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RESEARCH

Clinicopathological and Molecular Spectrum of Ewing Sarcomas/PNETs, Including Validation of EWSR1 Rearrangement by Conventional and Array FISH Technique in Certain Cases

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Abstract Over the years, a wide clinicopathological spectrum has been identified within Ewing family of tumors (EFTs). As these tumors are chemosensitive, their correct and timely identification is necessary. The aims of this study were (1) to present the diverse clinicopathological and molecular profile of EFTs in our settings, (2) to identify a pragmatic approach for diagnosing EFTs, especially for application of ancillary techniques, namely RT-PCR for specific transcripts (EWS-FLI1, EWS-ERG) and FISH for *EWSR1* gene rearrangement, in certain cases and (3) to show the utility of tissue microarray in establishing a new FISH test. Fifty-eight EFTs were identified in 38 males and 20 females within an age-range of 1–65 years (median, 16), mostly in lower extremities (14) (24.1 %). Therapeutically, most patients underwent neoadjuvant chemotherapy with subsequent surgery. Histopathologically, diagnosis of EFTs was initially offered in 41/58 (70.6 %) tumors. On review, 59 % tumors showed diffuse pattern, while 41 % displayed rosettes. Immunohistochemically, tumor cells were mostly diffusely positive for CD99 (48/52) (92.3 %); FLI-1 (17/18) (94.4 %); variably for BCL2 (16/18) (88.8 %), synaptophysin (6/20) (35 %), S100-P (2/7) (28.5 %), CD56 (2/5) (40 %), NSE (2/5) (40 %), calponin (3/4) (75 %), EMA (5/24) (20.8 %) and CK (3/24) (12.5 %), the latter two mostly focally. Fifty five tumors were *EWS-FLI1* positive, while a single tumor was *EWS-ERG* positive. Sensitivity for PCR was 61 %. *EWSR1* rearrangement was detected by FISH in 12/13 Ewing sarcomas/PNETs. Sensitivity for *EWSR1* test was 92.3 % and specificity was 100 %. Thirty-eight tumors, including 14 molecular confirmed EFTs and 21 other tumors were tested for *EWSR1* rearrangement. Among 21 unrelated tumors, *EWSR1* rearrangement was detected in few myoepithelial tumors, occasional desmoplastic small round cell tumor and an extraskeletal myxoid chondrosarcoma. Further, a tissue microarray with a separate set of 8 EFTs, confirmed at another laboratory was analysed for validation of *EWSR1* rearrangement test. 23/28 (82.1 %) tissue cores of the tissue microarray, stained by FISH were interpretable, including *EWSR1* rearrangement, detected in 20/28 tissue cores; not detected in 3 liver cores and uninterpretable in 5 (17.8 %) cores. Classical EFTs can be diagnosed with diffuse, membranous CD99 positivity, intranuclear FLI1 positivity and LCA negativity in malignant round cells. In unconventional cases, it is indispensable to reveal the concomitant fusion m-RNA by RT-PCR. In case of negative molecular results, it is necessary to prove *EWSR1* rearrangement by FISH. These tests should be interpreted with clinicopathological correlation. Tissue microarrays for FISH are useful during validation of a new test, especially when sarcomas like EFTs show less genetic heterogeneity within tumor cells.

Keywords Ewing sarcoma · PNET · *EWS-FLI1* · *EWSR1* rearrangement · FISH in soft tissue tumors · Molecular pathology of soft tissue sarcomas · Array FISH

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Introduction

Ewing family of tumors (EFT) include Ewing sarcoma (ES), mostly of bones and of soft tissues; peripheral primitive

neuroectodermal tumor (pPNET), mostly of soft tissues; Askin-Rosai's tumor of chest wall and primitive neuroectodermal tumor (PNET) of paravertebral tissues [1–3]. ES and PNET constitute high grade round cell sarcomas that display varying degree of neuroectodermal differentiation [4]. Both these entities form part of a common spectrum and are characterized by a recurrent, non-random chromosomal translocation t(11; 22)(q24; q12) that leads to formation of a specific transcript *EWS-FLI1*, in 85–90 % cases [1, 4–6]. Remaining cases harbour several variant translocations leading to specific chimeric transcripts such as t (21;22)(q22;q12) (*EWS-ERG*), t (7;22) (q22;q12) (*EWS-ETV1*), t (17;22) (q21;q12) (*EWS-E1AF*), t(2;22) (q36;q12) (*EWS-FEV*), inv(22) (q12;q12) (*EWS-PATZ1*), t(2;22) (q31;q12) (EWS-SP3). Besides, minority of cases harbour *FUS* gene rearrangements, such as t (16; 21) (p11; q22) (*FUS-ERG*) and t (2; 16) (q36; p11) (*FUS-FEV*) [4, 7–10].

Within the *EWS-FLI1* transcript, variations in the locations of the *EWS* and *FLI1* genomic breakpoints have been noted. Nearly 12 different in-frame *EWS-FLI1* chimeric transcripts have been observed containing different combinations of exons from *EWS* and *FLI1*, resulting in various combinations of *EWS* and *FLI1* genomic breakpoints. Predominantly, the two main types are *EWS* exon 7 to *FLI1* exon 6 (Type 1) and fusion of *EWS* exon 7 to *FLI1* exon 5 (Type 2) that account for about 60 % and 25 %, respectively [7, 11].

Considering ES/PNET is chemosensitive and treated by a specific protocol (EFT2001), at certain Institutions, its correct recognition assumes importance. Despite being a round cell sarcoma that invariably afflicts paediatric patients, ES/PNET displays a wide histopathological spectrum in form of epithelial-like or 'adamantioma-like' variant, cyst formations, 'vascular tumor-like' appearance; 'hyalinising/sclerosing-type'; with anaplastic cells, clear cells and rarely displays spindle cells [12–15] . At the same time, it can affect various musculoskeletal body sites, along with unconventional sites such as central nervous system (dural-based), abdomen, kidneys, female genital tract, to name but a few [16–21]. Diagnosis of ES/PNET is typically based on analysis of histopathological features and immunohistochemical markers, most important CD99 with FLI1. However, it becomes challenging in tumors with unusual histological features, occurring at uncommon body sites, especially on limited biopsy specimens. These lead to various differential diagnoses with overlapping histopathological and immunohistochemical features. In such cases of diagnostic dilemmas there is a necessity for an objective confirmation with molecular technique, namely reverse transcriptase polymerase chain reaction (RT-PCR) and/or molecular cytogenetic technique namely, fluorescence in-situ hybridization (FISH), as testified by various studies [13, 15, 22–30].

Whereas, RT-PCR detects specific chimeric transcripts, FISH is useful in confirmation of *EWSR1* rearrangement. At the same time, *EWSR1* rearrangement is also noted in other soft tissue tumors, not related to EFTs such as desmoplastic small round cell tumor (DSRCT), clear cell sarcoma of soft parts, myoepithelial tumors, myxoid liposarcoma and angiomatoid fibrous histiocytomas [23, 31]. Several studies have indicated application of both the techniques that are complementary to each other [23, 24, 26–30]. Cost constraints, especially in lower resource settings limit routine application of conventional FISH analysis in such cases. Lately, the tissue microarray technique is being utilised for FISH examinations in certain tumors. Considering soft tissue tumors less commonly harbour genetic heterogeneity, there is a possibility for FISH on tissue microarrays for identification of gene rearrangements in a cost-effective and high throughput manner [32]. Lately, apart from conventional single fused or single split signals, complex interphase FISH patterns of *EWSR1* have been identified within certain ESs/PNETs [33].

The aims of this study were to present the diverse clinicopathological profile of a series of ESs/PNETs, including the molecular results. Further, it was intended to identify a pragmatic approach for diagnosing EFTs especially for the usage of ancillary techniques, and to prove the tissue microarray technique as useful for establishing a new FISH test in a laboratory together with interlaboratory testing for quality assurance.

Methods

Fifty-six tumors were positive for *EWS-FLI1* or *EWS-ERG* since 2006 (5 years, 9 months), as per records of our pathology database. Two cases were confirmed with only *EWSR1* rearrangement by FISH technique. During this period, a total of 139 tumors were subjected for *EWS-FLI1* analysis and 12 tumors were subjected for *EWS-ERG* analysis. Clinical details were accessioned from case files, electronic medical record (EMR) and hospital Diagnostic Information System (DIS).

Histopathologically, diagnosis of ES/PNET, preceding molecular confirmation was offered in 41/58 (70.6 %) cases. In the remaining 17 (29.3 %) cases, initial histopathological diagnoses included differential diagnosis of ES/PNET versus synovial sarcoma (7) cases, including preference for the latter in 2; undifferentiated sarcoma/round cell tumor (4); DSRCT (2); ES/PNET versus neuroendocrine carcinoma (1); ES/PNET versus sex-cord stromal tumor (1); ES/PNET versus synovial sarcoma versus DSRCT (1) and ES/PNET versus neuroblastoma versus neuroendocrine carcinoma (1).

Conventional hematoxylin and eosin (H & E) stained microsections were available in all cases at the time of initial reporting. Immunohistochemical results were available and contributory in 55 (94.8 %) tumors. However, at the time of review (by B.R.), H & E stained microsections from 44 tumors were available, considering ours being a tertiary cancer centre with

our policy of returning back the H & E slides and or tissue blocks after ancillary testing in certain cases, whenever these are requested for returning.

Immunohistochemistry (IHC)

IHC was performed by immunoperxoidase method using MAC H2 Universal HRP-Polymer detection kit, Biocare, CA, USA, including 3′-3′-diaminobenzidine tetrahydrochloride (DAB) as the chromogen. Appropriate positive and negative controls were included. The details of the various antibody markers are enlisted in Table 1.

Fifty six (96.5 %) cases were confirmed by molecular test.

Molecular Testing by Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) Technique

Biopsy material in form of paraffin blocks was submitted for molecular analysis using ribonucleic acid (RNA) isolation and reverse transcription polymerase chain reaction (RT-PCR). Total RNA was isolated from formalin-fixed, paraffin-embedded tissue sections (FFPE tissue) using a Recover All Total Nucleic Acid Isolation kit (Ambion, USA). The extracted RNA was then treated with RNase-free DNase I before cDNA preparation. RNA was then reverse transcribed into cDNA using the Superscript First strand synthesis system (Invitrogen). A total of 500 nanograms of total RNA was briefly reverse transcribed into cDNA using random hexamers at 42 °C for 50 min, which was then followed by 70 °C for 15 min. The synthesized cDNA was then treated with RNase H for 20 min at 37 °C to remove the RNA-DNA hybrids.

A total of 2 μl from the reaction was PCR amplified using *EWS* 22.3 forward primer (5′-TCC TAC AGC CAA GCT CCA AGT C-3′) and FLI1 11.3 reverse primer (5′-ACT CCC CGT TGG TCC CCT CC-3′) in a 20-μl reaction volume containing 10 pmol each of the forward and reverse primers, for a total of 20 μl PCR master mix (Qiagen, Germany) [34, 35]. PCR conditions from the samples were as follows: 35 cycles of 94 °C for 30 s, 65 °C for 1 min, and 72 °C for 1 min. Amplified PCR products were checked in 10 % polyacrylamide gel and stained with silver nitrate. Two positive controls (*EWS-FLI1* type-I and type- II PCR product cloned into pTZ57R/T vector) and one water-only (no cDNA) negative control were included in each run. To check the quality and integrity of the cDNA, FOXO1A was amplified as a housekeeping gene (FKH-F: 5′ CAT CCC CTT CTC CAA GAT CA 3′; FKH-R: 5′ GCT GCC AAG AAG AAA GCA TC 3′). The chimeric gene *EWS-FLI1* was subtyped as Type I, *EWS* (exon 7) with *FLI1* (exon 6) of 330 bp size and Type II, *EWS* (exon 7) with *FLI1* (exon 5) of 394 bp size. The chimeric gene *EWS-ERG* as a result of t(21; 22) (q22; q12) was subtyped as Type I, *EWS* (exon 7) with *ERG* (exon 6) of 433 bp size and Type II, *EWS* (exon 7) with *ERG* (exon 7) of 364 bp size.

Molecular cytogenetic testing was performed in 38 tumors, for EWSR1 rearrangement. EWSR1 rearrangement was tested in 17 ESs/PNETs, including 14 cases confirmed by molecular testing (RT-PCR). Two cases (56 and 57) were only tested by molecular cytogenetic testing, apart from IHC.

Molecular Cytogenetic Analysis

Molecular cytogenetic analysis by fluorescence in situ hybridization (FISH) with Vysis LSI *EWSR1* dual color break apart,

Table 1 List of various antibody markers in the present study

Sr no.	Antibody marker	Clonality, clone	Dilution	Antigen retrieval	Manufacturer
1	MIC2/CD99	Monoclonal,12E7	1:100	Heat (Tris-EDTA) Pascal	Dako, Produkionsveg, Glostrup, Denmark
2	FLI1	Polyclonal	1:50	Heat (Sodium citrate) Pressure cooker	Biocare, CA, USA
3	BCL2	Monoclonal, 124	1:50	Heat (Tris-EDTA) Microwave	Dako
4	Synaptophysin	Polyclonal	1:100	Heat (Tris-EDTA) Pascal	Thermo Scientific, USA
5	S-100P	Polyclonal	1:1500	Heat (Tris-EDTA) Pascal	Dako
6	CD56	Monoclonal, Bc56C04	1:50	Heat (Tris-EDTA) Pascal	Dako
7	Neuron-specific enolase (NSE)	Monoclonal,BsNcHI4	1:100	Enzymatic, Pepsin	Dako
8	Calponin	Monoclonal, CALP	1:50	Heat (Tris-EDTA) Microwave	Dako
9	Epithelial membrane antigen (EMA)	Monoclonal, E 29	1:200	Heat (Tris-EDTA) Pascal	Dako
10	Cytokeratin (CK)	Monoclonal, MNF116	1:200	Heat (Tris-EDTA) Pascal	Dako
11	Desmin	Monoclonal,D33	1:200	Heat (Tris-EDTA) Pascal	Dako
12	Vimentin	Monoclonal, V9	1:400	Heat (Tris-EDTA) Microwave	Dako

probe (Abbott Molecular, Chicago, Illinois, USA)) was performed on 4 μ thick paraffin-embedded tissue sections in 39 different tumors, diagnosed at TMH, Mumbai. Processed sections were finally stained with 4′-6-Diamidino-2-phenylindole (DAPI) and examined under a fluorescent microscope (Carl Zeiss, Axio Imager Z1, Germany), using AxioCam MRc5 camera and AxioVision Rel 4.5 software. Tumor sample was considered positive for *EWSR1* rearrangement if more than 15 % of 100 nuclei showed rearrangement/"break-apart"/"split" [24]. A total of 100 tumor nuclei in each case were scored for analysis. All cases were interpreted by B.R.

FISH Tissue Microarray

A tissue microarray for *EWSR1* FISH testing was constructed from cases diagnosed as Ewing sarcoma/PNET at the Institute of Pathology, University Hospital Tuebingen, Eberhard-Karls-University, Germany. According to the technique of Wilkens (Wilkens L. German patent: DE 102 03 524 A1.) and Chen et al. [36], a double sided adhesive tape (Knutsel-tape, 2.75 m, 38 mm, Tesa AG, Hamburg, Germany) was attached to x-ray film. With a biopsy punch (Kai industries Co. Ltd, Japan) 2 mm in diameter the paraffin tissue cores were extracted from the routine paraffin blocks of Ewing sarcomas (donor blocks) and transferred to the adhesive tape by freehand. The position of the cores was registered in an Access file (Microsoft Inc., Redmond, WA, USA). Four lanes (1 to 4), comprising 7 tissue cores each (a to g) were included in the microarray block. First 3 cores of lane 1 (3a to 3c) were liver tissues. Thereafter, tumor cores from 8 cases were placed. The number of cores for individual case was 4, 2, 3, 6, 3, 1, 2 and 4, respectively. Most cores were representative of another tumor area from the donor block, along with cores from more than 1 block in certain cases, wherever available. Then the composite of the x-ray film, the adhesive tape and the upright standing paraffin tissue cores was put in an ordinary steel embedding mould filled with liquid paraffin and completely molten in order to get a strong contact between the paraffin core biopsies and the surrounding paraffin of the later TMA. The paraffin tissue cores remained in an upright position due to the fixation by the adhesive tape. After solidification of the paraffin at room temperature and then at 4 °C in the refrigerator, the TMA block was released from the steel mould and the x-ray film together with adhesive tape detached from the TMA block using a dissecting needle. Cutting and staining of the TMA block was done according to routine procedures.

Array slide was stained with H & E and FISH for *EWSR1*, at both the Institutes, as per aforementioned protocol and was analysed by two authors (U.V. and B.R.).

Sensitivity for both PCR and FISH were calculated, considering unequivocal clinicopathological diagnosis of ES/PNET. Specificity for *EWSR1* test was also calculated.

Results

Fifty-eight tumors occurred in 38 males and 20 females (M: F, 1.9:1) within age-range of 1–65 years (mean, 20.4, median, 16). Site-wise most tumors occurred in lower extremities (14) (24.1 %), including gluteal region, thigh, leg, foot; followed by head and neck region (10) (17.2 %), including neck, supraclavicular region, clavicular region, nose, temporal soft tissues; central nervous system (3) (5.1 %); upper extremities (8) (13.7 %), including axilla, arm, forearm, hand; chest wall (6) (10.3 %), including back, rib, lung, mediastinum; abdomen and pelvis, including ovary (5) (8.6 %); kidney (5) (8.6 %); spine, including paravertebral tissues, sacrococcyx (4) (6.8 %) and a single tumor, each in vagina, prostate and at an unspecified site, respectively. Tumor size, known in 19 (32.7 %) cases, varied from 2.2 to 14.3 cm (mean, 7.4, median, 6.9).

On histopathological review of 44 tumors, most showed diffuse and/or nesting arrangement (26) (59 %), while 18 (40.9 %) tumors displayed rosetting arrangement of tumor cells. Variable tumor necrosis and desmoplastic stroma was noted in certain tumors. Tumor cells were mostly round with scanty to moderate ill to well-defined cytoplasm and homogeneous to speckled nuclear chromatin with indistinct or barely discernible nucleoli in most tumors Variable histological features were conspicuous vascular tumor-like arrangement (5) (11.3 %), atypical-type with cells exhibiting prominent nucleoli and numerous mitoses (2) (4.5 %), sclerosing-type (1) (2.2 %), prominent cytoplasmic clearing in 10 (22.7 %) tumors, spindly cells in 4 (8.8 %), and anaplastic cells in 1 (2.2 %) Dystrophic calcification was noted in 4 and "azzopardi effect" defined as blood vessels in a necrotic tumor area stained and highlighted with DNA originating from the necrotic tumor cell nuclei was seen in a single tumor. Immunohistochemically, tumor cells were mostly diffusely positive for CD99 (48/52) (92.3 %), followed by FLI-1 (intranuclear positivity) (17/18) (94.4 %); variably positive for BCL2 (16/18) (88.8 %), synaptophysin (6/20) (35 %), S-100P (2/7) (28.5 %), CD56 (2/5) (40 %), NSE (2/5) (40 %), calponin (3/4) (75 %), EMA (5/24) (20.8 %) variably and CK (3/24) (12.5 %), latter two, mostly focally. Tumor cells were positive for either of the epithelial markers in (6/26) (23 %) cases.

Fifty five tumors were *EWS-FLI1* positive, while a single tumor was *EWS-ERG* positive. Overall sensitivity for PCR was 61 %.

Bone marrow involvement was noted in 1/30 (3.3 %) cases.

EWSR1 rearrangement was detected in 10/11 molecular confirmed tumors, where results were interpretable. It was detected in another 2 tumors that were clinicopathologically ESs/PNETs, but lacked molecular confirmation (cases 56 and 57). In another case that was clinicopathologically diagnosed

Table 2 Clinicopathological, immunohistochemical and of 58 Ewing sarcomas/PNETs, including molecular confirmation in 54 cases and *EWSR1* rearrangement results in select cases

Sr no.	Age/Sex	Site	IHC results	Diagnosis	Molecular result, *EWSR1* result	Treatment	Outcomes
1	37/M	Chest wall	MIC2-N, EMA-N, CK-N, Synap-N, NSE-N	Undiff. RCS, Ewing/PNET	*EWS-FLI1*P	R0 excision	AWD (12 mo.)
2	24/M	Spine	MIC2-P	Ewing/PNET	*EWS-FLI1*P	NK	NK
3	11/M	Neck	MIC2-P	Ewing/PNET	*EWS-FLI1*P	R0(Post CT)	FOD(52 mo.)
4	65/M	Gluteal	MIC2-P, BCL2-P, CK-N, EMA-N, Synap-N	Ewing/PNET	*EWS-FLI1*P	NK	NK
5	10/F	Tibia	NC	RCT	*EWS-FLI1*P	R0(Post CT)	NK
6	15/M	Supraclavicular	MIC2-P, Synap-N, CK-N, EMA-N	Ewing/PNET	*EWS-FLI1*P	NK	NK
7	17/M	Pleura	MIC2-P	Ewing/PNET	*EWS-FLI1*P	R0(Post CT)+RT	AWD (9 mo.)
8	19/M	Clavicular	MIC2-P	Ewing/PNET	*EWS-FLI1*P	R0(Post CT)	AWD (12 mo.)
9	10/M	Thigh	MIC2-P, BCL2-P, CK-N, EMA-N, Synap-N	Ewing/PNET VS Synovial sarcoma	*EWS-FLI1*P	Rx(Post CT)	NK
10	1/F	Axilla	MIC2-P, CK-FP, BCL2-P, Synap-N	Ewing/PNET	*EWS-FLI1*P	Rx(Post CT)	NK
11	15/M	Gluteal	MIC2-P, BCL2-P, Calponin-P	Ewing/PNET	*EWS-FLI1*P	Post CT	AWD (13 mo.)
12[a]	3/M	Rib	MIC2-P,FLI1-P, BCL2-P,CK-N, EMA-N, Calponin-N	Ewing/PNET VS Synovial sarcoma	*EWS-FLI1*P, Not worked	Rx(Post CT)+RT	FOD (39 mo.)
13	52/F	Forearm	NK	Ewing/PNET	*EWS-FLI1P*	R0(Post CT)	FOD (36 mo.)
14	17/M	Sole	EWS-FLI1P	Ewing/PNET	*EWS-FLI1*P	Rx(Post CT)+RT	FOD(16 mo.)
15	9/M	Abdomen	Desmin-P, NSE-P, S100-P-P, CK-N, EMA-N	DSRCT	*EWS-FLI1*P	NK	FOD(129mo)
16[a]	28/F	Ovary	MIC2-P, FLI1-P, CK-N, EMA-N, Synap-N	Ewing/PNET over sex-cord stromal	*EWS-FLI1*P, Detected	Rx(Post CT)+RT	DOD (15 mo.)
17	37/M	Arm	MIC2-P,FLI1-P, BCL2-P, CK-N, EMA-N, Synap-N, CD56-N, FLI1-P	Ewing/PNET over Synovial sarcoma	*EWS-FLI1*P	Rx(Post CT)	FOD (39 mo.)
18	20/F	Vagina	MIC2-P, BCL2-P, FLI1-P, EMA-N, CK-N	Ewing/PNET	*EWS-FLI1*P	Post CT+RT	FOD (9 mo.)
19	16/M	Toe	MIC2-P, S100-P-P, Vimentin-P, CK-N	Ewing/PNET	*EWS-FLI1*P	Rx(Post CT)+RT	AWD (131 mo)
20	25/F	Brain	MIC2-P, Vim-P, CK-P, EMA-P, Synap-N	Ewing/PNET	*EWS-FLI1*P	R0(Post CT)+RT	FOD(39 mo.)
21	33/M	Inguinal/Abdomen	MIC2-P	Ewing/PNET	*EWS-FLI1*P	R1(Post CT)	NK
22	35/F	Kidney	MIC2-P, CK-N, EMA-N, BCL2-N	Ewing/PNET VS Synovial sarcoma	*EWS-FLI1*P	NK	NK
23	25/M	Mediastinum	MIC2-P, FLI1-P, LCA-N, CD3-N	Ewing/PNET	*EWS-FLI1*P	NK	AWD (9 mo)
24	8/F	Lung	MIC2-P, LCA-N	Ewing/PNET	*EWS-FLI1*P	Rx(Post CT)+RT	FOD (36 mo)
25	25/F	Tibia	MIC2-P, FLI1-P	Ewing/PNET	*EWS-FLI1*P	R0(Post CT)+RT	FOD (9 mo.)
26	55/M	Prostate	MIC2-P, Synap-N, CK-N, EMA-N	Ewing/PNET	*EWS-FLI1*P	Post CT	FOD (9 mo.)
27[a]	28/F	Kidney	MIC2-P, CD56-P, EMA-P, CK-N, BCL2-N, S100-P-N	Ewing/PNET VS Neuroendocrine ca.	*EWS-FLI1*P, Detected	NK	NK
28[a]	3/M	Brain	MIC2-N, Synap-P, EMA-N	Ewing/PNET	*EWS-FLI1*P, Detected	Post CT	AWD (12 mo.)
29	6/M	Calcaneum	EWS-FLI1P	Ewing/PNET	*EWS-FLI1*P	Post CT	FOD (2 mo.)
30	1/F	Sacrococcygeal	MIC2-P, FLI1-N, Synap-N	Ewing/PNET	*EWS-FLI1*P	NK	On FU
31	6/M	Neck	MIC2-P,S100-P-N, CK-N, EMA-N	Ewing/PNET	*EWS-FLI1*P, Not worked	Post CT+RT	FOD (15 mo.)
32	27/M	Thigh	MIC2-P, Calponin-P, EMA-P, CK-N, S100-P-N	Synovial sarcoma favoured	*EWS-FLI1*P	Post CT	FOD(14 mo.)
33[a]	31/M	Abdomen	MIC2-P, EMA-P, CK-N, NSE-N	DSRCT favoured	*EWS-FLI1*P, Detected	Rx	NK
34[a]	18/F	Pelvis	MIC2-P, BCL2-P	Ewing/PNET	*EWS-FLI1*P, Detected	NK	NK
35	11/F	Oropharynx	MIC2-N, S100-P-N	Undiff. RCS	*EWS-ERG*P	NK	NK

Table 2 (continued)

Sr no.	Age/Sex	Site	IHC results	Diagnosis	Molecular result, *EWSR1* result	Treatment	Outcomes
36[a]	6/F	Neck	MIC2-P, EMA-P, CK-P, Synap-P	PD Synovial sarcoma favoured	EWS-FLI1P, Detected	NK	NK
37[a]	4/M	Humerus	MIC2-P, Vim-P	Ewing/PNET	EWS-FLI1P, Not worked	Rx(Post CT)	NK
38[a]	16/M	Ankle	MIC2-P, BCL2-P, EMA-N, CK-N, Calponin-P	RCT	*EWS-FLI1*P, Detected	Rx(Post CT)	AWD (17 mo.)
39	10/M	NK	MIC2-P, FLI1-P	Ewing/PNET	*EWS-FLI1*P	NK	NK
40	12/M	Radius	MIC2-P, FLI1-P, CD56-N, Synap-N	Ewing/PNET	*EWS-FLI1*P	R0(Post CT)	FOD (5 mo.)
41	20/M	Spine	MIC2-P, BCL2-P, Synap-N, CD56-N NSE-N	Ewing/PNET	*EWS-FLI1*P	NK	NK
42	8/M	Retrobulbar	NC	Ewing/PNET	*EWS-FLI1*P	NK	NK
43	30/F	Hand	MIC2-P, BCL2-P, FLI1-P, EMA-N, CK-N, S100-P-N	Ewing/PNET	*EWS-FLI1*P	NK	NK
44	18/M	Hand	MIC2-P, BCL2-P	Ewing/PNET	*EWS-FLI1*P	NK	NK
45	22/F	Brain	MIC2-P, FLI1-P, Synap-N	Ewing/PNET	*EWS-FLI1*P	NK	NK
46	37/F	Kidney	MIC2-P, BCL2-P, FLI1-P	Ewing/PNET	*EWS-FLI1*P	NK	NK
47	54/M	Kidney	MIC2-P, CK-N, EMA-N, BCL2-N	Ewing/PNET	*EWS-FLI1*P	NK	NK
48	18/M	Elbow	MIC2-P, BCL2-P, S100-P-N, CK-N, EMA-N	Ewing/PNET	*EWS-FLI1*P	Post CT	AWD (6 mo.)
40	18/F	Kidney	MIC2-P, BCL2-P, FLI1-P, EMA-N	Ewing/PNET	*EWS-FLI1*P	NK	NK
50[a]	35/M	Thigh	MIC2-P, FLI1-P, Synap-P	Ewing/PNET	*EWS-FLI1*P, Detected	Post CT	NK
51[a]	1/M	Hip	MIC2-P, EMA-P, NSE-P	Ewing/PNET, DSRCT, Synovial sarcoma	*EWS-FLI1*P, Not Detected	Rx(Post CT)	FOD (NK)
52[a]	33/M	Knee	MIC2-P, BCL2-P, FLI1-N, Synap-N	Synovial sarcoma VS. Ewing/PNET	*EWS-FLI1*P, Detected	NK	NK
53	5/M	Presacral	MIC2-P, FLI1-P	Ewing/PNET	*EWS-FLI1*P	Post CT	AWD (3 mo.)
54	21/M	Tibia	MIC2-P, BCL2-P, FLI1-P, EMA-N, CK-N	Ewing/PNET	*EWS-FLI1*P	Post CT	AWD (3 mo.)
55	24/F	Nose	MIC2-P, Synap-P, CD56-P, Chromo-P	Neuroendocrine, Neuroblastoma, Ewing/PNET	*EWS-FLI*1P	Ro(Post CT,RT)	AWD (12 mo.)
56[b]	4/F	Nose	MIC2-P, FLI1-P,Synap-P	Ewing/PNET	Detected	Ro(Post CT)	AWD (7 mo.)
57[b]	32/M	Scapula	MIC2-P, FLI1-P, Synap-FP	Ewing/PNET	Detected	Rx(Post CT)	NK
58[a]	14/M	Temporal	MIC2-P	Ewing/PNET	*EWS-FLI1*P, Detected	NK	NK

M male, *F* female, *P* positive, *N* negative, *NC* not contributory, *EWS-FLI1P* not performed, *NK* not known, *EMA* epithelial membrane antigen, *CK* cytokeratin, *Synap* synaptophysin, *NSE* neuron specific enolase, *S100-P* S100-Protein, *Chromo* chromogranin, *DSRCT* desmoplastic small round cell tumor, *RCT* round cell tumor, *Undiff. RCS* undifferentiated round cell sarcoma, *ca* carcinoma, *NA* not Available, *EWS-FLI1*P EWS-FLI1 positive, *EWS-ERG*P EWS-ERG positive, *CT* chemotherapy, *RT* radiotherapy, *AWD* alive with disease, *DOD* died of disease, *FOD* free of disease, *mo* months

[a] Case with molecular confirmation and *EWSR1* test

[b] Cases with *EWSR1* positive result

ES/PNET, without molecular confirmation, *EWSR1* test was uninterpretable. Overall, *EWSR1* rearrangement by FISH was detected in 12/13 ESs/PNET, forming sensitivity of 92.3 %. Its specificity, including tumors that do not form histopathological differential of ES/PNET was 100 %.

Among 21 other tumors, *EWSR1* rearrangement was detected in myoepithelial tumors (3/7), desmoplastic small round cell tumor (DSRCT) (1/1), extraskeletal myxoid chondrosarcoma (1/3) and was negative in other 10 unrelated tumors (negative controls) like infiltrating ductal carcinomas (2), alveolar rhabdomyosarcomas (2), synovial sarcomas (4), pleomorphic sarcoma (1) and prostate neuroendocrine carcinoma (1), the latter case that was suspected as PNET on histopathology (Table 2, Figs. 1, 2, 3, 4, and 5).

Most tumors with *EWSR1* rearrangement displayed single or 'double-split' signals. However, 3 tumors revealed complex patterns, including multiple split signals (2) and multiple red signals (1), apart from rearrangement in that same case (case33) (Figs. 4 and 5).

FISH Tissue Microarray Results

The results have been enlisted in Table 3. Overall, results were interpretable in 23/28 (82.1 %) cores, including *EWSR1* rearrangement that was detected in 20/28 tissue cores; was not detected in 3 cores of liver tissue and was uninterpretable in 5 (17.8 %) cores. Overall discrepancy between two authors was minor in 2 cases and major in a single case, all that were reviewed and finally resolved (Table 3, Fig. 6).

Treatment details were available in 36 (62 %) patients. Most patients (25) (69.4 %) underwent neoadjuvant chemotherapy (CT) with surgery, followed by adjuvant RT in 9 of the 25 cases. Nine patients underwent chemotherapy and remaining 2 patients underwent CT and RT, with unavailable details regarding further surgical intervention. Type of surgical resection was complete (R0) in 11 cases, was not known (Rx) in 13 cases and was marginal (R1), including microscopically positive margin in a single case.

Outcomes were known in 30(51.7 %) patients over 2–131 months (mean, 20.4, median, 12). These included 13 patients alive with disease (AWD) over 3 to 131 months (mean, 18.9, median, 12), 16 patients free of disease (FOD) over 2 to 52 months (mean, 22.1, median, 15) and a single patient, who died of disease (DOD) within 15 months (Table 2).

Discussion

The present study forms a sizable documentation of the Ewing family tumors (EFT: ESs/PNETs), confirmed by molecular and/or FISH technique and testifies a diverse clinicopathological spectrum of these tumors in form of wide age-range, various body sites of involvement and diverse morphological patterns [13–31]. Besides, this forms the second documentation on utility of microarray FISH for detecting *EWSR1* rearrangement during validation of this test [37].

Identification of a diverse clinicopathological spectrum within ESs/PNETs at a tertiary referral centre and at the same time, availability of limited resources necessitates formulation a pragmatic approach for application of ancillary testing.

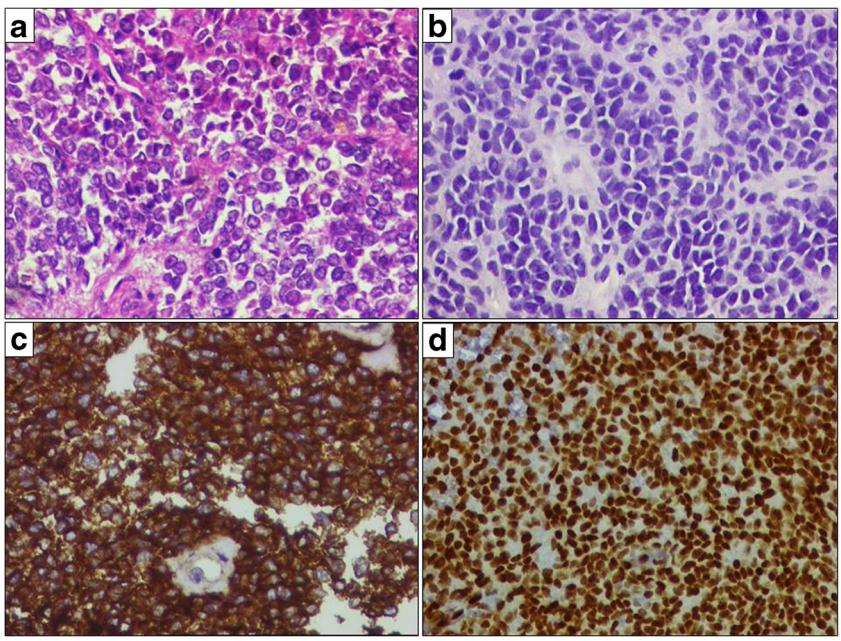

Fig. 1 **a** Case 36. Classical Ewing sarcoma (ES) comprising uniform round cells with fine chromatin, scanty indistinct cytoplasm, and inconspicuous nucleoli arranged in a diffuse matrix with minimal stroma. H & E×400. **b** Case 55. PNET displaying uniform round cells with hyperchromatic nuclei arranged in typical pseudorosettes. H & E×400. **c** Diffuse, membranous CD99 positivity in ES/PNET. Diaminobenzidine (DAB)×400. **d** Diffuse, intense, intranuclear FLI1 positivity in ES/PNET. DAB×400

Routinely, diagnosis of ES/PNET in our laboratory is finalized on histopathology in classical cases with diffuse, membranous CD99 positivity in a malignant round cell sarcoma, along with intranuclear FLI1 positivity and LCA negativity.

However, CD99 is also expressed by other tumors such as synovial sarcoma, rhabdomyosarcoma, lymphoblastic lymphoma, melanoma, to name, but a few. These entities are differentiated on the bases of other specific markers that they display. In our recent study, we observed CD99 positivity in 74.1 % synovial sarcomas. FLI1 is also expressed in other tumors like vascular tumors, lymphomas, and to some extent in rhabdomyosarcomas and synovial sarcomas. Earlier CD99 was a routine diagnostic marker for paediatric sarcomas [38]. Despite its reasonable sensitivity for EFTs, its lower specificity has been observed and documented over the years [39]. Among 58 molecular confirmed study cases, CD99 was positive in 92 % cases and FLI1 in 94 % cases. Sensitivity for these two markers in various studies ranged from 90 to 100 % for CD99 [13, 24, 26, 28, 40–42] and 71 to 94 % for FLI1 [13, 26, 39, 41, 42], respectively. It is noticeable that CD99 can be rarely negative in EFTs. In such cases, additional newer markers like caveolin-1 have been found to be useful in enhancing objectivity for diagnosis of ES/PNET [15].

In the present study, epithelial differentiation in certain tumors was reinforced with epithelial markers in 23 % cases. Earlier investigators have noted various epithelial markers like CK/MNF116, pan cytokeratin, high molecular weight cytokeratin and EMA in 5-32 % Ewing sarcomas/PNETs [13, 40]. Schuetz et al. [14] provided additional evidence for epithelial differentiation in certain Ewing sarcomas/PNETs by

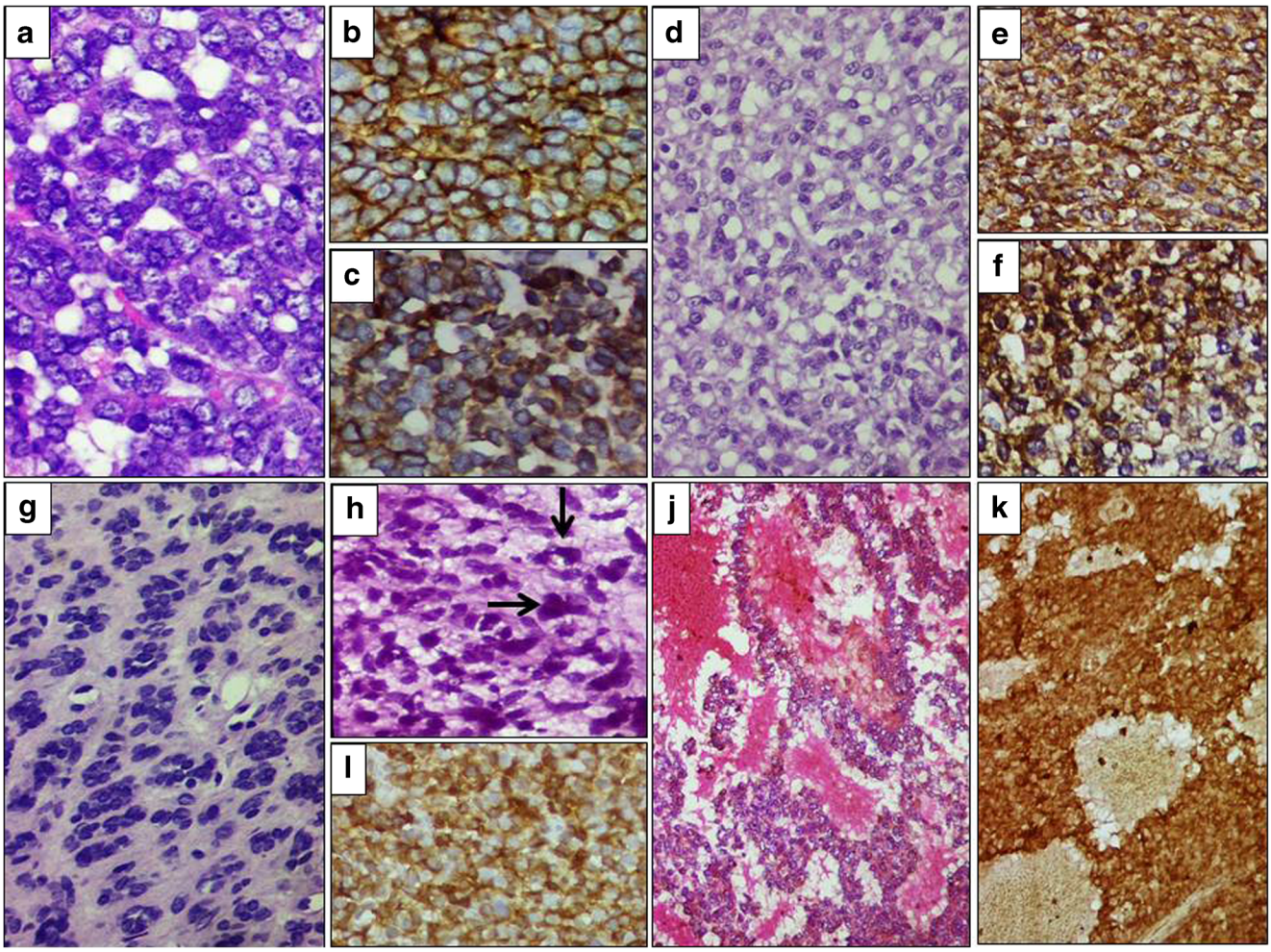

Fig. 2 Case 44 (**a**–**c**). **a** Atypical PNET displaying cells with vesicular to hyperchromatic nuclei and prominent nucleoli. H & E×400. **b** Diffuse, membranous CD99 positivity (same case). DAB×400. **c** Diffuse BCL2 positivity within tumor cells. DAB×400. Case 33 (**d**–**f**). **d** Tumor cells with well-defined cell membranes and abundant clear cytoplasm. H & E×400. **e** Diffuse EMA positivity. DAB×400. **f** Membranous CD99 positivity. DAB×400. **g** (Case 52). Hyalinizing/sclerosing variant of Ewing sarcoma/PNET with clusters of round cells in a sclerotic stroma. H & E×200. Case 21 (**h**–**i**). Tumor composed of spindly and anaplastic cells (*arrows*). H & E×400. **i** Diffuse CD99 positivity. DAB×400. Case 47 (**j**–**k**). **j** Tumor cells arranged in form of a vascular tumor. H & E×200. **k** Diffuse membranous CD99 positivity. DAB×200

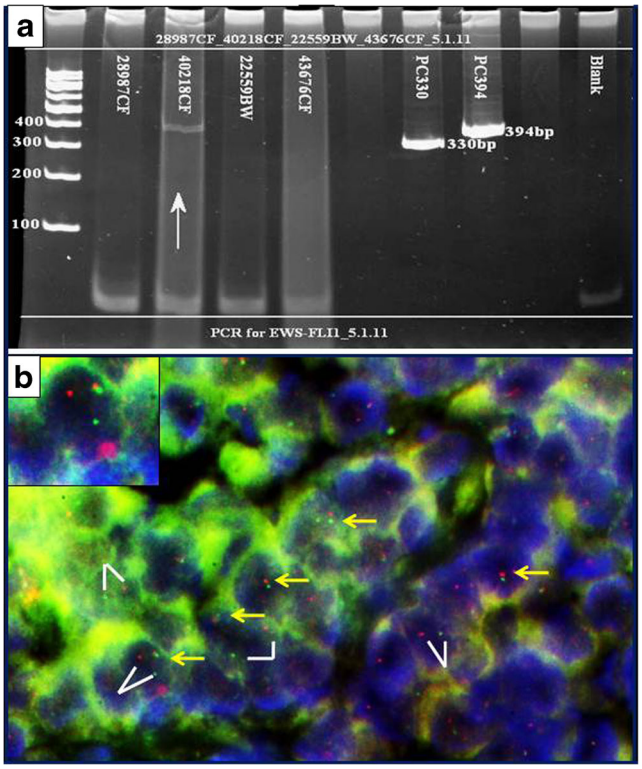

Fig. 3 Case 36. **a** ES comprising uniform round cells with fine chromatin, arranged in a diffuse pattern. H & E×400. **b** On FISH, *EWSR1* rearrangement was detected in form of single red-green split signals (*double lines*) in several tumor nuclei with single intact allele in form of fused signals (*single arrow*). DAB×1,000. *Inset* Single tumor nucleus displaying copy of intact allele (fused signal) and rearranged (red-green split signal)

demonstrating intercellular junctions with help of markers in claudin-1, occludin, ZO-1 and others in certain cases.

Apart from CD99 and FLI1 that comprise most important markers for diagnosing ES/PNET, synaptophysin NSE and CD56 constituted as additional surrogate markers. Kavalar et al. [40] observed 66.6 % positivity with NSE and 25.4 % positivity with S-100P in ESs/PNETs. BCL2 was one of the most commonly observed IHC marker in 88.8 % of our study cases, while it was found to be positive in 70.1 % cases in an earlier study [16]. BCL2 has a diagnostic value in substantiating diagnosis of synovial sarcomas that can form differential diagnoses of EFTs. In another study, we observed BCL2 positivity, mostly diffusely in 97.3 % synovial sarcomas. Considering variable expression of BCL2 is seen in several ESs/PNETs, its diffuse expression is more indicative of synovial sarcomas.

The most common differential diagnosis, necessitating application of additional molecular study in the present series was poorly differentiated synovial sarcoma. Other differentials include DSRCT, neuroblastoma, medulloblastoma (in supratentorial, dura-based PNETs), neuroendocrine carcinoma, epithelioid solitary fibrous tumor, rhabdoid tumors, and undifferentiated sarcomas, to name but a few. Invariably, optimal IHC markers are useful in sorting out these different tumor entities.

Within various documented studies, range of sensitivity and specificity for CD99 [13, 24, 26, 28, 38–41] was 90–100 % and 58.3 %; for FISH (*EWSR1* rearrangement) [23, 24, 26, 29, 37, 41], 50 %–96.3 % and 81.8 %–100 %, and for

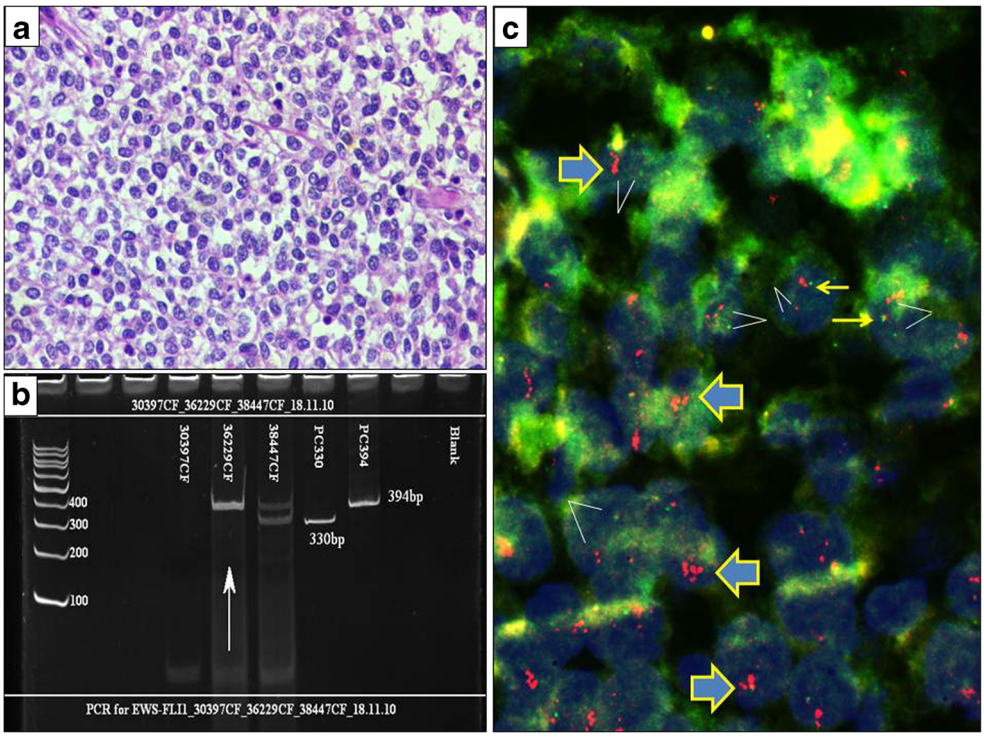

Fig. 4 **a** Case 33 (**a**–**c**). ES displaying cells with abundant clear cytoplasm arranged in a diffuse pattern. H & E×200. **b** PCR reaction displaying *EWS-FLI1* positivity in form of band (*arrow*) (Type II of 394 bp size). Positive controls on right-sided lanes of test case have been labeled as 330 bp (Type I) and 394 bp (Type II). Negative control in form of a negative case on left-sided lane of the test case and blank in the last column. **c** *EWSR1* rearrangement detected (*double lines*), along with complex signals in form of multiple red signals (*arrow heads*). DAPI×1,000

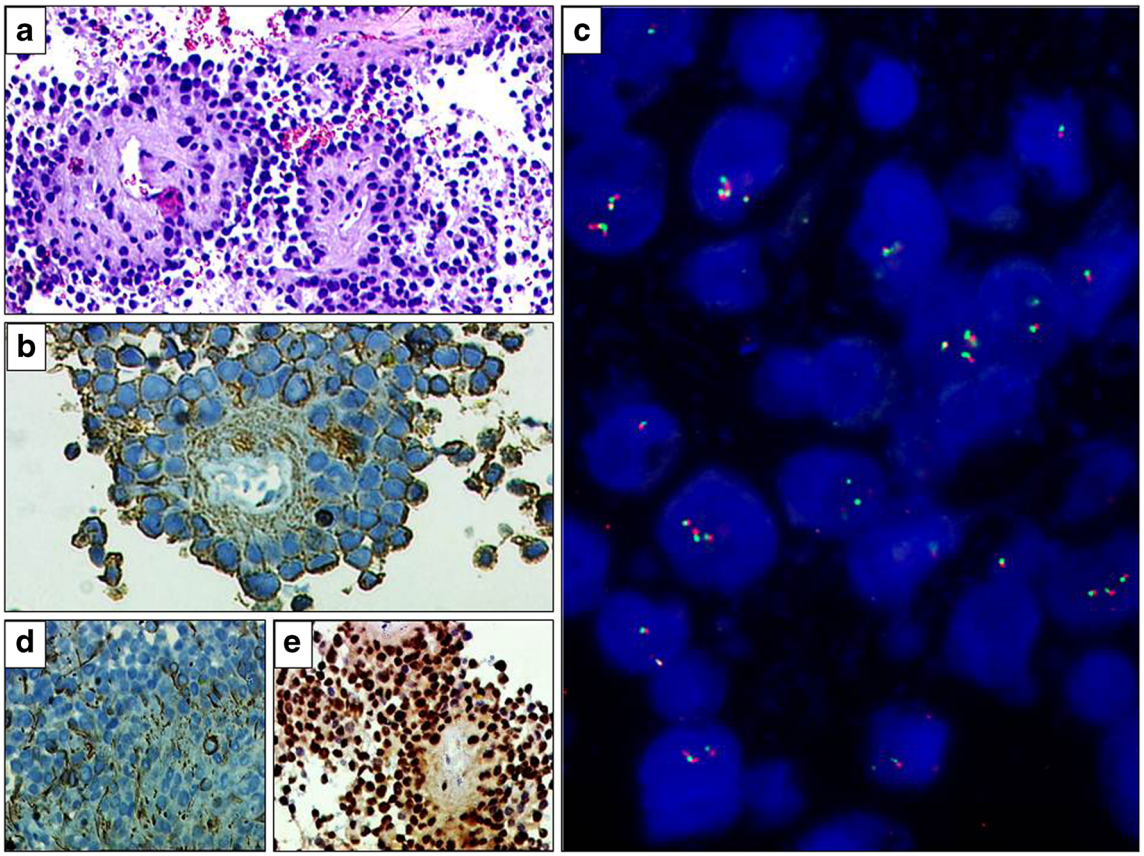

Fig. 5 Malignant round cell tumor with perivascular rosettes. H & E×200. **b** Membranous CD99 positivity led to initial diagnosis of ES/PNET. DAB×400. **c** *EWSR1* rearrangement was not detected on FISH. All the alleles were intact with fused signals including complex, multiple signals in some nuclei. DAPI×1,000. **d** On review, desmin was distinctly positive in tumor cells. DAB×400. **e** Diffuse intranuclear MyoD-1 positivity confirmed diagnosis of rhabdomyosarcoma. DAB×400

molecular confirmation [24, 29, 37, 43–45] by RT-PCR 54 %–97.5 % and 85–92.9 %, respectively.

The diagnostic value of molecular and cytogenetic testing cannot be overemphasized in EFTs in cases with equivocal IHC results and those occurring at unconventional sites. Objective confirmation with specific translocations has now become a part of diagnostic "work-up" of ES/PNET and also a prerequisite for inclusion of patients in ES/PNET therapeutic trials, for example EuroEwing [46]. This trial is a reason for molecular or cytogenetic testing for these tumors, at least in certain cases, even in limited resource settings wherein these patients similarly undergo a specific chemotherapy protocol (EFT 2001) that is expensive, and also has its own side-effects.

In the present study, out of 151 cases where molecular testing was requested, 56 (37 %) cases revealed *EWS-FLI* or *EWS-ERG* transcripts. Of these, definite diagnosis of ES/PNET was initially offered in 78/151 (51.6 %) cases. The included cases were the ones diagnosed at our hospital and substantial number of referrals from across the country. Although most cases (71 %), wherever ancillary testing was requested turned out to be ESs/PNETs; in 29 % cases unequivocal diagnosis of ES/PNET could not be confirmed on histopathology and IHC.

In our study, overall sensitivity for *EWSR1* test by FISH was 92.3 % and specificity, while including histopathologically overlapping tumors, was 100 %. At the same time, *EWSR1* rearrangement was detected in some unrelated tumors like myoepithelial carcinomas (42.8 %) and a single EMC and DSRCT, respectively as previously reported [31, 47]. The test could be performed in older blocks, but the number of interpretable cases was lesser than in relatively 'newer' cases. Reasons for uninterpretable test (10.5 % cases) included poor fixation, especially in referral cases and tiny biopsies leading to a lesser number of interpretable nuclei with clearer signals. Apart from single fused and/or split signals, few cases showed complex signals, including multiple fused or splits as well as multiple red signals in a tumor showing rearrangement. This could also be the 'garland-effect' that occurs as a result of multiple signals due to cutting artefacts or co-amplification apart from rearrangement. Recently, Chen et al. [33] documented complex signals in one-third of ESs and hypothesized that it relates to high-stage disease. Unfortunately, a case with multiple red signals in this study was lost to follow-up.

Table 3 Results of array analysis of eight cases, including liver comprising 28 tissue cores

Sr no.	Cases	Lane and core	*EWSR1* rearrangement
1	Liver	LIA	Not detected
2	Liver	LIB	Not detected
3	Liver	L1C	Not detected
4	Case 1	L1D	Detected
5	Case 1	L1E	Detected
6	Case 1	L1F	Detected
7	Case 1	L1G	Detected
8	Case 2	L2A	Detected
9	Case 2	L2B	Detected
10	Case 3	L2C	Not interpretable
11	Case 3	L2D	Detected
12[a]	Case 3	L2E	Not interpretable. Not detected in pneumocytes.
13	Case 4	L2F	Not interpretable
14	Case 4	L2G	Detected
15	Case 4	L3A	Not interpretable
16	Case 4	L3B	Detected
17	Case 4	L3C	Detected
18	Case 4	L3D	Detected
19	Case 5	L3E	Detected
20	Case 5	L3F	Detected
21	Case 5	L3G	Detected
22	Case 6	L4A	Detected
23	Case 7	L4B	Detected
24	Case 7	L4C	Detected
25	Case 8	L4D	Detected
26	Case 8	L4E	Not interpretable
27	Case 8	L4F	Detected
28	Case 8	L4G	Detected

[a] Tumor deposits in lung tissue. The core comprised lung alveoli and scanty tumor foci

Whereas FISH using break-apart probes has its advantages of high sensitivity and reasonable specificity with formalin-fixed paraffin embedded tissues, its limitations include cost and inability to identify specific transcript, unless fusion probes are also tested that further raises the cost, especially in our settings.

In the present study, on RT-PCR, 98 % tumors showed *EWS-FLI1* transcript. Previous studies also revealed higher number of *EWS-FLI1* positive tumors. However, the number of *EWS-ERG* positive tumors was higher than in the present study [24, 27, 35]. Sensitivity for PCR in the present study was lower (61 %), as was also noted of the order of 54 % by Bridge et al. [24]. Reasons for lower sensitivity include probable expression of our house keeping gene at a higher level than *EWS* fusion transcripts. Intriguingly, like Bridge et al. [23] we identified a single tumor displaying the *EWS-FLI* transcript, but lacking *EWSR1* rearrangement. As previously hypothesized, probably there was a tumor clone harbouring the ES/PNET transcript that could not be identified by FISH. At the same time, RT-PCR is sensitive for detecting rare events, despite its overall lesser sensitivity as noted in this study and by others [24]. Gamberi et al. [27] observed *EWSR1* rearrangement in all tumors that either contained insufficient quality RNA for RT-PCR, or were negative for *EW-S-FLI* and *EWS-ERG* transcripts. Other reasons for lower sensitivity in the present and few earlier studies [24] could also be larger base pair size of the designed primers used for that particular transcript identification. At the same time, with smaller base pair size primers, there is fear of cross reactivity. Besides, the cases, especially the ones referred from within the hospital that were subjected for RT-PCR were mostly diagnostic dilemmas after immunohistochemical analysis, including undifferentiated sarcomas. This probably lowered the overall number of "true positives". Although higher sensitivity has been observed with FISH, even in earlier studies [24], the latter incurs more cost that is an important issue within our settings. In order to make FISH cost-effective, we attempted FISH on a tissue microarray for interlaboratory comparison during test validation. In view of high percentage of detectable tumors as well as high sensitivity, it seems that FISH on tissue microarrays can be utilised for interlaboratory comparisons during the process of validation. Although substantial morphological and immunohistochemical heterogeneity exists within soft tissue sarcomas including EFTs, molecular heterogeneity, an anticipated formidable challenge, is less common [48]. Despite molecular heterogeneity in breast carcinomas, the tissue microarray technique has been found to be a robust and effective method for technical validation of FISH tests for HER2 [49]. However, challenge exits with smaller biopsies wherein double cores for each case that is ideal, can be a difficulty. At the same time this technique is useful for testing several available prognostic markers like p53, p16/p14ARF for ES/PNET. These markers have been found to be associated with aggressive behaviour and poor CT response within Ewing sarcomas, apart from recognized clinicopathological factors, including metastasis [50–52].

Although the present study was conducted on paraffin embedded tissue sections, fine needle aspiration cytology (FNAC) samples form good specimens for molecular and cytogenetic analysis [24, 47].

A feasible, pragmatic approach that can be followed in suspected cases of ES/PNET, necessitating confirmation by molecular and or cytogenetic techniques within our settings, is to initially test these tumors for known transcripts like *EWS-FLI1* and *EWS-ERG* by RT-PCR. In case these are positive, *EWSR1* testing by FISH may be obviated. In case of negative result, the case should be tested for *EWSR1* rearrangement and the final result should be interpreted with correlation of

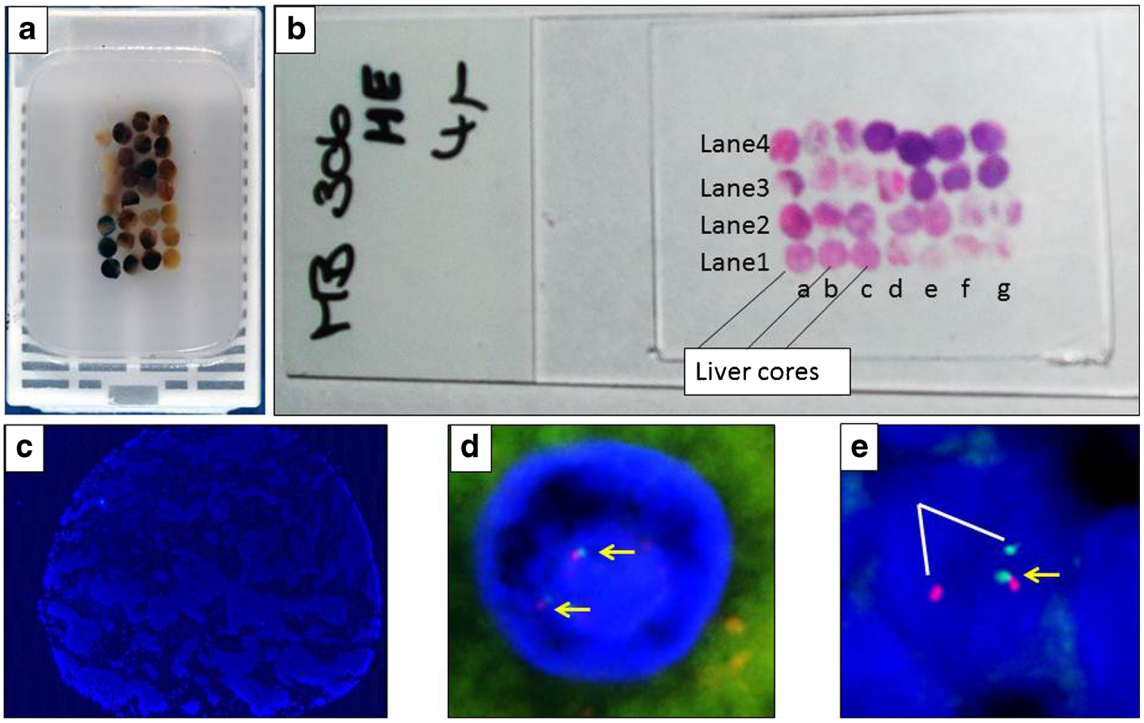

Fig. 6 Microarray FISH. **a**. Array block. **b**. Array slide marked with *lanes* and *rows*, including liver tissue cores (control and for identification). H & E. **c** single core in slide stained for FISH for *EWSR1* rearrangement. DAPI×100. **d** *Lane 1c*. Liver cells displaying intact alleles represented by fused signals. DAPI×1,000. **e** *Lane 3f*. Nuclei from one of the tumor cores displaying *EWSR1* rearrangement. DAPI×1,000

histopathological and immunohistochemical findings. In case of negative results and persisting clinical and/or pathological suspicion for ES/PNET, the case should be tested for *FUS* rearrangement, as suggested by Gamberi et al. [27]. *FUS* testing is presently lacking in our laboratory. Recently, two different studies [53, 54] have unravelled additional fusion genes in suspected cases of Ewing sarcoma/PNET that, in view of absent EWSR1-ETS translocations were classified as undifferentiated sarcomas. Pierron et al. [53] discovered a new subtype of bone sarcoma defined by BCOR-CCNB3 fusion gene, formed by BCOR (encoding the BCL6 co-repressor) and CCNB3 (encoding the testis-specific cyclin B3) on the X chromosome. They also identified CCNB3 as a useful immunohistochemical diagnostic marker for this subgroup of sarcoma. During the same time, Italiano et al. [54] identified CIC fusion with double-homeobox (DUX4) transcription factors in *EWSR1*-negative undifferentiated small blue round cell sarcomas. These authors observed variable expression of O13 in most of these cases.

To conclude, ES/PNET has a wide clinicopathological spectrum. Diffuse membranous MIC2 positivity, intense intranuclear FLI-1 positivity with LCA negativity in a malignant round cell tumor are highly diagnostic for ES/PNET. Molecular and or cytogenetic testing is necessary for a precise diagnosis of ES/PNET in unconventional cases. RT-PCR and FISH testing are complimentary and may be used in form of a pragmatic approach as stated, especially in limited resource settings. Tumors with histopathological suspicion of EFT, but negative for usual transcripts should necessarily be tested for *EWSR1* rearrangement by FISH. FISH on tissue microarrays can be a cost-effective technique. A negative molecular/cytogenetic test does not rule out an ES/PNET. Molecular or cytogenetic results should be interpreted in a clinicopathological context [27]. Introduction of a new test in a molecular laboratory should necessarily include simultaneous interlaboratory testing for quality assurance. Unexpected results should be repeated and confirmed with another test [55]. Newer gene fusions are being identified, leading to identification of newer genetically defined sarcomas in cases that were suspected to be Ewing sarcomas/PNETs, but were diagnosed as undifferentiated round cell sarcomas, in view of lack of classical fusion transcripts for EFTs [27, 53, 54]. Finally, this study also testifies the currently widely accepted neoadjuvant chemotherapy in most cases of EFTs, therefore need for its correct identification.

Certain prognostic molecular markers such as p53, p16/p14ARF for ES/PNET and still newer markers may be further tested, especially in metastatic cases, utilizing high throughput technique like tissue microarrays, to shed light on improvement in management of this aggressive, yet chemosensitive tumor.

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Conflict of Interest None.

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