# Differential diagnosis of small round cell tumours (SRCT), fluorescence in situ hybridization (FISH) and immunohistochemical (IHC) study

Konrad Ptaszyński[1], Anna Szumera-Ciećkiewicz[1], Monika Pękul[1], Zbigniew Nowecki[2]

[1]Department of Pathology, Maria Skłodowska-Curie Memorial Cancer Center and Institute of Oncology, Warsaw
[2]Department of Soft Tissue/Bone Sarcoma and Melanoma, Maria Skłodowska-Curie Memorial Cancer Center and Institute of Oncology, Warsaw

**Introduction:** Small round cell tumours (SRCT) of bone and soft tissue constitute a heterogeneous group of neoplasms with similar histological and cytological features. Immunohistochemical studies with panels of antibodies are necessary in order to make the diagnosis. A molecular testing is helpful in many cases.
**Aim of the study:** To assess the value of IHC and FISH tests in the differential diagnosis of SRCT.
**Material and methods:** The material was obtained from patients diagnosed and treated at the Maria Skłodowska-Curie Memorial Cancer Center-Institute in Warsaw between February 2003 and March 2009. One hundred and thirty one patients with the initial diagnosis of SRCT of bone or soft tissue were qualified to the investigation. The material from the primary tumour was obtained by an open or core biopsy in all the patients. During the treatment the patients were monitored, the local recurrence and the distant metastases were reported. The IHC study was performed routinely using wide panels of antibodies. FISH tests: *EWSR1*, *SS18* (*SYT*), *FKHR* (*FOXO1A*) and *FUS* were carried out using dual colour, break-apart probes.
**Results:** IHC tests for CD99 and FLI-1 showed low specificity, had low sensitivity, myogenin staining revealed high specificity and sensitivity. A "lymphoma" panel with LCA, CD20, CD79a, TdT, CD3 showed acceptable specificity and sensitivity. There were 28 (21.37%) uninformative FISH results showing no acceptable signals.
**Conclusions:** Diagnostic assessment of SRCT requires IHC studies as an introductory method. FISH is necessary in many cases of SRCT for the final diagnosis but it requires well-fixed and processed tissue, otherwise there is a high percentage of uninformative results. A diagnostic algorithm including IHC and FISH tests has been proposed.

**Key words:** small round cell tumours, differential diagnosis, fluorescence *in situ* hybridization, immunohistochemistry.

## Introduction

Small round cell tumours (SRCT) of soft tissue and bones consist of a heterogeneous group of neoplasms characterized by similar histopathological and cytological features. SRCTs are composed of uniform small round cells with round nuclei containing fine chromatin, scanty clear or eosinophilic cytoplasm. In some cases, the tumour cells are larger, ovoid or spindle, with prominent nucleoli and irregular contours.

In differential diagnosis of SRCT, the classical SRCT and the group of malignancies with primitive, small, cell morphology should be included. Among classical

SRCTs, the most common is an Ewing sarcoma family of tumours (EFT) with a neuroectodermal or mesenchymal stem cell origin and a various degree of neural differentiation. Ewing's sarcoma (ES) and its morphological variants – peripheral primitive neuroectodermal tumour (PNET) and Askin's tumour (AT) belong to EFT. Furthermore, classical SRCT includes desmoplastic small round cell tumour (DSRCT), melanocytic neuroectodermal tumour (MNT), neuroblastoma (NBL), olfactory neuroblastoma (ON), rhabdomyosarcoma (RMS), poorly differentiated synovial sarcoma (SyS), small cell osteosarcoma (SCO) and mesenchymal chondrosarcoma (MChS). Tumours with small cell morphology similar to SRCT include non-Hodgkin lymphoma (NHL), round cell liposarcoma (RCLS), extraskeletal myxoid chondrosarcoma (EMC), poorly differentiated malignant peripheral nerve sheath tumour (MPNST), malignant melanoma (MM), rhabdoid tumour (RT), germ cell tumours (GCT), small cell carcinoma (SCC) and Merkel cell carcinoma (MCC) [1].

The histopathological assessment of SRCT is the initial step of the diagnostic procedure because of appreciable similarity of SRCT morphological picture. In differential diagnosis the immunohistochemical studies are essential. The CD99 protein is expressed in almost all cases of EFT, nevertheless it is not specific. The neuroectodermal differentiation may be evaluated using neurospecific enolase (INSE) antibody or CD56. These markers are nonspecific as well. Genetic testing is the other diagnostic tool facilitating correct classification of SRCT. The characteristic cytogenetic features of the majority of classical SRCTs are chromosomal aberrations, mostly translocations and multiplication of chromosome fragments [2]. It is regarded that translocations are primary, tumour-specific chromosomal aberrations among soft tissue neoplasms leading to a recombination and fusion of protein coding genes as well as regulatory genes originating from different chromosomes. Fusion of a regulatory gene with a protein coding gene may result in protein expression with a normal structure acting similarly to the oncogene. Whereas a combination of two protein coding genes contributes to fusion gene formation, which is transcribed, translated into fusion (chimeric) protein. These two mechanisms may lead to a neoplastic transformation and uncontrolled cell proliferation due to disturbance of signalling protein cell cascade [3]. In particular types of tumours, the cytogenetic variants of translocations and molecular variants of fusion genes are described. Some of them differ in the clinical outcome [4]. In this study, we assessed utility of combined immunohistochemistry (IHC) and fluorescence in situ hybridisation (FISH) in the differential diagnosis of cases of SRCT.

## Materials and methods

### Patients

The material was obtained from patients diagnosed and treated at the Maria Skłodowska-Curie Memorial Cancer Center and Institute of Oncology in Warsaw between February 2003 and March 2009. One hundred and thirty one patients (66 men and 65 women) with the initial diagnosis of small round cell tumour (SRCT) were qualified to the investigation. Material from the primary tumour was obtained by an open biopsy or less frequently by a core biopsy. During the treatment all patients were monitored, the local recurrences and the distant metastases were recorded.

### Immunohistochemistry

All specimens were fixed in 10% buffered formalin and embedded in paraffin (FFPE) according to standard procedures. Serial sections (4 $\mu$m in thickness) were used for haematoxylin and eosin staining (HE), immunohistochemistry (IHC) and FISH analysis. An IHC study was performed using panels of antibodies and is summarized in Table I. Briefly, paraffin-embedded sections of the tumour were deparaffinized, dehydrated and heat-treated for antigen retrieval in a water bath at 96°C for 20 minutes in TRIS/EDTA buffer, pH 9.0 (Target Retrieval Solution, Dako, S2367). Subsequently, all sections were blocked in 0.3% $H_2O_2$ in methanol for 30 minutes and incubated with the primary antibody for 30 minutes at room temperature in a humidity chamber. For detection, the Dako REAL EnVision Detection System, Peroxidase/DAB+, Rabbit/Mouse (K5007) were used. Immunohistochemical stainings were evaluated following the criteria recommended by the manufacturer.

### FISH analysis

FISH analysis of *EWSR1*, *SS18* (*SYT*), *FKHR* (*FOXO1A*) and *FUS* was carried out using the Dual Colour Break-Apart Probes (Vysis-Abbott Laboratories) according to the manufacturer's protocol. Sections were incubated at 65°C overnight, deparaffinized in xylene 2 × 10 minutes, dehydrated (99.98% ethanol 2 × 5 minutes) and air-dried. Tissue sections were treated with Sodium Thiocyanate-NaSCN (Pretreatment Solution, Abbott Vysis) for 30 minutes at 80°C and then enzymatically digested in Protease Solution for 25 minutes at 37°C in a humidity chamber. The sections were fixed in 4% buffered formalin (10 minutes at room temperature), washed in Standard Saline Citrate buffer (2 × SSC, 5 minutes in room

**Table I.** An immunohistochemical study: antibodies used and the results

| ANTIBODIES | CLONE | MANUFACTURER | DILUTION |
|---|---|---|---|
| Smooth Muscle Actin (SMA) | 1A4 | Dako | 1 : 100 |
| Caldesmon | h-CD | Dako | 1 : 100 |
| CD3 | – | Do | 1 : 50 |
| CD19 | LE-CD19 | Dako | 1 : 50 |
| CD20cy | L26 | Dako | 1 : 100 |
| CD31, Endothelial Cell | JC70A | Dako | 1 : 50 |
| CD34 Class II | QBEnd/10 | Dako | 1 : 50 |
| CD45 (LCA) | 2B11+PD7/26 | Dako | 1 : 500 |
| CD56 | 1B6 | Novocastra | 1 : 50 |
| CD79α | JCB117 | Dako | 1 : 100 |
| CD99 (MIC2 Gene Products) | 12E7 | Dako | 1 : 100 |
| Chromogranin A | DAK-A3 | Dako | 1 : 100 |
| Cytokeratin (AE1/AE3) | AE1/AE3 | Dako | 1 : 100 |
| Cytokeratin (CAM 5.2) | CAM 5.2 | Becton Dickinson | Stock |
| Cytokeratin 7 | OV-TL 12/30 | Dako | 1 : 100 |
| Cytokeratin 20 | Ks20.8 | Dako | 1 : 100 |
| Desmin | D33 | Dako | 1 : 100 |
| Epithelial Membrane Antigen (EMA) | E29 | Dako | 1 : 100 |
| FLI-1 | RB9295-P | Lab Vision | 1 : 50 |
| Melan-A | A103 | Dako | 1 : 100 |
| Melanosome | HMB-45 | Dako | 1 : 100 |
| Microphthalmia Transcription Factor (MITF) | 34CA5 | Novocastra | 1 : 40 |
| Myogenin | F5D | Dako | 1 : 100 |
| S100 Protein | – | Dako | 1 : 2000 |
| Synaptophysin | – | Dako | 1 : 150 |
| Terminal Deoxynucleotide Transferase (TdT) | SEN28 | Novocastra | 1 : 50 |
| Wilms' Tumour (WT-1) Protein | 6F-H2 | Dako | Stock |

temperature) and air-dried. 10 $\mu$l of a cocktail containing mixture of Dual Colour Break-Apart Probe (EWSR1 22q12, SS18 18.q11.2, FOXO1A 13q14 or FUS 16p11) was applied to the sections. In order to prevent vaporization the slides were covered with a cover slip and sealed with rubber cement. Specimen and probe DNA were denatured by placing the samples in Thermo-Brite (5 minutes at 73°C). Hybridization was carried out under the cover slip (overnight, 37°C). An unbound probe was washed away with Post-Hybridization Solution 1 (0.4 × SSC/0.3% NP-40, 10 seconds at 72°C) followed by wash in Post-Hybridization Solution 2 (0.2 × SSC/0.1% NP-40, 20 seconds at room temperature). Tissue sections were then air-dried in the darkness and counterstained with 4',6-diamidino-2-phenylindole (DAPI, Abbott-Vysis). Slides were evaluated for EWSR1, SS18 (SYT), FKHR (FOXO1A) and FUS status using Olympus BX40 microscope equipped with filters: Spectrum Orange, FITC, DAPI monofilters and triple-band pass (rhodamine/FITC/DAPI) filter. Before reviewing the FISH assay, the appropriate tumour areas were confirmed by using a parallel HE stained section. Hybridization signals were assessed in 60 interphase nuclei with strong, well-delineated signals and distinct nuclear borders at 1000 × magnification by two individuals. The percentages of green, orange, and fused signals were calculated and the images were acquired using F-View CCD Camera and Cell-F Image Analysis System (Olympus). For the FISH break-apart approach, a positive interpretation was defined as > 15% of nuclei with split signals (> 1 signal diameter apart). That cut-off was based on counts in non-neoplastic controls. Hybridizations where signals were either lacking or too weak to be interpreted were repeated using higher probe concentrations. Those still non-interpretable were considered uninformative. In summary, the results were classified as positive, negative or uninformative.

In the cases of positive IHC staining for CD99 but negative FISH result the case was classified as SRCT and re-evaluated using new sections from the paraffin block or further evaluated with IHC and FISH on a new material obtained from the case.

## Results

The average age of patients was 36.74 years (range 17-84 years, SD 28.53) and there was no statistically significant difference between groups of women and men. The material from the primary tumour was obtained by an open biopsy in most of the patients. In some cases only, a core biopsy material was available. The most frequent regions of malignancy occurrence were: thigh (25.95%), retroperitoneum (12.21%) and lower leg, chest wall, pelvic bones (9.92% each). The localizations were also classified according to their relation to the bone vs. soft tissue. The greatest percentage of bone vs. soft tissue localization was identified in the forearm (75 vs. 25%) and the lowest in the thigh (17.65 vs. 82.35%). During the treatment the patients were monitored, the local recurrence and the distant metastases were recorded. The mean time of follow-up was 20.78 months (1-240 months, SD 28.53). Nineteen (14.5%) patients under observation deceased and the mean time of survival in that group was 14.32 months (1-44 months, SD 10.61). In the carcinoma (CA) category, cases of MCC, SCC and poorly differentiated carcinoma (PDC) were included.

### Immunohistochemistry results

The results of significant immunohistochemical observations are presented in Table II (A, B) and Fig. 1 (C-F). A typical histological appearance of a SRCT is depicted in Fig. 1 (A, B).

### FISH results

The summary of FISH results including percentages of positive and negative results is presented in Table III and illustrated in Fig. 1 (G, H).

There were 28 (21.37%) cases showing no acceptable signals designated as cases with uninformative FISH results. In this group, clinical, histopathological and immunohistochemical studies were re-evaluated and additional IHC studies were performed. Among the cases with uninformative results, 67.86% were classified as EFT, SyS or other entities after further additional assessment with IHC and a review of the clinical features. The number of informative vs. uninformative FISH test results was 103 (78.63%) vs. 28 (21.37%). In 13 cases, two or more FISH tests with *EWS*, *SYT*, *FKHR* or *FUS* probes were performed, therefore the total number of tests is 122.

A list of final clinico-histopathological diagnoses substantiated with immunohistochemistry and FISH results is depicted in Table IV.

## Discussion

Differential diagnosis of small round cell tumours (SRCT) of soft tissues and bones constitutes frequently a difficult diagnostic problem. In some cases, routine morphological and immunohistochemical studies are not sufficient for making a decision on tumour classification and determination of its line of differentiation. It appears that biopsy specimens of various neoplasms may present morphology of small round cell tumour (SRCT).

Diagnostic assessment of SRCTs requires IHC studies as an initial modality. IHC diagnostic studies of EFT show consistent staining with CD99 antibody with approximately 90% sensitivity. However, it is present in a growing number of other tumours including NHL and SyS. Therefore, specificity of the IHC staining for CD99 is low [5]. In addition, the following markers have been found positive in cases of EFT: chromogranin, synaptophysin, CD56, S100 protein, neuron specific enolase (NSE), desmin and epithelial membrane antigen (EMA) [6]. Recently Folpe *et al*. showed that reaction with the transcription factor antibody FLI-1 was positive in 94% of EFT cases with t(11;22)(q24;12) [6]. Cases of DSRCT are characterized by IHC positive staining with cytokeratin and punctate paranuclear staining with desmin [7]. Poorly differentiated SyS may not exhibit an IHC pattern typical of SyS. Cytokeratin (CK) and EMA staining can be weak or completely lost and in addition there is reported a positive staining with CD99 in 62% of classic and poorly differentiated SyS [8]. These findings may cause diagnostic problems if only morphology and IHC is utilized. Cases of poorly differentiated SyS can be distinguished from RMS with the IHC study. Lack of immunoreactivity with desmin, actin and myogenin is helpful. Muscle specific actin (SMA) is present in virtually all cases of RMS although the staining is frequently dim with some background stain. Desmin shows positive staining in the vast majority of RMS, nevertheless it is frequently focal. Some of EFT cases may show desmin immunoreactivity as well. Myogenin nuclear staining is considered very sensitive and specific for all variants of RMS [9]. Cytokeratin (CK) staining is a characteristic feature of epithelial neoplasms but not all cases of SCC or PDC show positive reactivity. Interestingly, as mentioned above, most cases of DSRCT are positive for CK. Characteristically, cases of MCC are positive for CK20. CK is positive in some soft tissue tumours including SyS and epithelioid sarcoma but also epithelioid variants of other soft tissue tumours and in some cases of MM, EFT and

**Table IIA.** The results of immunohistochemical study

| Diagnosis | SMA | DES | EMA | Myogenin | WT-1 | CD3 | CD19 | CD20 | CD31 | CD34 | CD45 | CD56 | CD79 | CD99 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| AS | – | – | – | – | – | – | – | – | + | + | – | – | – | – |
| ASPS | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| CB | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| CCS | – | – | – | – | – | – | – | + | – | – | – | – | – | – |
| DLBCL | – | – | – | – | – | – | + | + | – | – | + | – | – | – |
| DSRCT | – | + | + | – | + | – | – | – | – | – | – | – | – | + |
| MChS | – | – | – | – | – | – | – | – | – | – | – | – | – | + |
| MM | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| ON | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| PL | – | – | – | – | – | – | – | – | – | – | – | + | – | – |
| SCO | – | – | – | – | – | – | – | – | – | – | – | – | – | + |
| SFT | – | – | – | – | – | – | – | – | – | + | – | – | – | + |
| EMC | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| RCLS | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| MPNST | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| GCT | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| CA | – | – | 1/5 | – | – | – | – | – | – | – | – | – | – | 1/5 |
| RMS | 5/6 | 6/6 | – | 6/6 | – | – | – | – | – | – | – | – | – | – |
| SyS | – | – | 20/26 | – | – | – | – | – | – | – | – | – | – | 10/26 |
| SRCT | – | 3/25 | – | – | – | – | – | – | – | – | – | – | – | 6/25 |
| EFT | – | 5/38 | – | – | – | – | – | – | – | – | – | 4/38 | – | 36/38 |

**Table IIB.** The results of immunohistochemical study

| Diagnosis | CALD | CHR | SYN | AE1/AE3 | CAM 5.2 | CK7 | CK20 | FLI-1 | HMB45 | Melan-A | MIFT | S100 | TTF1 | TdT |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| AS | – | – | – | – | – | – | – | + | – | – | – | – | – | – |
| ASPS | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| CB | – | – | – | – | – | – | – | – | – | – | – | + | – | – |
| CCS | – | – | – | – | – | – | – | – | + | – | + | + | – | – |
| DLBCL | – | – | – | – | – | – | – | + | – | – | – | – | – | – |
| DSRCT | – | – | – | + | – | – | – | + | – | – | – | – | – | – |
| MchS | – | – | – | – | – | – | – | – | – | – | – | + | – | – |
| MM | – | – | – | – | – | – | – | – | + | + | + | + | – | – |
| ON | – | + | + | – | – | – | – | – | – | – | – | + | – | – |
| PL | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| SCO | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| SFT | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| EMC | – | – | – | – | – | – | – | – | – | – | – | + | – | – |
| RCLS | – | – | – | – | – | – | – | – | – | – | – | + | – | – |
| MPNST | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| GCT | – | – | – | + | – | – | – | – | – | – | – | – | – | – |
| CA | – | 2/5 | 2/5 | 3/5 | – | 1/5 | 2/5 | – | – | – | – | – | 2/5 | – |
| RMS | 5/6 | – | – | – | – | – | – | – | – | – | – | – | – | – |
| SyS | – | – | – | 19/26 | 23/26 | 19/26 | – | – | – | – | – | – | – | – |
| SRCT | – | 2/25 | 3/25 | – | – | – | – | 4/10 | – | – | – | 3/20 | – | – |
| EFT | – | – | 7/38 | – | – | – | – | 30/32 | – | – | – | 4/22 | – | – |

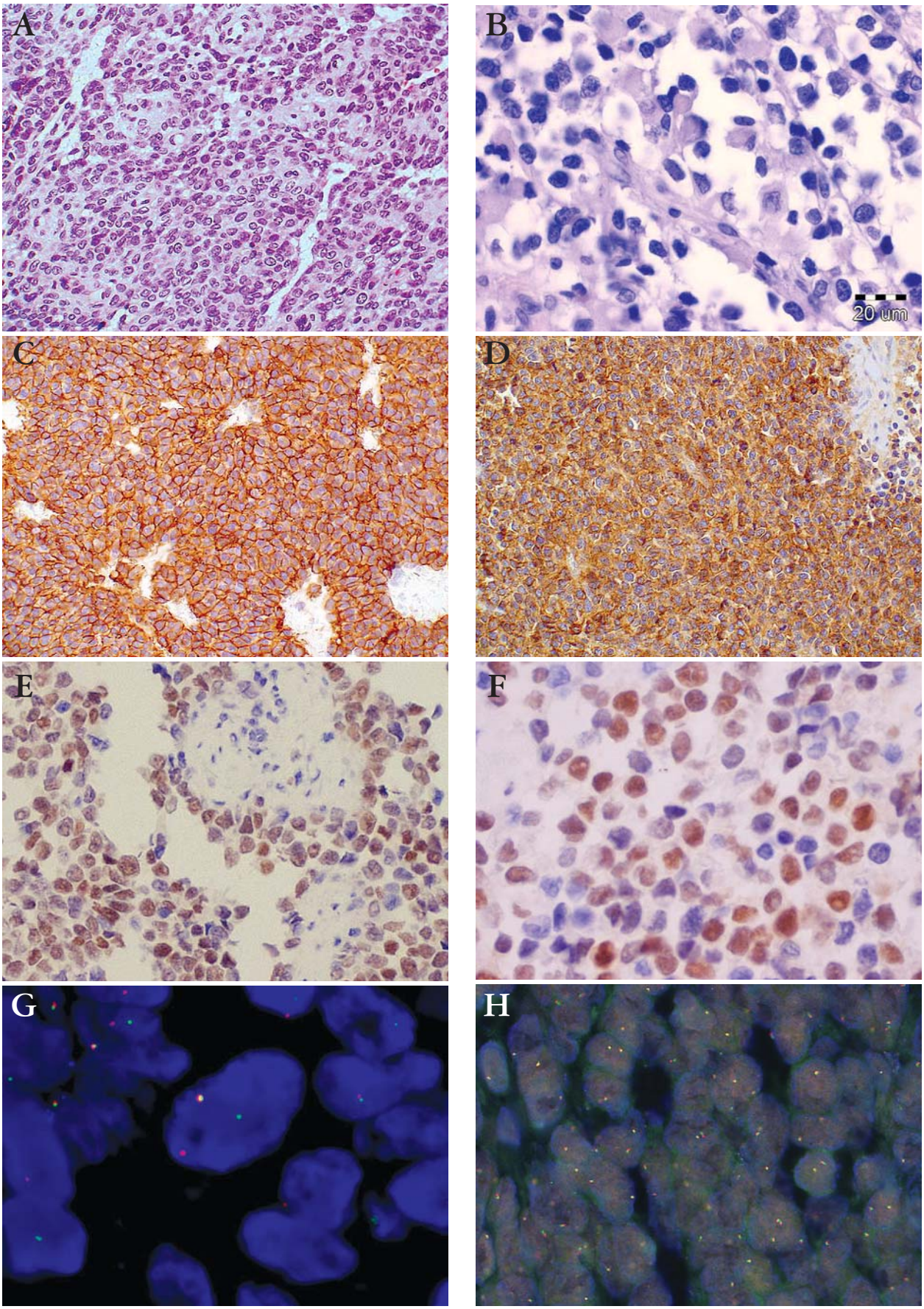


**Fig. 1.** Examples of IHC and FISH results (A – SRCT, HE, 200×; B – ARMS, HE, 400×; C – EFT, CD99, membranous reaction, 200×; D – EFT, CD99, membranous-cytoplasmic reaction, 400×; E – EFT, FLI-1, 400×; F – ARMS, myogenin, 200×; G – positive and H – negative FISH result, translocation of EWSR1 in EFT)

**Table III.** The summary of the results of FISH break-apart tests

| FISH | Positive | % | Negative | % | Total |
|---|---|---|---|---|---|
| EWS | 35 | 52.23 | 32 | 47.77 | 67 |
| SYT | 23 | 51.11 | 22 | 48.89 | 45 |
| FOXO1A | 2 | 40 | 3 | 60 | 5 |
| FUS | 1 | 20 | 4 | 80 | 5 |
| Total | 60 | | 62 | | 122 |

**Table IV.** A list of final clinico-histopathological diagnoses of SRCTs

| Final clinico-histopathological diagnosis | | N | % |
|---|---|---|---|
| Angiosarcoma | AS | 1 | 0.76 |
| Alveolar soft part sarcoma | ASPS | 1 | 0.76 |
| Chondroblastoma | CB | 1 | 0.76 |
| Clear cell sarcoma | CCS | 1 | 0.76 |
| Diffuse large B-cell lymphoma | DLBCL | 1 | 0.76 |
| Desmoplastic small round cell tumour | DSRCT | 1 | 0.76 |
| Mesenchymal chondrosarcoma | MchS | 1 | 0.76 |
| Malignant melanoma | MM | 1 | 0.76 |
| Olfactory neuroblastoma | ON | 1 | 0.76 |
| Plasmocytoma | PL | 1 | 0.76 |
| Small cell osteosarcoma | SCO | 1 | 0.76 |
| Solitary fibrous tumour | SFT | 1 | 0.76 |
| Extraskeletal myxoid chondrosarcoma | EMC | 1 | 0.76 |
| Round cell liposarcoma | RCLS | 2 | 1.53 |
| Malignant peripheral nerve sheath tumour | MPNST | 3 | 2.29 |
| Germ cell tumour | GCT | 4 | 3.05 |
| Carcinoma | CA | 5 | 3.82 |
| Rhabdomyosarcoma | RMS | 6 | 4.58 |
| Synovial sarcoma | SyS | 26 | 19.85 |
| Small round cell tumour | SRCT | 34 | 25.95 |
| Ewing family of tumours | EFT | 38 | 29.01 |
| Total | 131 | 100.00 | |

RMS [10]. IHC with a lymphoma panel of antibodies is a necessary test in most of SRCT cases.

In our study, some SRCT cases were evaluated with two rounds of IHC. Immunohistochemical tests for CD99 showed 100% sensitivity since all cases of EFT were positive, however it revealed inferior specificity, the other entities presented with mixed membranous and cytoplasmic staining. FLI-1 test had a low sensitivity in our study due to strong cytoplasmic, background staining and frequently ambiguous results. Some cases of EFT showed clearly negative staining with this antibody. It had a moderate specificity since cases of other entities exhibited positive reaction. Myogenin staining revealed 100% specificity and sensitivity in cases of RMS. A lymphoma panel with LCA, CD20, CD79a, TdT and CD3 showed good specificity and sensitivity.

It seemed that using molecular methods focusing on specific genetic alterations it would be possible to determine more accurately the biology and clinical course of soft tissue tumours, especially SRCTs [4]. Reports of the same spectrum of genetic changes in ES and PNET, as well as AT, constituted the basis for qualification of these tumours to a single group with common pathogenesis. This group was designated as EFT. However, a large number of cytogenetic and molecular variants has been reported lately, and an interpretation of those test results has become more complicated. Also, cases of various types of tumours, not only sarcomas, have

**Table V.** Cytogenetic variants of EFT and other gene fusions with *EWSR1*

| Ewing Family of Tumours – cytogenetic variants | |
|---|---|
| *EWSR1 – FLI1* | t(11;22)(q24;q12) |
| *EWSR1 – ERG* | t(21;22)(q22;q12)<br>t(19;der(ins,inv(21;22))) |
| *EWSR1 – ETV1* | t(7;22)(q22;q12) |
| *EWSR1 – ETV4* | t(17;22)(q12;q12) |
| *EWSR1 – FEV* | t(2;22)(q33;q12) |
| *EWSR1 – SP3* | t(2;22)(q31;q12) |
| *EWSR1 – POU5F1* | t(6;22)(p21;q12) |
| *EWSR1 – PATZ1* | t(1;22)(q36;q12) |
| **Clear cell sarcoma** | |
| *EWSR1 – ATF1* | t(12;22)(q13;q12) |
| *EWSR1 – CREB1* | t(2;22)(q33;q12) |
| **Angiomatoid fibrous histiocytoma** | |
| *EWSR1 – ATF1* | t(12;22)(q13;q12) |
| *EWSR1 – CREB1* | t(2;22)(q33;q12) |
| **Extraskeletal myxoid chondrosarcoma** | |
| *EWSR1 – CHN1* | t(9;22)(q22–31;q11–12) |
| *EWSR1 – NNR4A3* | t(9;22)(q22;q12) |
| **Acute leukemia** | |
| *EWSR1 – CIZ1* | t(12;22)(p13;q12) |
| **Myxoid type and round cell type liposarcoma** | |
| *EWSR1 – ATF1* | t(12;22)(q13;q12) |
| **Desmoplastic small round cell tumour** | |
| *EWSR1 – WT1* | t(11;22)(p13;q12) |

been described showing the same cytogenetic and molecular changes (Table V) [11].

In EFT cases, t(11;22)(q24;q12) with chromosome 22 breakpoint within one of 4 introns and chromosome 11 breakpoint within one of 6 introns is the most frequently found translocation. t(21;22)(q22;q12) is another common translocation giving a similarly heterogeneous population of transcripts and proteins. A breakpoint within one of 3 introns on chromosome 22 and one of 4 introns on chromosome 21 was reported [12]. Therefore, several cytogenetic and molecular variants of those translocations are described. There is a combination of several fusion transcripts and fusion (chimeric) proteins of variable length. Other, less frequent translocations have been reported recently, including t(2;22)(q33;q12), t(7;22)(p22;q12), t(17;22)(q12;q12) with translocation of the *EWSR1* gene to the area of genes coding next transcription factors of the ETS family: *FEV*, *ETV1* and *E1AF* [13]. Some recently reported cytogenetic variants include translocations involving the *FUS* gene located on chromosome 16. *FUS* is a gene coding a RNA-binding protein and is homologous to the *EWSR1* gene. Another partner of those translocations is the ERG gene located on chromosome 21, and the *FEV* gene on chromosome 2 [14, 15]. Translocation t(16;21)(p11;22) with formation of fusion gene *FUS-ERG* was also noted in cases of myeloid leukaemia [16, 17].

Understanding biology of these tumours and determination of criteria for evaluation of molecular tests is further complicated by the fact that SRCT cases with some features of EFT but a different genetic profile have been recently reported. They were described as EFT-like tumours and show gene rearrangements previously not encountered in EFT. A few genes involved in these translocations have been described including: *NFAT.2*, *SP3*, *ZNF278*, *POU5F1*, *CIC*, *DUX4* [13, 18, 19].

It was postulated that FISH test with an EWSR1 probe is highly specific [20]. However, there are several reports including the current report showing that there are entities other than EFT including CCS, DSRCT, EMC with a positive FISH test for the *EWSR1* rearrangement. Sensitivity of the test as high as 91% was reported by Bridge *et al.* [21]. Provided that the received material was optimally formalin-fixed and paraffin-embedded, sensitivity of the test for EWSR1 rearrangement was reaching 100%. Some rare cases are not recognised by this test due to variants not involving the *EWSR1* gene. Cases with no signal due to suboptimal material were put into a non-diagnostic category.

Rearrangements of the *FUS* gene detected by FISH studies with a double colour, break-apart strategy are proved to be a hallmark of myxoid liposarcoma (MLS) and its variant: RCLS. It is rearranged due to translocation t(12:16)(q13:p11) [22]. However, this test appeared to be nonspecific due to reported rearrangements of the *FUS* gene in rare cases of EFT. In some cases of SRCT, only a small biopsy specimen is available and no MLS component is present. RCLS showing positive FUS rearrangement by FISH can be erroneously classified as EFT with rare translocation involving the *FUS* gene.

There are documented examples of poorly differentiated round cell lesions with the presence of fusion gene products characteristic of EFT, which tend to differentiate towards other tumours, most commonly RMS. The diagnosis of a typical alveolar (ARMS) or embryonic (ERMS) RMS is not difficult. In the majority of cases that diagnosis can be confirmed immunohistochemically with myogenin or MyoD1 staining. There are, however, cases of poorly-differentiated, solid RMS, which are closely related to the classic alveolar type (called a solid form of ARMS), and which are indistinguishable from the EFT family tumours under a light microscope [23]. It is believed that those tumours are related to the EFT family and show polyphenotypic differentiation

[24-26]. Differential diagnosis of a RMS case from sarcoma belonging to the EFT family is not always unequivocal, even if genetic techniques are used.

There are two different variants of cytogenetic translocations seen in cases of ARMS [27]. Translocation t(2;13)(q35;q14) occurs in 70% of ARMSs and leads to rearrangements of transcription factor *PAX3* and *FOXO1A* (*FKHR*) [28, 29]. Variant translocation t(1;13)(p36;q14) with *PAX7* and *FOXO1A* (*FKHR*) is less common [30]. There is no specific translocation described to date in cases of ERMS.

Rearrangements of the *FOXO1A* gene, the common partner in both cytogenetic variants in ARMS, can be evaluated with the FISH test using a dual colour break-apart *FOXO1A* probe which was found to be highly sensitive and specific. All tested cases of ARMS showed this specific rearrangement by FISH. It is a very good complementary method to IHC testing with myogenin or MyoD1 antibody. It was shown that the prognosis of RMS cases with translocation t(2;13)(q35;q14) is worse than of those, in which translocation t(1;13)(p36;q14) was found, because there is a different biological potential of fusion gene products: PAX3-FOXO1A and PAX7-FOXO1A, respectively. Studies indicated a higher proliferation potential and more massive deregulation of the cellular cycle by PAX3-FOXO1A [31-33]. It was also found that a total 4-year survival coefficient of metastatic patients was lower in cases with a variant involving the *PAX3-FOXO1A* gene and constituted a bad prognostic factor [34, 35]. FISH method with the *FOXO1A* break apart probe is unable to distinguish between these two variants. It is suggested that a RT-PCR test should be performed in order to determine the prognostic markers.

A characteristic cytogenetic feature of SyS is translocation t(X;18)(p11;q11) with fusion of the *SS18* (*SYT*) gene located on chromosome 18 with one of the genes of the *SSX* family from chromosome X [36]. There are several molecular variants of this translocation with *SSX1* or *SSX2* as most frequent partners of the *SS18* gene. In addition, there are frequent internal additions of various numbers of nucleotides which increase the fusion heterogeneity. A correlation between the clinical course and cytogenetic translocation variant was found. The worse prognosis with a shorter metastasis-free period and larger proliferation coefficient was associated with cases showing the presence of the SS18-SSX2 product, compared to those cases, in which SS18-SSX1 was detected. Correlation between a given cytogenetic variant and clinical course has not been fully confirmed [37-41]. Molecular testing using FISH with SS18 (SYT) break-apart probe is a very reliable test with 100% sensitivity. No other tumour types showed positive FISH test with SS18 probe. However, in order to determine cytogenetic variants with different prognosis features, the RT-PCR test is required.

Molecular diagnostic tests are routinely performed using RT-PCR or FISH method. Both methods have their advantages and disadvantages. RT-PCR is best performed on fresh or –70°C-frozen material which allows examination of the full length of transcripts of the fusion genes encountered in the tumours. RT-PCR tests may be also performed using formalin-fixed and paraffin-embedded material for RNA extraction, due to recently introduced technologies [42, 43]. However, that kind of material allows only for examination of short transcripts, due to a degradation of long RNA fragments, a part of which is not available for analysis, and the test is burdened with a high rate of false negative results. In cases of tumours clinically consistent with EFT, a RT-PCR test is aimed at detection of the most common aberrations *EWS-FLI1* and *EWS-ERG* [44]. In the case of a negative result, encountered in 5-9% of cases, the second set of primers is used in order to detect less frequent translocations in a single RT-PCR reaction: *EWS-FLI1*, *EWS-ERG*, *EWS-ETV1*, *EWS-E1AF* [13]. If those tests also give a negative result it is necessary to perform additional reactions using primers designed to detect rearrangements of the *FUS* gene and one gene of the *ETS* family. A less difficult approach is recommended in cases of ARMS and SyS due to a few known cytogenetic variants.

The FISH method greatly simplifies this procedure; it seems to be more useful for a diagnostic review of material; it is able to detect simultaneously many cytogenetic and molecular variants. The material for the tests may be fresh, frozen or FFPE paraffin block. Thus, archival material is suitable for a FISH testing. FISH allows for detection of specific DNA sequences in metaphase chromosomes, interphase nuclei or cytological specimens. There are two different strategies of that technique used in cases characterised by a translocation, a dual colour-single fusion technique and much more frequently used dual colour-break apart strategy which allows for testing cytogenetic variants with a single reaction and obtain more clear unequivocal results, two probes are currently used surrounding a group of all known breakpoints of one of frequently encountered translocation partners [21, 45, 46]. The drawback of the strategy is the fact that it does not provide any information concerning the other translocation partner. It seems to be prognostically important information. Negative results are due to the presence of cytogenetic or molecular variants of neoplasms. Cryptic translocations and inversions of small fragments of genetic material constitute another cause. These genetic alterations are only available to

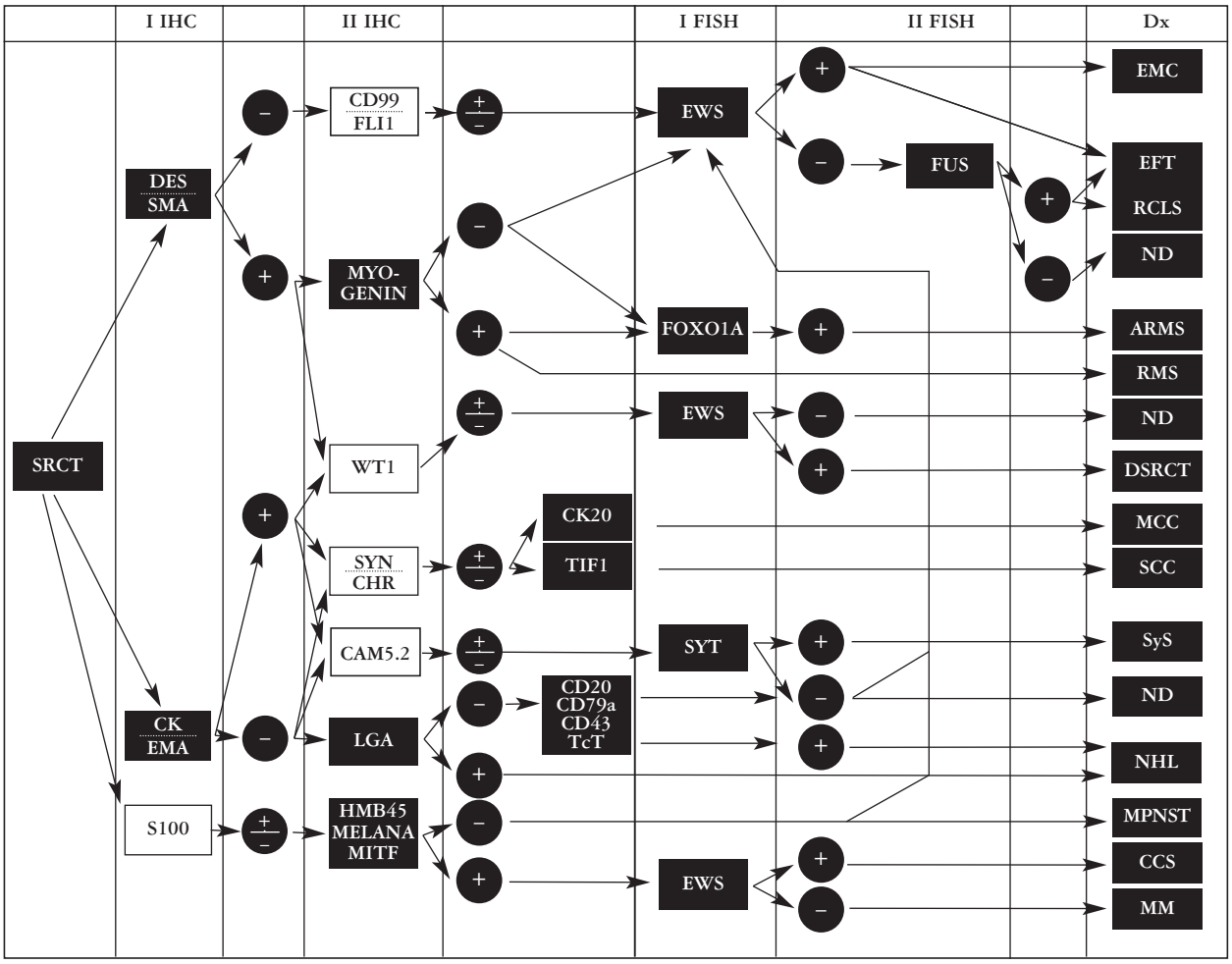


**Fig 2.** Diagnostic algorithm of SRCT. Clinical information, especially location of the tumour is one of the most important clues in finding a correct path of the algorithm. Negative results of the IHC with antibodies shown in white boxes are non-informative so regardless of the result next step of the algorithm can be followed. In case of boxes with more than one antibody a positive IHC test with any of the antibodies leads to the next step. Abbreviations are explained in Table IV and in the text of the article. Abbreviations non explained elsewhere: ND – non-diagnostic, Dx – diagnosis

multiple PCR, sequencing or may be detected using a spectral karyotyping (SKY) method. The FISH method requires well fixed material in buffered formalin. A non-diagnostic result of a FISH test means that no signals indicating hybridisation of molecular probes with a complementary DNA sequence were found in a specimen. That is frequently a result of problems with fixation or an improper FISH procedure. Due to various structures of individual tumour types, the digestion time should be individually selected. Tumour made of loosely arranged cells with regular oval shapes and scant cytoplasm requires shorter enzymatic digestion and reduction of the incubation time in buffers.

Bridge *et al*. reported 12% of cases of EFT as non-diagnostic [21]. In another report by Mhawech there was 8.5% of cases considered uninformative and non-diagnostic [20]. In our material, 23.6% of various SRCTs showed no signals or signals difficult to interpret. Many cases included in our study were consultations from other hospitals. It was presumed that the standards of fixation and preservation of the material were difficult to control and suboptimal. In cases of non-diagnostic FISH results, clinical data were re-analysed, histopathological examinations and IHC tests were reviewed or repeated. In some cases that procedure has led to determination of the final diagnosis other than SRCT.

We propose an algorithm (Fig. 2) based on our data and data acquired from the literature useful for a differential diagnosis of SRCTs with rounds of IHC and FISH [20, 23]. An initial IHC study includes cytokeratin/EMA, Des/MSA and S100 immunohistochemical evaluation. Cases negative for Des/MSA were tested with CD99/FLI-1 antibodies in order to include the cases to the group suspected for EFT. The test with CD99 antibody shows a low specificity. In our material FLI1 antibody was not

specific and gives high background staining. These stainings are included in the algorithm due to variations of the specificity and sensitivity in different laboratories. Therefore, the next step including a FISH test with an EWSR1 probe is an important test to be performed. A positive FISH test confirms the diagnosis of EFT or EMS. Other neoplasms with *EWSR1* gene rearrangement should be considered and clinical data evaluated. Those cases that are FISH negative for EWSR1 and still suspected for EFT can be tested with a FUS probe. This test can show additional cases of EFTs with rare FUS rearrangement or a case of RCLS. A review of the clinical data is an important adjunct of the evaluation. Cases positive for Des/MSA are stained with a myogenin antibody. It presents a reliable nuclear marker with 100% specificity and sensitivity. Positive staining is consistent with the diagnosis of one of the variants of RMS, however cases with positive staining but equivocal clinically and negative cases for myogenin are tested with the *FOXO1A* FISH probe.

CK or EMA positive cases presenting with specific clinical features are usually tested with Des and WT1 antibody. In order to confirm the diagnosis of DSRCT, a FISH study with the *EWSR1* probe is performed regardless of the status of WT1 staining. *EWSR1* negative cases are evaluated further with clinical and immunohistochemical data. In the specific clinical setting, synaptophysin and chromogranin stainings are performed in order to evaluate metastatic tumours in the soft tissues, namely CK20 positive MCC or TTF1 positive SCC. A soft tissue SRCT, positive for CK or EMA in an appropriate clinical setting should have the FISH test with a *SYT* probe performed in order to confirm SyS diagnosis. An EWSR1 test is done in *SYT* negative cases to exclude tumours associated with rearrangements of *EWSR1*, primarily EFT. It is imperative to exclude NHL with a panel of antibodies. S100 protein is a useful introductory marker of MM, however the diagnosis is confirmed with HMB45, Melan A and MITF antibodies. *EWSR1* rearrangement allows for differentiation of MM and CCS. Cases of SCRT showing S100 or desmin staining are tested with *EWSR1* FISH to exclude or confirm EFT. Clinical data are necessary to exclude a small cell variant of MPNST in S100 positive as well as negative.

## References


1. Meis-Kindblom JM, Stenman G, Kindblom LG. Differential diagnosis of small round cell tumors. Semin Diagn Pathol 1996; 13: 213-241.
2. Rabbitts TH. Chromosomal translocations in human cancer. Nature 1994; 372: 143-149.
3. Xia SJ, Barr FG. Chromosome translocations in sarcomas and the emergence of oncogenic transcription factors. Eur J Cancer 2005; 41: 2513-2527.
4. Ladanyi M. The emerging molecular genetics of sarcoma translocations. Diagn Mol Pathol 1995; 4: 162-173.
5. Fellinger EJ, Garin-Chesa P, Glasser DB, et al. Comparison of cell surface antigen HBA71 (p30/32MIC2), neuron-specific enolase, and vimentin in the immunohistochemical analysis of Ewing's sarcoma of bone. Am J Surg Pathol 1992; 16: 746-755.
6. Folpe AL, Goldblum JR, Rubin BP, et al. Morphologic and immunophenotypic diversity in Ewing family tumors: a study of 66 genetically confirmed cases. Am J Surg Pathol 2005; 29: 1025-1033
7. Lae ME, Roche PC, Jin L. Desmoplastic small round cell tumour: a clinicopathologic, immunohistochemical and molecular study of 32 cases. Am J Surg Pathol 2002; 26: 8232-8235.
8. Dei Tos AP, Wadden C, Calonje E, et al. Immunohistochemical demonstration of glycoprotein p30/32MIC2 (CD99) in synovial sarcoma. A potential cause of diagnostic confusion. Appl Immunohistochem 1995; 3. 168-173.
9. Kumar S, Perlman E, Harris CA, et al. Myogenin is a specific marker for Rhabdomyosarcoma: An immunohistochemical study in paraffin-embedded tissues. Mod Pathol 2000. 13: 988-993.
10. Chu PG, Weiss LM. Keratin expression in human tissues and neoplasms. Histopathology 2002; 40: 403-439.
11. Ordonez JL, Osuna D, Herrero D, et al. Advances in Ewing's sarcoma research: Where are we now and what lies ahead? Cancer Res 2009; 69: 7140-7150.
12. Zucman J, Melot T, Desmaze C, et al. Combinatorial generation of variable fusion proteins in the Ewing family of tumours. Embo J 1993; 12: 4481-4487.
13. Wang L, Bhargava R, Zheng T, et al. Undifferentiated Small Round Cell Sarcomas with Rare EWS Gene Fusions: Identification of a Novel EWS-SP3 Fusion and of Additional Cases with the EWS-ETV1 and EWS-FEV Fusions J Mol Diagn 2007; 9: 498-509.
14. Ng TL, O'Sullivan MJ, Pallen CJ, et al. Ewing sarcoma with novel translocation t(2;16) producing an in-frame fusion of FUS and FEV. J Mol Diagn 2007; 9: 459-463.
15. Shing DC, McMullan DJ, Roberts P, et al. FUS/ERG Gene Fusions in Ewing's Tumors. Cancer Res 2003; 63: 4568-4576.
16. Panagopoulos I, Aman P, Fioretos T, et al. Fusion of the FUS gene with ERG in acute myeloid leukemia with t(16;21)(p11;q22). Genes Chromosomes Cancer 1994; 11: 256-262.
17. Shimizu K, Ichikawa H, Tojo A, et al. An ets-related gene, ERG, is rearranged in human myeloid leukemia with t(16;21) chromosomal translocation. Proc Natl Acad Sci U S A 1993; 90: 10280-10284.
18. Szuhai K, Ijszenga M, de Jong D, et al. The NFATc2 Gene Is Involved in a Novel Cloned Translocation in a Ewing Sarcoma Variant That Couples Its Function in Immunology to Oncology. Clin Cancer Res 2009; 15: 2259-2268.
19. Kawamura-Saito M, Yamazaki Y, Kaneko K, et al. Fusion between CIC and DUX4 up-regulates PEA3 family genes in Ewing-like sarcomas with t(4;19)(q35;q13) translocation. Hum Mol Genet 2006; 15: 2125-2137.
20. Mhawech-Fauceglia P, Herrmann F, Penetrante R, et al. Diagnostic utility of FLI-1 monoclonal antibody and dual-colour, break-apart probe fluorescence in situ (FISH) analysis in Ewing's sarcoma/primitive neuroectodermal tumour (EWS/PNET). A comparative study with CD99 and FLI-1 polyclonal antibodies. Histopathology 2006, 49: 569-575.
21. Bridge RS, Rajaram V, Dehner LP, et al. Molecular diagnosis of Ewing sarcoma/primitive neuroectodermal tumor in routine processed tissue: a comparison of Two FISH strategies and RT-PCR in malignant round cell tumors. Mod Pathol 2006, 19: 1-8.
22. Limon J, Turc-Carel C, Dal Cin P, et al. Recurrent chromosome translocations in liposarcoma. Cancer Genet Cytogenet 1986; 22: 93-94.
23. Tsokos M. The diagnosis and classification of childhood rhabdomyosarcoma. Semin Diagn Pathol 1994; 11: 26-38.

24. de Alava E, Lozano MD, Sola I, et al. Molecular features in a biphenotypic small cell sarcoma with neuroectodermal and muscle differentiation. Hum Pathol 1998; 29: 181-184.
25. Sorensen PH, Shimada H, Liu XF, et al. Biphenotypic sarcomas with myogenic and neural differentiation express the Ewing's sarcoma EWS/FLI1 fusion gene. Cancer Res 1995; 55: 1385-1392.
26. Thorner P, Squire J, Chilton-MacNeil S, et al. Is the EWS/FLI-1 fusion transcript specific for Ewing sarcoma and peripheral primitive neuroectodermal tumor? A report of four cases showing this transcript in a wider range of tumor types. Am J Pathol 1996; 148: 1125-1138.
27. Barr FG. Gene fusions involving PAX and FOX family members in alveolar rhabdomyosarcoma. Oncogene 2001; 20: 5736-5746.
28. Galili N, Davis RJ, Fredericks WJ, et al. Fusion of a fork head domain gene to PAX3 in the solid tumour alveolar rhabdomyosarcoma. Nat Genet 1993; 5: 230-235.
29. Seidal T, Mark J, Hagmar B, et al. Alveolar rhabdomyosarcoma: a cytogenetic and correlated cytological and histological study. Acta Pathol Microbiol Immunol Scand [A] 1982; 90: 345-354.
30. Davis RJ, D'Cruz CM, Lovell MA, et al. Fusion of PAX7 to FKHR by the variant t(1;13)(p36;q14) translocation in alveolar rhabdomyosarcoma. Cancer Res 1994; 54: 2869-2872.
31. Anderson J, Ramsay A, Gould S, et al. PAX3-FKHR induces morphological change and enhances cellular proliferation and invasion in rhabdomyosarcoma. Am J Pathol 2001; 159: 1089-1096.
32. Barr FG, Biegel JA, Sellinger B, et al. Molecular and cytogenetic analysis of chromosomal arms 2q and 13q in alveolar rhabdomyosarcoma. Genes Chromosomes Cancer 1991; 3: 153-161.
33. Collins MH, Zhao H, Womer RB, et al. Proliferative and apoptotic differences between alveolar rhabdomyosarcoma subtypes: a comparative study of tumors containing PAX3-FKHR or PAX7-FKHR gene fusions. Med Pediatr Oncol 2001; 37: 83-89.
34. Anderson J, Gordon T, McManus A, et al. Detection of the PAX3-FKHR fusion gene in paediatric rhabdomyosarcoma: a reproducible predictor of outcome? Br J Cancer 2001; 85: 831-835.
35. Sorensen PH, Lynch JC, Qualman SJ, et al. PAX3-FKHR and PAX7-FKHR gene fusions are prognostic indicators in alveolar rhabdomyosarcoma: a report from the children's oncology group. J Clin Oncol 2002; 20: 2672-2679.
36. Limon J, Dal Cin P, Sandberg AA. Translocations involving the X chromosome in solid tumors: presentation of two sarcomas with t(X;18)(q13;p11). Cancer Genet Cytogenet 1986; 23: 87-91.
37. Guillou L, Benhattar J, Bonichon F, et al. Histologic grade, but not SYT-SSX fusion type, is an important prognostic factor in patients with synovial sarcoma: a multicenter, retrospective analysis. J Clin Oncol 2004; 22: 4040-4050.
38. Mezzelani A, Mariani L, Tamborini E, et al. SYT-SSX fusion genes and prognosis in synovial sarcoma. Br J Cancer 2001; 85: 1535-1539.
39. Nilsson G, Skytting B, Xie Y, et al. The SYT-SSX1 variant of synovial sarcoma is associated with a high rate of tumor cell proliferation and poor clinical outcome. Cancer Res 1999; 59: 3180-3184.
40. Clark J, Rocques PJ, Crew AJ, et al. Identification of novel genes, SYT and SSX, involved in the t(X;18)(p11.2;q11.2) translocation found in human synovial sarcoma. Nat Genet 1994; 7: 502-508.
41. Shipley JM, Clark J, Crew AJ, et al. The t(X;18)(p11.2;q11.2) translocation found in human synovial sarcomas involves two distinct loci on the X chromosome. Oncogene 1994; 9: 1447-1453.
42. Dagher R, Pham TA, Sorbara L, et al. Molecular confirmation of Ewing sarcoma. J Pediatr Hematol Oncol 2001; 23: 221-224.
43. Meis JM, Osborne BM, Butler JJ. A comparative marker study of large cell lymphoma, Hodgkin's disease, and true histiocytic lymphoma in paraffin-embedded tissue. Am J Clin Pathol 1986; 86: 591-599.
44. Dockhorn-Dworniczak B, Schafer KL, Blasius S, et al. Assessment of molecular genetic detection of chromosome translocations in the differential diagnosis of pediatric sarcomas. Klin Padiatr 1997; 209:156-164.
45. Kumar S, Pack S, Kumar D, et al. Detection of EWS-FLI-1 fusion in Ewing's sarcoma/peripheral primitive neuroectodermal tumor by fluorescence in situ hybridization using formalin-fixed paraffin-embedded tissue. Hum Pathol 1999; 30: 324-330.
46. Qian X, Jin L, Shearer BM, et al. Molecular diagnosis of Ewing's sarcoma/primitive neuroectodermal tumor in formalin-fixed paraffin-embedded tissues by RT-PCR and fluorescence in situ hybridization. Diagn Mol Pathol 2005; 14: 23-28.

## Address for correspondence

**Anna Szumera-Ciećkiewicz** MD
Department of Pathology
Maria Skłodowska-Curied Memorial
Cancer Center and Institute of Oncology
ul. Roentgena 5
02-781 Warszawa
phone +48 22 546 27 26
e-mail: annacieckiewicz@coi.waw.pl