and (D) representative fluorescence in situ hybridisation (FISH) image showing EWSR1 rearrangement in 72% of cells. Predominant clone (34%) showed, standard break-apart signal pattern of one fusion and separated 3' orange (centromeric) and 5' green (telomeric) signal pattern (indicated by arrow). Two additional clones showed atypical break-apart pattern with one clone (24%) showing break and deletion of 3' centromeric orange signal (1 fusion and one 5' green) (indicated by arrow), and another clone 14% with break followed by deletion of 5' (telomeric) green signal. Additional clones with atypical signal pattern demonstrate submicroscopic deletion of centromeric and telomeric regions of the EWSR1 gene, respectively. The same is represented as per International System for Human Cytogenetic Nomenclature (2016). nuc ish (EWSR1 $\times 2$) (5' EWSR1 sep 3' EWSR1 $\times 1$) [17/50]/nuc ish (5' EWSR1 $\times 2$, 3' EWSR1 $\times 1$) (5' EWSR1 con 3' EWSR1 $\times 1$) [12/50]/nuc ish (5' EWSR1 $\times 1$, 3' EWSR1 $\times 2$) (5' EWSR1 con 3' EWSR1 $\times 1$) [7/50]. ESFT indicates Ewing sarcoma family of tumours; FLI1, Friend leukaemia integration.

included arm (3), forearm (1), thigh (20), leg (4), popliteal fossa (2), gluteal region (5), cheek (2), orbit (2), nose and nasopharynx (2), scalp (1), neck (7), parapharyngeal (2), chest wall (29), mediastinum and thorax (5), paraspinal (20), abdomen and retroperitoneum (14). Visceral tumours were noted in 34 (22.22%) patients and the sites included ovary (4), cervix (3), vagina (1), kidney (7), bladder (2), prostate (1), lung (4), breast (5), lymph node (3), intestine (2), and brain (2). Immunohistochemistry for both CD99 and FLI1 was positive in 127 (16 confirmed by FISH for EWSR1 rearrangement and 19 for EWS-FLI1 by RT-PCR), CD99 and EWSR1 rearrangement by FISH in 4, FLI1 and EWSR1 rearrangement by FISH in 1, EWSR1 rearrangement by FISH in 2, only CD99 in 17, and only FLI1 in 2. Pan CK, desmin,

and Bcl2 were variably positive and LCA was negative. Neuroendocrine markers (synaptophysin, chromogranin, CD56, and NSE) were present in 7. Fluorescence in situ hybridisation for EWSR1 rearrangement was positive in 23 and EWS-FLI1 by RT-PCR in 19 (including both in one) (Figure 1).

Comparison of skeletal and extra-skeletal ESFT: The skeletal ESFT showed predominance (67.79%) in men, whereas extra-skeletal ESFT had no gender predilection; this difference in sex distribution was not statistically significant ($P = .0691$). The median age was 16 in skeletal and 25 in extra-skeletal location, and this was statistically significant ($P < .001$). Number of children in skeletal location were 64 (42.95%) and 41 (26.80%) in extra-skeletal location

Table 1. Comparison of demographic features and sites of involvement in skeletal and extra-skeletal ESFT (n=302).

PARAMETER	SKELETAL ESFT N = 149	EXTRA-SKELETAL ESFT N = 153	P VALUE
Male: Female	101: 48	79:74	.0691
Age (median), y	3-61 (16)	7 months-65 (25)	<.001
Children <15y	64	41	.0032
Site	Upper limb: 24 Lower limb:50	Upper limb: 04 Lower limb: 31	.019
Diagnosis CD99+FLI1 (FISH for <i>EWSR1</i> and EWS-FLI1 by RT-PCR) CD99/FLI1+FISH for <i>EWSR1</i> /only FISH/only IHC	109 (20 + 0) 39	127 (16 + 19) ^a 26	.048
Molecular tests performed for diagnosis	31(20.81%) 31 (FISH)	42 (27.45%) 23 (FISH) + 19 (RT-PCR) Visceral (47.06%)	

Abbreviations: CD99, cluster of differentiation 99; ESFT, Ewing sarcoma family of tumours; FISH, fluorescence in situ hybridisation; FLI1, Friend leukaemia integration; IHC, immunohistochemistry; RT-PCR, reverse-transcriptase polymerase chain reaction.

^aIncludes one which was positive for both *EWSR1* by FISH and EWS-FLI1 by RT-PCR.

and this was statistically significant ($P = .0032$). Location in extremities was 74 (49.66%) in skeletal compared with 35 (29.41%) in soft tissue ESFT; this was statistically significant ($P = .019$) (Table 1). Molecular tests were more frequently performed (27.45%) for the diagnosis of extra-skeletal ESFT, particularly more for visceral (47.06%), when compared with skeletal ESFT (20.85%). The FISH technique was performed more frequently (73.97%) than RT-PCR (26.03%).

Ewing sarcoma family of tumour in viscera (n=34): The most common organ involved was kidney, followed by ovary and others. Fluorescence in situ hybridisation was more frequently (47.06%) performed for the diagnosis. The demographic details, system involved, details of IHC, and *EWSR1* rearrangement by FISH are presented in Table 2.

Ewing sarcoma family of tumour in female genital tract (n=8): Most common site was the ovary in 4 (50%), cervix (3/8), and vagina (1/8). CD99 and FLI1 was positive in all 8 and FISH for *EWSR1* was positive in 5 (2/4 ovary and 3/3 cervix) (Figure 2A and B).

Ewing sarcoma family of tumour in genitourinary tract (n=10): Tumours were more frequent in the kidney (7/10), whereas bladder (2/10) and prostate (1/10) were rare. The median age of patients with renal involvement was 30 (range: 17-40) years. CD99 and FLI1 expression was noted in all 10 and FISH for *EWSR1* was positive in 5 (4/7 kidney and 1/2 bladder) (Figure 2C to F).

Ewing sarcoma family of tumour in breast (n=5): The median age of patients with breast involvement was 31 (range: 16-50) years. Immunohistochemistry for CD99 and FLI1 was positive in 4 and FISH for *EWSR1* was positive in 2. The one sample that was negative for both CD99 and FLI 1 was positive for *EWSR1* gene rearrangement by FISH.

Ewing sarcoma family of tumour in lung (n=4): The median age of patients with involvement of lung was 33.5 (range: 32-41) years. Immunohistochemistry for CD99 and FLI1 was positive in all 4 and FISH for *EWSR1* was positive in 1. All were primary ESFT.

Ewing sarcoma family of tumour in lymph node (n=3): The median age of patients with lymph node involvement was 44 (range: 21-61) years. Immunohistochemistry for CD99 and FLI1 was positive in 2 and one had the *EWSR1* rearrangement. In one sample, where CD99 was not done, FLI1 was positive, and *EWSR1* was positive for the rearrangement. All were primary ESFT.

Ewing sarcoma family of tumour in intestine (n=2): The patients were aged 11 and 37 years. CD99 was positive in both but FLI1 was positive in 1. Fluorescence in situ hybridisation was not done in both.

Ewing sarcoma family of tumour in brain (n=2): Two patients aged 28 and 40 years had ESFT of brain. CD99 was positive in both and FLI1 was focal positive in both. One was a *EWSR1* rearranged tumour and FISH for *EWSR1* was inconclusive in one.

Discussion

Ewing sarcoma family of tumours are aggressive mesenchymal neoplasms and constitute 6% to 8% of primary bone tumours in children and young adults with a peak incidence in the second decade and male predominance.^{7,12} In this study of 302 ESFT, male predominance (59.6%) was noted in skeletal ESFT versus the extra-skeletal ESFT, and this difference was statistically significant. Further ESFT was noted in 105 (34.77%) tumours occurring in children less than 15 years. The median age was higher (25 years) in patients with extra-skeletal tumours compared with those with skeletal (16 years) tumours, which was statistically significant. Similar observations were made earlier.^{12,13,16}

Table 2. ESFT of viscera: demographic details, site, immunohistochemical features, and FISH for EWSR1 status (n=34).

S. NO.	ORGAN	GENDER/AGE IN YEARS	IMMUNOHISTOCHEMISTRY	FISH FOR EWSR1 BREAK
1	Ovary	F/18	CD99:P; FLI1:P; LCA: N; pan CK: N; WT1: N	P
2	Ovary (post chemotherapy)	F/40	CD99:P; FLI1:P; Vimentin:P; EMA: N; Calretinin, S100, SMA: N	P
3	Ovary	F/19	CD99:P; FLI1: P	ND
4	Ovary	F/20	CD99:P; FLI1:P; LCA: N; Tdt: N; Desmin: P; Bcl2: P; SMA: N	ND
5	Cervix	F/34	CD99: P; FLI1:P; pan CK: weak	P
6	Cervix	F/5	CD99: P; FLI1:P; Desmin: N; Bcl2: P	P
7	Cervix	F/21	CD99: P; FLI1: FP; Desmin: N; Chromogranin, CD 56: FP; Synaptophysin: N; CD 34: N	P
8	Vagina	F/20	CD99: P; FLI1:P; LCA: N; pan CK: N; Desmin: N; Bcl2: N; CD 10: N	ND
9	Kidney	F/17	CD99: P; FLI1:P	P
10	Kidney	F/31	CD99: P; FLI1:P; LCA: N; Desmin: N; Bcl2: P; WT1: P; Vimentin: P	P
11	Kidney	M/25	CD99: P; FLI1:P; LCA: N; pan CK: N; WT1: N; Desmin: P; Bcl2: N; Vimentin: P	P
12	Kidney	F/30	CD99: P; FLI1:P; pan CK: N; WT1: N	P
13	Kidney	M/40	CD99: P; FLI1:P; LCA: N; pan CK: N; WT1: N; CD 10: N	ND
14	Kidney	M/30	CD99: P; Vimentin: P; CK7: N; PAX8: N	ND
15	Kidney	F/26	CD99: P; FLI1:P	ND
16	Bladder	F/4	CD99: P; FLI1:P; LCA: N; Desmin: N; Myogenin: N; MELAN A: N	P
17	Bladder	M/22	CD99: P; FLI1: FP; LCA: N; Bcl2: N	ND
18	Prostate	M/33	CD99: P; FLI1: FP; TLE1: N; Inhibin: N; SALL4: N; CD 34: N	ND
19	Breast	F/50	CD99: P; pan CK: N; Vimentin: P	P
20	Breast	F/31	CD99: P; FLI1: FP; LCA: N; pan CK: N; Vimentin: P	ND
21	Breast	F/16	CD99: P; FLI1: P; LCA: N; pan CK: N; Bcl2: N; S100: N	ND
22	Breast	F/20	CD99: P; FLI1: FP; LCA: N; pan CK: N; Vimentin: P	ND
23	Breast	M/50	CD99: N; FLI1: N; pan CK: N	P
24	Lung	M/33	CD99: P; FLI1: P; ERG: N	P
25	Lung	F/32	CD99: P; FLI1: P; TLE1: N; CD34: N	ND
26	Lung	M/34	CD99: P; FLI1: FP; CD56: N	ND
27	Lung	M/41	CD99: P; FLI1: P; Bcl2:P; TLE1: N; CD56: N	ND
28	Axillary lymph node	M/44	CD99: P; FLI1: FP; LCA: N; pan CK: N	ND
29	Cervical lymph node	M/61	CD99: ND; FLI1: P	P
30	Inguinal lymph node	M/21	CD99: P; FLI1: FP; LCA: N; Tdt: N	ND
31	Rectosigmoid colon	M/37	CD99: P; FLI1: P	ND
32	Intestine	M/11	CD99: P; FLI1: N; Chromogranin: N; CK7: N; LCA: N; CK20: N	ND
33	Brain	M/28	CD99: P; FLI1: FP; WT1: N	P
34	Brain	F/40	CD99: P; FLI1: FP; LCA: N; TLE1: N; CK7: N; CK20: N; TTF1: N; Synaptophysin: N	Failed/ inconclusive

Abbreviations: CD99, cluster of differentiation 99; CK, cytokeratin; FISH, fluorescence-in-situ hybridisation; FLI1, Friend leukaemia integration; LCA, leukocyte common antigen; N, negative; ND, not done; P, positive; TLE1, transducin-like enhancer of split 1; WT, Wilms tumour gene 1.

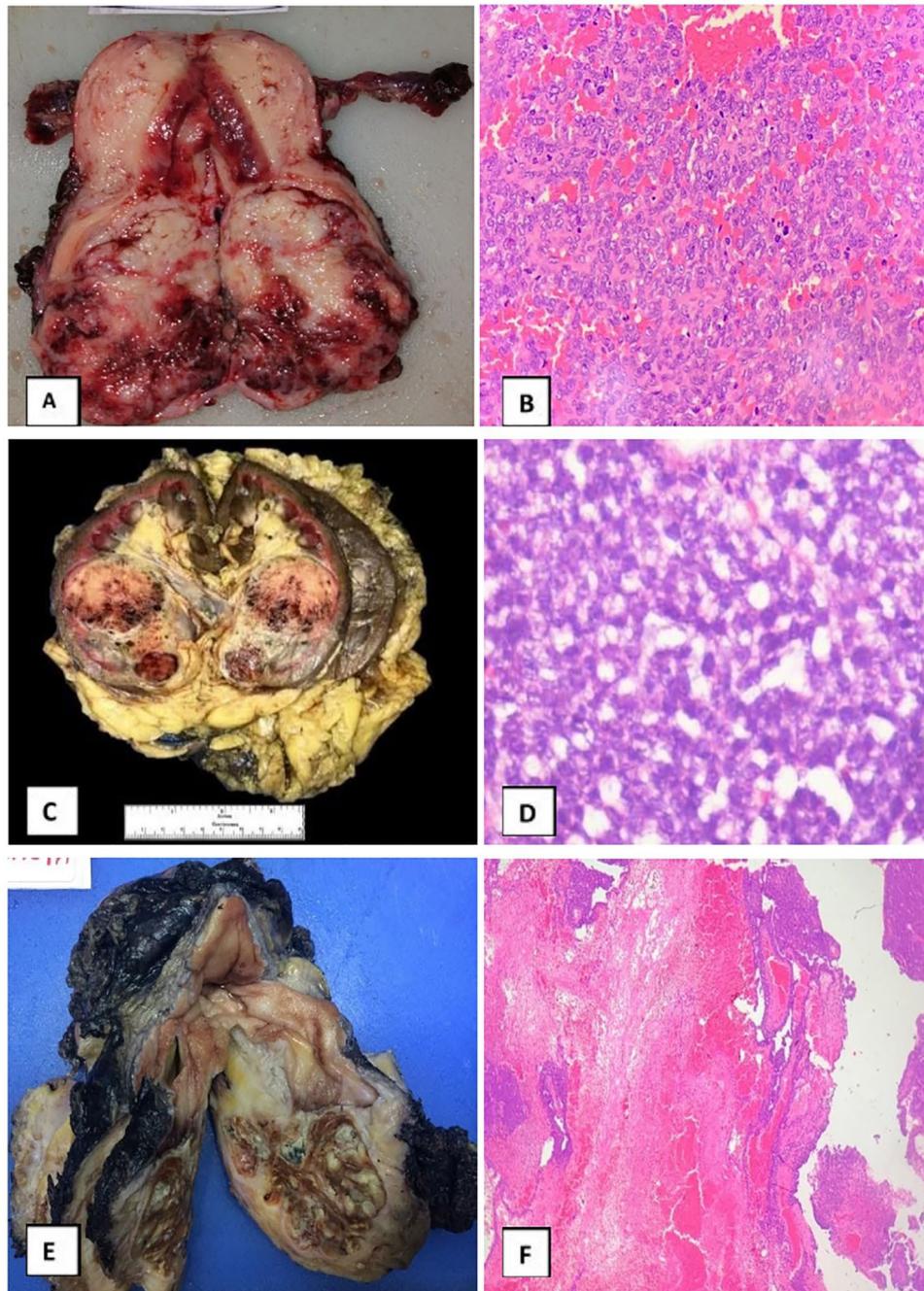


Figure 2. (A) Gross specimen of a fleshy to firm mass in cervix and lower uterine segment, (B) photomicrograph showing diffuse sheets of small round cells (hematoxylin-eosin, original magnification $\times 100$), (C) gross specimen of kidney with a circumscribed firm lesion extending from middle region to lower pole with haemorrhage and cystic areas, (D) photomicrograph showing diffuse nests and sheets of oval cells with smoky chromatin and moderate amounts of cytoplasm (hematoxylin-eosin, original magnification $\times 400$), (E) gross specimen showing polypoid mass arising from mucosal surface of urinary bladder, and (F) photomicrograph showing urothelium with submucosal sheets of round cells interspersed by areas of fibrosis (hematoxylin-eosin, original magnification $\times 100$).

The reported frequency of extra-skeletal location of tumours in various series ranged from 21% to 31% in cohort ranging from 120 to 2202 patients.^{12,14,15} Reports from India described extra-skeletal tumours in small sample size of 51 to 58 patients and described the frequency to range from 23% to 29%.¹⁹⁻²¹ In this study of 302 patients, ESFT of extra-skeletal location was seen in 50.7% with diagnosis in visceral organs being more. The relatively high frequency was probably related

to the referral bias, subspecialty reporting, and use of ancillary techniques in the diagnosis. The sites of involvement for skeletal ESFT are diaphysis or metaphyseal-diaphyseal portion of long bones such as femur, tibia or humerus, flat bones of pelvis, and rib and less commonly, bones of skull, vertebra, scapula, and short tubular bones of hands and feet.^{7,17} The number of tumours in the long bones of the extremities was 74 (49.66%), and those in axial skeleton involving flat bones was 75

Table 3. Demographic features in skeletal and extra-skeletal ESFT in different series compared with present series.

AUTHOR(S), YEAR, (REFERENCE); NUMBER, DURATION	SKELETAL	EXTRA-SKELETAL	
		SOFT TISSUES	VISCERAL
Applebaum et al ¹² ; n=2202 1973-2007	1519 (69%) M:F 63.3%:36.7% Mean age: 16.3 (0-39) y	683 (31%) M:F 53.4%:46.6% Mean age: 19.5 (0-39) y	NA
Salah et al ¹⁴ ; n=120 January, 2006 to June, 2018	91 (76%)	23 (19.17%)	6 (5%)
Cash et al ¹⁵ ; n=1029	816 (79.30%)	213 (20.70%)	NA
Rekhi et al ¹⁹ ; n=58 M: F: 65.52%: 34.48% Median age: 16 (1-65) y	Not specified	Not specified	12 (20.69%) Ovary:5 Kidney:5 Prostate:1 Vagina:1
Priya et al ²⁰ ; n=51 August 2009-April 2014 M: F: 53%:47% Mean age: 10 (2-14) y	36 (71%)	12 (23%)	3 (6%) Kidney:2 Adrenal:1
Mittal et al ²¹ ; n=58 January 2014-May 2016 M:F: 63%:37% Mean age: 20 (3-65) y	35 (61%)	17 (29%)	6 (10%) Spinal: 2 Brain: 2 Nose and nasopharynx: 1 Urinary bladder: 1
Present study n=302 September 2009 to December 2019	149 (49.34%) Median age: 16 (3-61) y	119 (39.40%) Median age: 25 (7 months-65) y	34 (11.26%)

Abbreviation: ESFT, Ewing sarcoma family of tumours.

(50.34%). Applebaum reported that axial and nonpelvic locations were more frequent in extra-skeletal tumours when compared with skeletal location, which were statistically significant.^{12,15} In this study, the extra-skeletal sites constituted 153 (50.66%) of ESFT including soft tissues in 119 (39.40%) and viscera in 34 (11.26%); however, the age and sex did not differ significantly between soft tissue and visceral location. The common sites of soft tissues involved were chest wall, extremities, paraspinal, abdomen, and others as reported in literature.^{7,12,18-22} Furthermore, involvement of the extremities between skeletal and extra-skeletal tumours was observed to be statistically significant in our study. Salah et al¹⁴ reported soft tissue location in 23 (79%). Visceral ESFTs have been described in studies with small sample size, ranging from 29 to 58 with percentage of such cases varying from 6% to 21% (Table 3).^{14,19-21}

The significant number of ESFTs in visceral location in this study may probably be due to heightened awareness and application of IHC, FISH, and RT-PCR as diagnostic methods. Molecular tests were more frequently (47.06%) performed to establish the diagnosis in visceral ESFT, when compared with skeletal or soft tissue location, highlighting the importance of ancillary techniques for accurate diagnosis.

The morphology of ESFT may be varied; however, skeletal and extra-skeletal ESFTs are indistinguishable and are defined

by the same molecular alteration. However, depending on the age, site, morphology, and differentiation, especially in extra-skeletal location, the differential diagnosis varies, necessitating a panel of IHC markers. The diagnosis of round cell sarcoma as ESFT is routinely established by diffuse membrane positivity of CD99 along with nuclear positivity of FLI1 and negative LCA.¹⁹ NKX2.2 has been established as a highly sensitive but only moderately specific marker for ES.²³ In this study, positivity of both CD99 and FLI1 along with other IHC markers was considered for diagnosing a tumour as ESFT when diagnosis was based on IHC. NKX2-2 is a new marker and was not done during that study period. FISH positivity for *EWSR1* rearrangement confirmed the diagnosis of ESFT in 31 skeletal and 23 in extra-skeletal location and in 15/34 (44.12%) tumours in the visceral location. The RT-PCR for *EWS-FLI1* fusion was positive in 19 of the extra-skeletal ESFT including one in visceral location.

Primary ESFT of the female genital tract is very rare with the most common site being the ovary, followed by the uterine corpus and rarely cervix, vagina, and vulva.²⁴⁻³² Chao et al²⁵ reported 4 cases involving the ovary and reviewed 15 published cases in the period 1980 to 2017. In the last decade of review of our cohort of 8 cases, 4 involved the ovary, 3 cervix, and 1 vagina. Diagnosis was established by morphology and IHC in all with confirmation by FISH studies in 5. The use of IHC

and FISH in the diagnosis of ESFT in the female genital tract was stressed earlier.^{24,25,29}

Primary ESFT of the kidney is rarely reported.³³⁻⁴¹ However, in the present series, involvement of kidney was seen in 7 (median age of presentation: 30 years) patients. Further ESFTs in bladder and prostate noted in this cohort were rarely reported.⁴²⁻⁴⁷

Metastatic disease involving lung, lymph nodes, and several organs has been described^{12,48,49}; however, primary ESFTs of breast,⁵⁰⁻⁵² lung,⁵³⁻⁵⁵ intestine,^{56,57} and brain^{22,58-62} were rarely reported.

Significant differences were reported in clinical presentation, treatment strategy, and outcomes for extra-skeletal ESFT compared with skeletal tumours.¹² Although the key tumour genomic features are same, the differences may arise due to subtle biologic differences in the tumour microenvironment between skeletal and extra-skeletal location.^{12,15} Extra-skeletal ESFT is an important subtype of Ewing sarcoma that may require different treatment strategies, and the prognosis is more favourable compared with skeletal tumours, independent of age, race, and primary site.¹⁵

Accurate diagnosis of ESFT is challenging when patients present with unusual clinical features, disease onset in older age, visceral location, rare sites, inconclusive, or aberrant expression of IHC markers. Confirmation of diagnosis by molecular studies may involve demonstration of gene rearrangement by FISH or chimeric fusion transcript by RT-PCR. Interphase FISH is a simple, robust, sensitive, and reliable ancillary technique, with a targeted approach, to detect the presence or absence of EWSR1 gene rearrangement, in the FFPE specimen as 82% to 92% ESFT show EWSR1 rearrangement.^{5,11} The RT-PCR has better yield on fresh tissue and has the advantage of identifying the fusion partner.¹¹ However, both the techniques can be applied to FFPE tissues, are complementary to each other, and a combination of the techniques was recommended to enhance the accuracy of the diagnosis.^{5-8,11} Both FISH and RT-PCR were applied in this study to establish diagnosis, especially for tumours in visceral and unusual locations. However, molecular techniques may not be available or are too expensive for routine use. Therefore, not all patients were subjected to molecular testing in this study. In addition, molecular techniques also have several limitations in technique and interpretation and in more than 10% of cases may not yield good-quality DNA or RNA.⁶³ A targeted enrichment strategy for library preparation for targeted next-generation sequencing (NGS) of soft tissue sarcomas has been reported to have higher sensitivity and specificity in soft tissue sarcomas.⁶⁴

The use of multiple technologies and knowhow of applying these in the right context is an intellectual and time-driven exercise for the pathologists. Accurate diagnosis and timeliness of the report are essential for appropriate management of the disease. The utilisation of these techniques in an algorithmic manner (assuming a turnaround time for histopathology and IHC to be 4 days, FISH additional 5 days, and RT-PCR where

necessary 5-7 days) may add up to the time for diagnosis significantly. This may be shortened with a planned molecular profiling by NGS which would take about 10 to 15 days but with more information and specificity. Therefore, all the techniques (IHC, FISH, RT-PCR) complement each other especially in settings where access to more sensitive technologies like anchored multiplex PCR-based NGS testing is not available.⁶⁴ Next-generation sequencing testing incorporating other fusion partners (*CIC-DUX4* and *BCOR*) may help in picking up the rare subset of tumours as well.

Conclusions

This study of a large cohort of 302 ESFT demonstrated statistically significant differences between skeletal and extra-skeletal tumours. Male sex predominance, younger age, and extremity location were noted in skeletal tumours. However, no gender predilection, older age, and axial location were seen in extra-skeletal tumours. Molecular tests were more frequently performed and contributed to the diagnosis of ESFT more frequently in extra-skeletal sites including soft tissues and viscera. High index of suspicion and appropriate use of molecular techniques help in the diagnosis of ESFT even in uncommon locations.

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