

## Fluorescence in Situ Hybridization (FISH) for Differential Diagnosis of Soft Tissue Sarcomas

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### Abstract

**Introduction:** Soft tissue sarcomas are rare tumors comprising 1 percent of solid malignancies. The latest edition of WHO soft tissue pathology lists 94 benign and malignant soft tissue tumors. Many of these show a large degree of morphological overlap. Immunohistochemistry has been shown to be reliable in many cases for differential diagnosis of lesions, although cytogenetic tests are considered the gold standard for many entities. Fluorescence in-situ hybridization (FISH) is a cytogenetic technique that uses fluorescent probes that bind to only those parts of the chromosome which have a high degree of sequence complementarity. Many soft tissue tumors show recurrent genetic mutations that are now being used as diagnostic markers. Knowledge of the molecular identity allows prediction of behavior, prognosis and treatment response. **Objective:** The aim of this study was to identify genetic mutations in soft tissue sarcomas using FISH testing and to assess correlations with histological diagnosis. **Material and methods:** A total of 25 cases of different soft tissue sarcomas diagnosed on histology with the help of immunohistochemical staining and for which FISH studies were requested were included in this study. Three pathologists with a special interest in soft tissue sarcomas reviewed the cases. FISH tests for EWS, the X:18 translocation, FOXO1 and MDM2 were respectively applied for 8 cases of Ewing sarcoma, 8 cases of synovial sarcoma, 2 cases of rhabdomyosarcoma and 7 cases of dedifferentiated liposarcoma and atypical lipomatous tumors/well differentiated liposarcomas. **Results:** EWS gene fusion was detected in 7 out of 8 cases of Ewing sarcoma and the X:18 translocation was positive in 3 of the 8 cases of synovial sarcoma. FOXO1 was not detected in either of the two rhabdomyosarcomas. MDM2 by FISH was detected in only one out of 5 cases of atypical lipomatous tumors and 1 out of 2 dedifferentiated liposarcomas. **Conclusion:** FISH is a useful adjunct in the diagnostic assessment of different types of soft tissue sarcomas. It is easy to set up, is relatively inexpensive and has the ability to diagnose sarcomas with great accuracy, especially in cases which can not be accurately classified even after thorough histological and immunohistochemical evaluation. It may play a very important role in the accurate diagnosis and correct management of patients.

**Keywords:** Ewing sarcoma- dedifferentiated liposarcoma- rhabdomyosarcoma- synovial sarcoma- soft tissue sarcomas

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### Introduction

Soft tissue sarcomas are a heterogeneous group of solid malignancies. These tumors are mesenchymal derivatives and comprise 1% of all adult malignancies and 15% of pediatric tumors. Biological behavior of these tumors is dependent on its specific type (Burningham et al., 2012).

Soft tissue sarcomas are diagnosed based on their clinical, morphological and cytogenetic features. Many soft tissue sarcomas show overlapping morphological features on histology. Immunohistochemical stains also show overlapping results and cannot determine a particular lineage in a large number of cases. The new WHO classification of soft tissue sarcomas has included cytogenetics as diagnostic criteria in many tumors including Ewing sarcoma, Low grade fibromyxoid

sarcoma, Dedifferentiated liposarcoma, Alveolar rhabdomyosarcoma, synovial sarcoma, epithelioid sarcoma and infantile fibrosarcoma (Fletcher, 2014).

Many soft tissue tumors are associated with recurrent chromosomal rearrangements including most commonly translocations. Isolation and sequencing of these translocations has led to identification of highly specific gene sequences involved in causation of these tumors (Bridge, 2014).

Commonly used genetic approaches in clinical testing include conventional cytogenetic analysis, Fluorescence in situ hybridization, reverse transcription PCR and sequencing.

Fluorescence in situ hybridization (FISH) is a cytogenetic method using fluorescent probes that binds to parts of chromosomes showing high degree of

sequence complementarity. It was developed in 1980s by biomedical researchers and can be used to detect and localize the presence or absence of specific DNA sequences. Fluorescence microscope is used to detect the probe bound to the chromosomes (Yin et al., 2015).

FISH is used in our department for both diagnostic and prognostic purposes. FISH studies related to soft tissue sarcomas which we use in our department are EWSR1 gene fusion for Ewing sarcoma family of tumors, FOXO1 for Alveolar rhabdomyosarcoma, MDM2 gene amplification for atypical lipomatous tumors/well differentiated liposarcoma and dedifferentiated liposarcoma and X:18 translocation for synovial sarcoma.

EWSR1 gene fusion is mainly used for confirmation of Ewing sarcoma. Ewing sarcoma is the second most common tumor occurring in children and young adults and also comprises of 10-15% of primary bone tumors. Other tumors with similar histology also arise in soft tissue. On light microscopy the tumor usually comprises cells arranged in nests and sheets. The tumor expresses increased cytoplasmic glycogen detected by Periodic acid-Schiff stain and shows membranous staining for CD 99 and nuclear staining for FLI-1 gene protein product. However, both these immunohistochemical markers are non-specific and can be expressed in many other round blue cell tumors arising in similar clinical scenario including lymphoblastic lymphoma and small cell variant of osteosarcoma. Similarly a poorly differentiated synovial sarcoma may mimic a Ewing sarcoma on histology and also expresses CD 99. EWSR1-ETS fusion gene can help differentiate these tumors (Burchill, 2003; Balamuth and Womer, 2010).

Synovial sarcoma can arise in any age in deep soft tissues of upper and lower extremities and occurs most commonly in teenagers and young adults. The tumor is either biphasic or monophasic. Biphasic tumors show epithelial and spindle cell components in varying proportion and can therefore be easily diagnosed on routine microscopy. However, majority of the tumors show monophasic spindle cell morphology composed of cells arranged in fascicles and dense cellular sheets. The differential diagnoses include Ewing sarcoma and malignant peripheral nerve sheath tumors. The tumors show positivity for CD99, EMA, high molecular weight cytokeratin and TLE-1. Synovial sarcoma is responsive to chemotherapy and its identification is of both therapeutic and prognostic significance. Detection of X:18 translocation, specific to synovial sarcoma can help in differentiating it from its mimickers (Foo et al., 2011; Terry et al., 2005).

Atypical lipomatous tumors are locally aggressive mesenchymal neoplasms and occur most frequently in deep soft tissues of the limbs, retroperitoneum, paratesticular areas and mediastinum. These lesions occur in middle aged adults with peak incidence in 6th decade. Morphologically they are composed of relatively mature adipocytic proliferation with focal atypia and hyperchromasia. Differential diagnosis include benign adipocytic tumors including spindle cell lipoma/pleomorphic lipoma (Mentzel et al., 2010).

Dedifferentiated liposarcoma is a malignant adipocytic

tumor arising mostly from retroperitoneum. The tumor shows abrupt transition from Atypical lipomatous tumor/well differentiated liposarcoma to non-lipogenic sarcoma which in most cases is of high grade. These high grade areas can resemble any high grade sarcoma. Immunohistochemical stains CDK4, MDM2 and P16 are used to differentiate these tumors from other sarcomas but are not always useful (Kim et al., 2010).

MDM2 gene amplification is present in these two tumors (atypical lipomatous tumors/well differentiated liposarcoma and dedifferentiated liposarcoma) and can help distinguish them from their histological mimickers (Weaver et al., 2008).

Alveolar rhabdomyosarcoma is a highly cellular malignant neoplasm with a monomorphous population of primitive cells with round nuclei. It most commonly arises in extremities and occurs most commonly in adolescent and young adults. It is characterized by PAX3-FOXO1 or PAX7-FOXO1 fusion. It is not always possible to differentiate it from Embryonal rhabdomyosarcoma on histology which carries a much better prognosis and FOXO1 detected through FISH is of prognostic importance (Linardic, 2008).

These genetic testing techniques are not available in routine histology laboratories. We have undertaken this study at this hospital to highlight the role of these techniques in the diagnosis of soft tissue sarcomas in developing country.

## Materials and Methods

It is a descriptive, cross sectional study. After approval from the Institutional Review Board a total of 25 cases of soft tissue sarcomas diagnosed between January 2014 to December 2016 at SKMCH and RC were retrieved from computerized database. All those cases were included on which immunohistochemical stains had already been performed on formalin fixed paraffin embedded sections, so that FISH could be performed. All ages, genders and sites were selected. Cases with poorly fixed and scanty tissues were excluded. Cases were reviewed by three pathologists with a special interest in soft tissue sarcomas. Diagnoses were unchanged after histological and immunohistochemical review of the cases. Cases included 8 Ewing sarcomas, 8 synovial sarcomas, 2 rhabdomyosarcomas, 5 lipomatous tumors and 2 dedifferentiated liposarcomas. Tissue blocks with adequate tumor material were selected for FISH evaluation. 4-5  $\mu$ m thick paraffin sections were mounted on positively charged slides (Super Frost). The tissues were subjected to FISH analysis according to the instructions mentioned in the FISH probe literature. FISH probes used were Vysis LSI EWSR1 (22q120 Dual Color, break Apart Rearrangement Probe (Part No.30-190059) for Ewing sarcoma, Vysis LSI MDM2 Spectrum Orange/CEP 12 Spectrum Green Probes (Part No.30-231098) for well differentiated and dedifferentiated liposarcomas, Vysis LSI FOXO1 (13q14) Dual color, break Apart Rearrangement Probe (Part No.30-231023) for rhabdomyosarcomas and Vysis LSI SS18 (18q11.2) Dual Color, break Apart Rearrangement Probe (Part No.30-231018) for synovial

sarcomas. The FISH slides were analyzed on an Olympus BX61 microscope using DAPI/Green/Red triple band filter set at 100x magnification. Ewing sarcoma break apart was reported positive if more than 14 out of 50 cells were seen to show break apart signals. Case was labelled as X:18 translocation positive if more than 10 cells showed break apart for X:18. MDM2 was reported as amplified if ratio of red to green signals was more than 2. FOXO1 gene rearrangement was said to be observed when more than 10 out of 50 cells showed break apart signals.

Mean, mode and median were calculated for quantitative variables such as patient's age. Frequencies and percentages were calculated for qualitative variables like gender, sites, histological types of sarcoma and FISH results.

## Results

Out of 8 cases of Ewing sarcoma (diagnosed on the basis of histology and immunohistochemical results) 2 were male patients and 6 were females. Age range was between 5 to 20 years (mean patient age 11.25 years). The commonest tumor site was femur (4 cases). Humerus, maxilla, chest wall and iliac crest were the sites of presentation in one case each. All these tumors revealed diffuse membranous staining for CD99 and negative expression for LCA, Desmin, Myogenin, Synaptophysin and CK. The main reason for applying FISH was to

differentiate between Ewing sarcoma and small cell variant of osteosarcoma. Out of all these cases, 7 cases showed EWSR gene rearrangement Figure 1. One case which did not show gene translocation was labeled as undifferentiated round cell sarcoma as it did not fit into any other category even after applying a large panel of immunohistochemical stains Table 1.

There were 8 cases diagnosed as Synovial sarcoma on the basis of histology and immunohistochemical stains. These cases had shown focal positivity for CK, EMA and CD99 and negative expression for Desmin, S100 and CD34. About 7 patients were males and 1 was female. Age range was between 21 to 35 years (mean patient age 26.4 years). Most common site was lower limb (5 cases) followed by upper limb (3 cases). X:18 translocation was detected in 3 out of 8 cases Figure 2. An extensive panel of immunohistochemical stains including SMA (smooth muscle actin), Caldesmon, High molecular weight cytokeratin (HMWCK), p16, CDK4, MUC4 was applied on the tumors which did not show gene translocation. All immunostains turned out to be negative and were reported as undifferentiated sarcomas Table 1.

A total of 5 cases were diagnosed as lipomatous tumors with differential diagnoses of atypical lipomatous tumors and lipoma. Final diagnosis was deferred for FISH analysis for MDM2 amplifications. There were 2 male patients and 3 female patients. Age range was 30 to 44

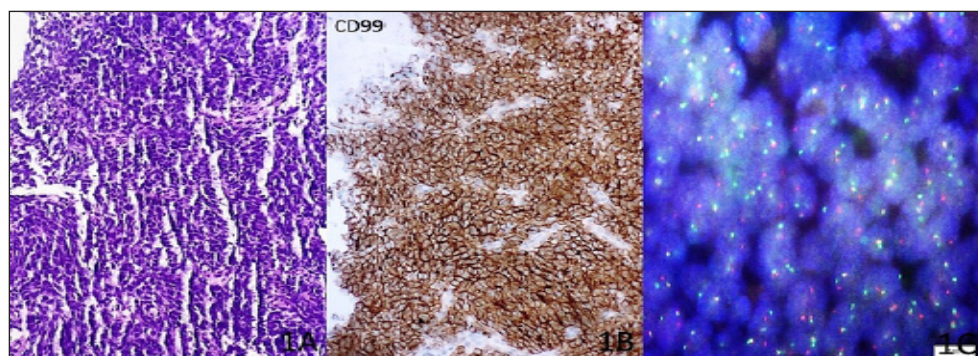


Figure 1. Light Microscopic Appearance of Ewing's Sarcoma Showing Diffuse Sheets of Small Sized Round to Oval Hyperchromatic Cells (H & E 10X). 1B: Diffuse strong membranous staining for CD99. 1C, FISH technique showing break apart signal representing EWSR1 gene rearrangement.

Table 1. Age, Gender, and Diagnosis (Before and After Applying FISH Results)

Diagnosis before applying FISH on the basis of histology and immunohistochemistry	Age Range	Gender		FISH results	Diagnosis (after FISH results)
		Male	Female		
8 cases of Ewing Sarcoma	5 to 20 years	2	6	Positive for EWSR break apart : 7 Negative : 1	Ewing Sarcoma Undifferentiated round cell sarcoma
8 cases of Synovial Sarcoma	21 to 35 years	7	1	Positive for X:18 break apart : 3 Negative : 5	Synovial Sarcoma Undifferentiated Sarcoma
5 cases of lipomatous tumors	30 to 44 years	2	3	Positive for MDM2 amplification : 1 Negative: 4	Atypical lipomatous tumor/well differentiated liposarcoma Lipoma
2 cases of pleomorphic sarcoma	45 to 55 years	0	2	Positive for MDM2 amplification : 1 Negative: 1	Dedifferentiated liposarcoma Undifferentiated pleomorphic sarcoma
2 cases of rhabdomyosarcoma	1 to 9 years	1	1	Positive for FOX O1 break apart : 0 Negative: 2	Embryonal Rhabdomyosarcoma

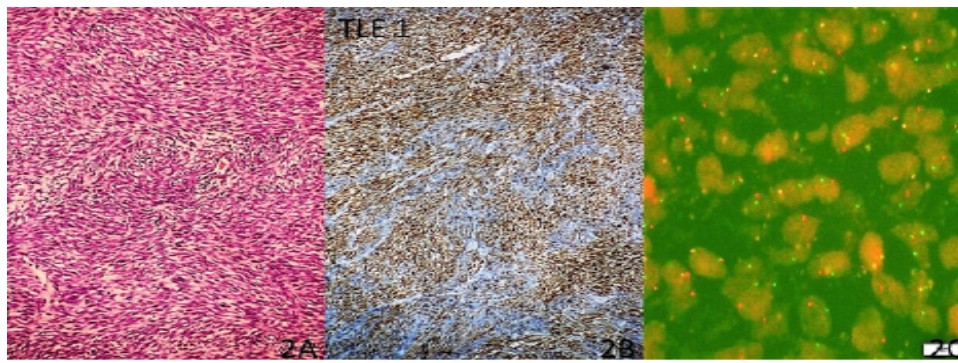


Figure 2. Light Microscopic Appearance of Monophasic Synovial Sarcoma (H & E 10X) 2B, TLE1 Nuclear Expression; 2 C, X;18 Break Apart Signals by FISH

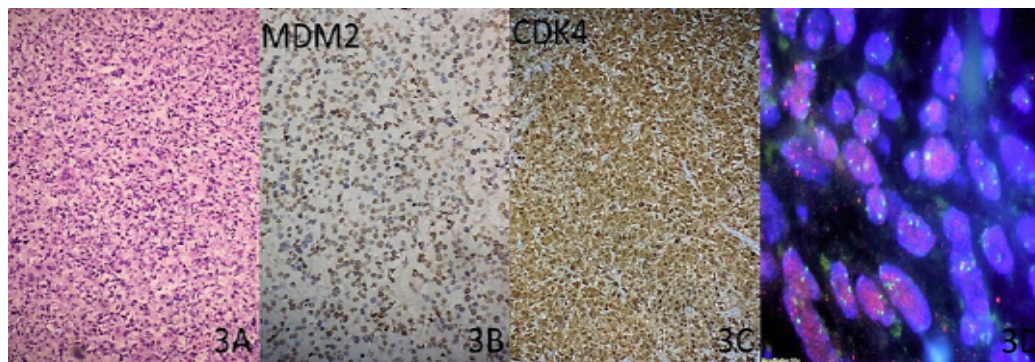


Figure 3. A, Light Microscopic Appearance of De-differentiated Liposarcoma; B and C, MDM2 and CDK4 Staining; D, MDM2 Amplification by FISH

years (mean patient age 34.8 years). Most common site was the lower limb (right thigh 1 case, left thigh 1 case), followed by pelvis (1 case), stomach and retroperitoneum (1 case each). The reason for performing FISH on these tumors was to differentiate atypical lipomatous tumors/well differentiated liposarcoma from lipomas including spindle cell or pleomorphic lipoma Figure 3. MDM2 gene amplification was detected in only 1 case which presented as lipomatous mass in right thigh. Rest of the cases were labelled as lipomas Table 1.

Two cases were suspected dedifferentiated liposarcomas on the basis of histology and immunohistochemical results as both of these cases had shown negative results for Desmin, SMA, CK, CD34, S100, HMB45, EMA and positive results for p16, CDK4 and MDM2. However one case showed strong and the other case showed focal positivities for p16, CDK4 and MDM2. Both patients were females. One patient was 45 and the other was 55 years old. Tumor sites were left scapular and left inguinal region respectively. Reason for performing FISH was to differentiate between undifferentiated pleomorphic sarcoma and dedifferentiated liposarcoma Figure 3. 1 out of these 2 cases showed MDM2 gene amplification and was labeled as dedifferentiated liposarcoma (left scapular region) and the other case was diagnosed as undifferentiated pleomorphic sarcoma Table 1.

Out of the two cases which were diagnosed as rhabdomyosarcomas, one patient was 1 year female child and the other was a 9 years old boy. Tumor sites were upper arm and orbit respectively. Reason for performing FISH was to distinguish between embryonal and alveolar

rhabdomyosarcoma. FOXO1 gene translocation was not detected in either of the two cases and they were labelled as embryonal rhabdomyosarcoma on the basis of histological features Table 1.

## Discussion

Soft tissue sarcomas are rare tumors comprising 1 percent of all solid malignancies. These tumors show variable biological behavior and therefore their correct diagnosis is essential for appropriate treatment and determination of prognosis. These tumors are sometimes difficult to diagnose due to overlap in histological and immunohistochemical features. FISH and other genetic techniques including PCR and next generation sequencing now play a very important role in final diagnosis. (Bunningham et al., 2012).

These techniques are not easily available at routine laboratories. It is better to refer these cases to specialized labs which conduct these tests.

Fluorescent in situ hybridization is comparatively an easy and cheaper technique and not very difficult to develop in tertiary care hospital labs. Our institute has recently acquired this technique. Probes related to soft tissue sarcomas being used in our lab are EWSR1, MDM2, FOXO1 and X:18 (details of probes are given in materials and methods).

Many tumors come under the differential diagnosis of Ewing Sarcoma including small cell variant of osteosarcoma, rhabdomyosarcoma, lymphoma, neuroblastoma and desmoplastic small round cell

tumor. Although immunohistochemical stains are helpful for final diagnosis, overlapping histological and immunohistochemical results make FISH for detection of EWSR1 translocation an important test for definitive diagnosis. (Machado et al., 2009).

EWSR1 gene rearrangement is most commonly observed in Ewing Sarcoma. However, other tumors such as clear cell sarcoma, desmoplastic small round cell tumor, extraskeletal myxoid chondrosarcoma can also show this gene rearrangement. Therefore FISH probe for EWSR1 is useful if used in correct clinical context. (Burchill 2003 ; Balamuth and Womer 2010).

In our study, seven out of eight cases tested for EWSR1 re-arrangement showed break apart signals. One case which did not show EWSR1 gene rearrangement was diagnosed as undifferentiated round cell sarcoma. These results are consistent with a large study of 382 cases where 92% of the histologically diagnosed cases of Ewing sarcoma were confirmed by FISH (Machado et al., 2009).

Likewise many spindle cell tumors come under the differential diagnosis of synovial sarcoma like malignant peripheral nerve sheath tumor, leiomyosarcoma, fibrosarcoma and solitary fibrous tumor. Therefore, FISH for detection of X:18 translocation is a valuable test to have in a lab. Synovial sarcoma shows a much better response to chemotherapy than other soft tissue sarcomas, therefore its detection is extremely important (Foo et al., 2011; Terry et al., 2005).

FISH for X:18 was applied on 8 cases which were histologically and immunohistochemically diagnosed as synovial sarcoma. These cases were positive for EMA, CD99 and TLE1. Three cases in our study showed break apart of SYT gene confirming the diagnosis of synovial sarcoma. The other five cases which showed negative results were further analyzed with further immunostains as explained in the results and were finally labelled as undifferentiated sarcomas. Terry et al found X:18 chromosomal translocation in 22 out of 23 synovial sarcomas diagnosed histologically, while none of the other sarcomas tested were positive for this translocation. (Terry et al., 2005). Therefore it is important to include FISH analysis as a part of diagnosis process before signing out of report. In our institute we ask the oncologists to wait for FISH results before start of treatment as this approach is very helpful for correct diagnosis and management.

Moving on to lipomatous tumors, sometimes it is very difficult to differentiate between atypical lipomatous tumor/well differentiated liposarcomas and lipomas especially when one is suspecting pleomorphic and spindle cell lipoma at unusual locations. In our experience sometimes even after extensive sampling of well differentiated lipomatous tumors, it is very hard to find hallmark lipoblasts or it is very hard to differentiate between true lipoblasts and lipoblast like cells. In this situation, a panel of immunohistochemical stains p16, CDK4 and MDM2 is very helpful as atypical lipomatous tumors/well differentiated liposarcomas show good expression for these immunostains whereas lipomas are either negative or show focal expression. Even then there are times, when results are not unequivocal and one needs further testing.

In this scenario, MDM2 amplification study by FISH is very helpful as it is very much specific for dedifferentiated and well differentiated liposarcoma/atypical lipomatous tumors (Mendoza et al., 2014). Its importance cannot be overemphasized because it is difficult to distinguish well differentiated liposarcoma from benign lipomatous tumors and dedifferentiated liposarcomas from other high grade sarcomas by using morphological criteria only (Kimura et al., 2013). Moreover dedifferentiated liposarcoma is associated with less aggressive clinical outcome as compared to other high grade pleomorphic sarcomas and therefore this distinction is of prognostic importance (Sirvent 2007).

We had this difficulty of differentiating between atypical lipomatous tumor/well differentiated liposarcomas in five cases as mentioned in the result. We had to apply FISH to detect MDM2 amplification. Only 1 case turned out to be MDM2 amplified whereas rest of the 4 cases were negative and we called them lipomas. Similarly FISH for MDM2 amplification was used in 2 cases with the differential diagnoses of dedifferentiated liposarcoma and undifferentiated pleomorphic sarcoma. MDM2 amplification was detected in one case as mentioned in the results and this case was labelled as dedifferentiated liposarcoma and other case which turned out to be negative was called undifferentiated pleomorphic sarcoma. The latter is usually a diagnosis of exclusion when no specific lineage can be identified after application of an exhaustive panel of immunohistochemical markers and FISH techniques. In the study conducted by Takeshi et al 81 out of 178 lipomatous tumors and 18 out of 20 dedifferentiated liposarcoma showed MDM2 gene amplification (Takeshi et al., 2012).

FISH also has limited but important role in rhabdomyosarcomas when used in correct clinical context. Because of prognostic difference between different types of rhabdomyosarcomas we need to classify them correctly into embryonal, alveolar, spindle cell, sclerosing and pleomorphic rhabdomyosarcomas. Histology and immunohistochemical stains are usually enough for accurate classification but sometimes problem arises when rhabdomyosarcoma shows focal alveolar and nesting pattern making it difficult to differentiate alveolar rhabdomyosarcoma from embryonal and other rhabdomyosarcomas. Moreover the prognosis of alveolar rhabdomyosarcoma is worse than that of embryonal rhabdomyosarcoma (Fenghai et al., 2012). Detection of FOXO1 gene rearrangement by FISH is useful to differentiate between embryonal and alveolar rhabdomyosarcoma. We applied FOXO1 gene rearrangement FISH on 2 cases with borderline histological features between alveolar and embryonal rhabdomyosarcomas. Both cases did not show break apart signals for FOX O1 gene and hence they were labeled as embryonal rhabdomyosarcoma.

Therefore in our limited experience we consider FISH analysis useful especially in those cases where histological and immunohistochemical evaluation cannot accurately classify tumors. It is important to develop a uniform diagnostic algorithm in the departments through discussion with other fellow pathologists and oncologists. This is very helpful for accurate diagnosis and correct

management of patients.

In conclusion, FISH is a useful adjunct in the diagnostic approach for various soft tissue sarcomas. It is easy to set up, is a cheaper technique and has the ability to diagnose sarcomas with great accuracy especially in those sarcoma cases which are not accurately classified even after thorough histological and immunohistochemical evaluation. It plays a very important role in the accurate diagnosis and correct management of patients.

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