Fluorescence In Situ Hybridization in the Diagnosis of Soft Tissue Neoplasms: A Review

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Abstract: This paper presents an overview of the role of fluorescence in situ hybridization (FISH) in the diagnosis of soft tissue neoplasms. Many soft tissue neoplasms harbor characteristic translocations or amplification of gene regions, which can be assessed by FISH, and can be used to assist in their diagnosis. We discuss the major morphologic categories in which FISH has come to be used including high-grade round cell sarcomas, spindle cell sarcomas, low-grade myxoid neoplasms, adipocytic neoplasms, and malignant melanocytic neoplasms on the basis of a recent review of soft tissue neoplasms which were analyzed by FISH. We also review the molecular alterations (translocations and amplification of gene regions), which have come to define many of these diagnostic entities and the most effective way to evaluate them with FISH with attention to potential pitfalls. Finally, we discuss the advantages and disadvantages of FISH as a technique when appraising soft tissue neoplasms.

Key Words: soft tissue neoplasms, fluorescence in situ hybridization, mesenchymal neoplasms

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More than any other subset of solid tumors, the diagnosis of soft tissue neoplasms has become heavily reliant on molecular analysis as an adjunct to light microscopic and immunohistochemical evaluation. This is because, in part, of the challenging nature of the discipline, and the difficulty of sorting through diagnostic entities with overlapping histologic and immunohistochemical features. In addition, a number of difficult diagnostic entities have characteristic molecular alterations, and as such, methods, which allow for detection of these alterations are attractive (Table 1).

Several methods exist to evaluate for these genetic events, including conventional cytogenetics, reverse transcriptase-polymerase chain reaction (RT-PCR), and fluorescence in situ hybridization (FISH). For a variety of reasons (discussed below) FISH has become the molecular test of choice at many institutions including our own. We recently reviewed 235 consecutive soft tissue consultations over a 30-month period where FISH was performed at our institution.[1] Herein, we describe the results from our study, expound upon how FISH is used in the diagnosis of individual soft tissue neoplasms, and provide a review of the literature with regards to the molecular alterations present in these diagnostic entities.

Five break-apart probes are used at our institution to evaluate the large majority of translocations including probes to the *EWSR1*, *FUS*, *DDIT3*, *SYT*, and *FOXO1A* genes (Abbott Molecular/Vysis, Des Plaines, IL). These are dual color, break-apart probes which hybridize to targets which flank the most common breakpoints in a given gene. Separation of the 2 differently colored probes indicates a rearrangement of the gene, which in turn indicates the presence of a translocation involving that gene (Fig. 1). A dual-color MDM2 FISH probe, which hybridizes to the MDM2 gene region (12q13 to 15) has been developed at the Cleveland Clinic to aid in the diagnosis of atypical lipomatous tumor/well-differentiated liposarcoma (ALT/WDLPS). This probe is compared with the number of signals present for a centromeric reference probe for chromosome 12 (CEP 12). A ratio of the average number of MDM2 and CEP 12 signals counted in the neoplastic cells is calculated. A ratio of ≥ 2.0 indicates an amplification of the MDM2 gene region, which is considered a sensitive and specific molecular signature of ALT/WDLPS in the differential diagnosis of well-differentiated lipomatous tumors.[2]

Major morphologic patterns emerged from our study in which FISH was used including high-grade round cell sarcomas, spindle cell sarcomas, low-grade myxoid neoplasms, adipocytic neoplasms, and melanocytic neoplasms. Below, we discuss the most common diagnostic entities for which FISH was used within each morphologic category, followed by a review of the literature, and a more detailed discussion of diagnostic strategies utilizing FISH.

USE OF FISH WITHIN MAJOR MORPHOLOGICAL CATEGORIES

High-grade Round Cell Sarcomas

At our institution, FISH was most commonly used in the evaluation of high-grade round cell sarcomas. This diagnostic category includes many of the sarcomas which fall under the rubric of "small round blue cell tumors", characterized by primitive appearing round cells with little to no distinguishing features by light microscopic evaluation. Diagnostic entities include Ewing sarcoma/primitive neuroectodermal tumor (ES/PNET), poorly differentiated synovial sarcoma, alveolar rhabdomyosarcoma (ARMS), and desmoplastic small round cell tumor (DSRCT). Immunohistochemistry could be used to help distinguish between many of these entities (eg, CD99 for ES/PNET, desmin or myogenin for ARMS). However, as these markers are not absolutely specific, molecular studies often play an essential role in arriving at the correct diagnosis. In our study (results highlighted in Table 2), the proportion of

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TABLE 1. Molecular Alterations in Mesenchymal Neoplasms

Diagnostic Entity	Cytogenetic Alteration	Genes Involved
Ewing sarcoma/peripheral neuroectodermal tumor	t(11;22)(q24;q12)	*FLI1-EWS*
	t(21;22)(q22;q12)	*ERG-EWS*
Alveolar rhabdomyosarcoma	t(2;13)(q35;q14)	*PAX3-FKHR*
	t(1;13)(p36;q14)	*PAX7-FKHR*
Myxoid/round cell liposarcoma	t(12;16)(q13;q11)	*CHOP-FUS*
	t(12;22)(q13;q11-12)	*CHOP-EWS*
Desmoplastic small round cell tumor	t(11;22)(p13;q12)	*WT1-EWS*
Synovial sarcoma	t(X;18)(p11.2;q11.2)	*SSX1-SYT*
		SSX2-SYT
Clear cell sarcoma	t(12;22)(q13;q12)	*ATF1-EWS*
Extraskeletal myxoid chondrosarcoma	t(9;22)(q22;q12)	*NOR1-EWS*
Low-grade fibromyxoid sarcoma	t(7;16)(q34;p11)	*CREB3L2-FUS*
	t(11;16)(p11;p11)	*CREB3L1-FUS*
Atypical lipomatous tumor/well-differentiated liposarcoma	12q rings and giant markers	*MDM2* (*amplified*)

positive FISH results for the diagnostic entities varied from near 100% positivity [rearrangement of gene(s) involved in their characteristic translocation] to those such as ARMS, which was characterized by rearrangement of the *FOXO1A* in 66% of cases, which approximate what has been reported in the literature for these entities.[3–12] A more detailed summary of these diagnostic entities and their molecular alterations is discussed below.

Ewing Sarcoma/Primitive Neuroectodermal Tumor

Ewing sarcoma/primitive neuroectodermal tumor is defined by the presence of translocations involving the *EWSR1* gene (22q12) resulting in a fusion of this gene with several members of the ETS family of transcription factors (*FLI-1*, *ERG*, *ETV1*, *ETV4*, *FEV*, and *ZSG*). The translocations are similar in that they are restricted to introns 7 to 10 of the *EWS* gene and introns 3 to 9 of the *ETS*-related genes. By far the most common translocation found in the ES/PNET family of tumors is the t(11;22)(q24;q12) resulting in the *EWS-FLI-1* fusion gene, representing approximately 90% of cases. The next most common translocation is t(21;22)(q22;q12) resulting in the fusion of *EWSR1* to *ERG* (21q22). The remaining translocations represent less than 5% of cases and includes the fusion of *EWSR1* to *ETV1* (7p22), *ETV4* (E1AF), *FEV* (2q33), or *ZSG* [resulting in an inv(22)].[13] We found 96% of our cases of ES/PNET to harbor rearrangements of *EWSR1*, which is in keeping with the above data. As *FUS* and *EWSR1* are characterized by a high degree of homology, rarely, ES/PNET may be characterized by *FUS-ERG*[14] or *FUS-FEV* fusions.[15] Thus, it may be helpful in selected circumstances (eg, CD99-positive neoplasms with a strong histologic resemblance to ES/PNET, but *EWSR1* FISH negative) to follow-up with FISH for *FUS* in the evaluation of ES/PNET.

Despite the many different translocations that can be found in ES/PNET, nearly all harbor a rearrangement of *EWSR1*; consequently a single set of FISH probes to *EWSR1* can be used to detect essentially all of these translocations, without necessarily knowing the translocation partner. One pitfall of this approach is that other small round blue cell neoplasms including DSRCT, round cell liposarcoma, and extraskeletal myxoid chondrosarcoma (EMC) also harbor rearrangements of *EWSR1*. FISH for *EWSR1* in this setting must be correlated with the light microscopic and immunohistochemical features for accurate diagnosis.

Poorly Differentiated Synovial Sarcoma

Poorly differentiated synovial sarcoma is discussed below in the sections containing spindle cell sarcomas. Briefly, 100% of poorly differentiated synovial sarcomas in our study harbored rearrangements of *SYT*.

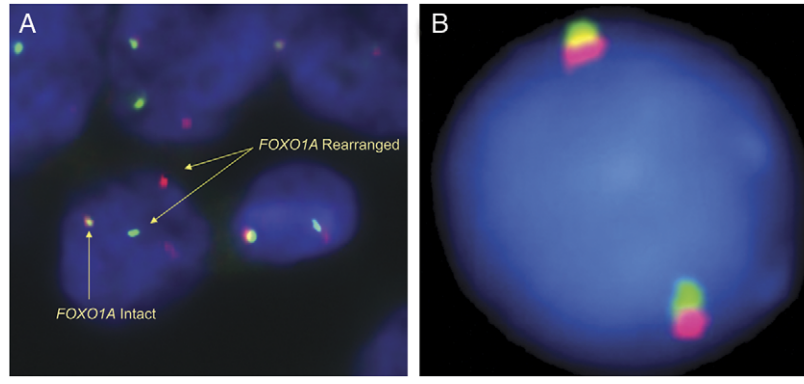

FIGURE 1. Split-apart FISH probes. A, Split-apart probes for the *FOXO1A* gene region (13q14) in alveolar rhabdomyosarcoma. Note that only 1 copy of *FOXO1A* is rearranged, as indicated by the spatially separated red rhodamine-labeled probe and green-FITC-labeled probes. The intact FOXO1A gene region is characterized by the adjoined red and green-labeled probes, which produce a yellow signal. B, A normal cell for comparison. Note that both copies are intact.

TABLE 2. Summary of FISH Results for Major Morphological Categories

	FISH Probes					
Diagnosis	**EWSR1**	**SYT**	**FOXO1A**	**DDIT3**	**FUS**	**MDM2**
High-grade round cell sarcomas (N = 67)						
Ewing sarcoma/PNET	(22/23) 96%*	—	—	—	—	—
Poorly differentiated synovial sarcoma	—	(5/5) 100%	—	—	—	—
Alveolar rhabdomyosarcoma	—	—	(4/6) 66%	—	—	—
Rhabdomyosarcoma, NOS	—	—	(0/5) 0%	—	—	—
Round cell liposarcoma	—	—	—	(3/3) 100%	—	—
Desmoplastic small round cell tumor	(2/2) 100%	—	—	—	—	—
High-grade round cell sarcoma, NOS	(1/17) 6%	(0/5) 0%	(0/1) 0%	(0/3) 0%	(0/1) 0%	—
Spindle cell sarcomas (N = 40)						
Monophasic synovial sarcoma	—	(26/27) 96%	—	—	—	—
Malignant peripheral nerve sheath tumor	—	(0/7) 0%	—	—	—	—
Spindle cell sarcoma, NOS	—	(0/6) 0%	—	—	—	—
Low-grade myxoid neoplasms (N = 34)						
Low-grade fibromyxoid sarcoma	—	—	—	—	(10/11) 91%	—
Myxoma	—	—	—	—	(0/4) 0%	—
Myxofibrosarcoma	—	—	—	(0/2) 0%	(0/3) 0%	—
Myxoid Liposarcoma	—	—	—	(5/5) 100%	(2/2) 100%	—
Low-grade myxoid neoplasm, NOS	—	—	—	(0/1) 0%	(0/12) 0%	—
Adipocytic neoplasms (N = 20)						
Lipoma	—	—	—	(0/2) 0%	(0/3) 0%	(0/7) 0%
Atypical lipomatous tumor	—	—	—	—	(0/1) 0%	(3/3) 100%
Dedifferentiated liposarcoma	—	—	—	—	—	(4/4) 100%
Pleomorphic liposarcoma	—	—	—	—	—	(0/1) 0%
Malignant melanocytic neoplasms (N = 19)						
Malignant melanoma	(0/11) 0%	—	—	—	—	—
Clear cell sarcoma	(7/8) 88%	—	—	—	—	—

*Numbers represent the proportion/percentage of cases demonstrating rearrangement (or amplification for *MDM2*) of that particular gene region by FISH split-apart probes. Totaling numbers from each individual entity within a morphological category may not equal the total number of cases within each category as multiple FISH tests were used on the same case in some instances.

FISH indicates fluorescence in situ hybridization; NOS, not otherwise specified; PNET, primitive neuroectodermal tumor.

Alveolar Rhabdomyosarcoma

Alveolar rhabdomyosarcoma can be distinguished from other round cell sarcomas by morphologic clues and its immunohistochemical profile, but also because of 2 characteristic translocations, t(2;13) and t(1;13), found in approximately 80% of cases. These 2 translocations involve the *FOXO1A* gene on chromosome 13q14 and either *PAX7* on chromosome 1p36 or *PAX3* on chromosome 2q35. *PAX3/FOXO1A* is the more common of the 2 fusion genes, occurring in approximately 70% of cases whereas *PAX7/FOXO1A* occurs in about 10% of cases.[13] We found roughly 70% of ARMS diagnosed at our institution to harbor rearrangements of *FOXO1A*, indicating the presence of a t(2;13) or t(1;13). PAX3 and PAX7 are transcription factors, which are expressed during the development of the dorsal neural tube and somites; PAX3 has been shown to play an important role in myoblast migration to the limbs. The PAX3-FOXO1A and PAX7-FOXO1A fusion proteins have been shown to enhance transcription of PAX-binding sites.[16]

As both translocations in ARMS involve the *FOXO1A* gene, the same set of probes for *FOXO1A* can be used to detect both translocations. In addition to being important diagnostically, detection of these translocations is also important prognostically, as ARMS has a worse prognosis than embryonal rhabdomyosarcoma (which does not harbor these translocations). Furthermore, preliminary studies show that the *PAX7/FOXO1A* gene fusion carries a better prognosis than the *PAX3/FOXO1A* gene fusion. Importantly, about 1 in 5 (20%) ARMS are translocation negative; thus a negative *FOXO1A* FISH result does not necessarily exclude the diagnosis of ARMS. In these cases, the diagnosis of ARMS rests primarily on characteristic morphologic features (and an immunophenotype which indicates skeletal muscle differentiation).[13]

Desmoplastic Small Round Cell Tumor

Desmoplastic small round cell tumor is a highly aggressive neoplasm which is characterized by a t(11;22)-(p13;q12), present in greater than 90% of DSRCT.[9] We found *EWSR1* to be rearranged in all examples of DSRCT diagnosed at our institution. Although its characteristic translocation superficially resembles that found in ES/PNET (the fusion gene involves *EWSR1* on 22q12), its translocation partner on chromosome 11 is *WT1*, rather than *FLI-1*. Most commonly, the breakpoint of the translocation involves exon 7 of *EWSR1* and exon 8 of *WT1*, joining the activation domain of EWSR1 with the DNA binding domain of WT1, resulting in an aberrant transcription factor. Although classically WT1 is a tumor suppressor, the fusion protein functionally behaves as an oncogene. Significantly, the carboxyl terminus of WT1 is present in the fusion protein, resulting in nuclear immunoreactivity for WT1 (using antibodies directed against the carboxyl terminus). Thus, essentially all DSRCT show strong and diffuse nuclear staining for the antigen, an important factor in distinguishing this entity from ES/PNET and other round cell sarcomas harboring rearrangements of *EWSR1*.[13,16]

Although *EWSR1* FISH is a very sensitive test for DSRCT, other ancillary studies including immunohistochemistry for desmin and WT1 (carboxyl terminus), additional FISH studies (eg, for *DDIT3* for round cell liposarcoma), or possibly RT-PCR (to determine the identity of the other gene fused to *EWSR1*) may be helpful.

Extraskeletal Myxoid Chondrosarcoma

Extraskeletal myxoid chondrosarcoma is a relatively uncommon sarcoma, which can also enter the differential diagnosis of round cell sarcomas, particularly in more cellular areas of the neoplasm. EMC is notable for the fact that there is no immunohistochemical profile that can be reliably used in making this diagnosis; it has essentially a null immunophenotype, occasionally showing focal immunoreactivity for S100 protein. A recent study showed that 89% of EMC harbor either a t(9;22) or t(9;17) translocation.[17] In about 70% of cases, a balanced translocation t(9;22)(q22;q12) can be found which fuses *EWSR1* on 22q12 with *NOR1* (also known as *NR4A3* or *TEC*) on 9q22. Approximately another 20% of cases have a t(9;17)(q22;q11) which adjoins *NOR1* with *RBP56* on 17q11. An additional translocation t(9;15)-(q22;q21) has also been described.[13] A FISH strategy using probes for *NOR1* would be ideal, as theoretically all 3 described translocations could be identified. However, these probes are not widely available. Hence, current approaches at many institutions involves using FISH probes for *EWSR1*, acknowledging that as many as 30% of cases will not have a translocation detectable by this approach. As with ARMS, diagnosis of EMC in these cases is dependent on recognition of its characteristic morphology and also the exclusion of other entities in the differential diagnosis by immunohistochemical means, or additional FISH testing.

SPINDLE CELL SARCOMAS

The next most common use of FISH in our practice is in the morphologic category of spindle cell sarcomas (Table 2). The main use is in the differential diagnosis of nonmyogenic spindle cell sarcomas, where the major diagnostic considerations include monophasic synovial sarcoma and malignant peripheral nerve sheath tumor (MPNST); entities which have a similar histologic appearance (Figs. 2A, B). The difficulty in differentiating between these 2 entities is because of the lack of a consistently robust marker, which can be used to distinguish them; synovial sarcoma typically contains only focal immunoreactivity for epithelial markers (cytokeratins and epithelial membrane antigen) whereas MPNST usually exhibits focal S100 protein. In addition, the 2 sarcomas have overlapping immunophenotypes. MPNST is occasionally immunoreactive (about 30%) at least focally for cytokeratins/epithelial membrane antigen, and synovial sarcoma can be focally positive (up to 40%) for S100 protein.[18] Thus, molecular studies play an important role in this differential diagnosis, discussed below.

Synovial Sarcoma

Synovial sarcoma is an aggressive sarcoma which exists in 2 main morphologic subtypes: biphasic and monophasic. The biphasic type, which contains epithelial gland-like structures, can occasionally be confused with sarcomatoid carcinomas or biphasic mesotheliomas. However, of the 2 subtypes, it tends to be easier to recognize. The monophasic subtype of synovial sarcoma can be more diagnostically challenging. It is essentially an intermediate to high-grade spindle cell sarcoma with subtle distinguishing features morphologically and without a consistently positive, widely available, immunohistochemical marker. The difficulty in distinguishing synovial sarcoma from MPNST is well recognized (for reasons mentioned above), particularly when the latter is not associated with a neurofibroma or nerve. Thus, cytogenetic studies can be critical in distinguishing between these 2 entities.

As mentioned above, synovial sarcoma is characterized by a t(X;18)(p11;q11) which is present in virtually all examples. This translocation fuses *SYT* on chromosome 18 with either *SSX1* (66%) or *SSX2* (33%), both located on chromosome Xp11. More rarely, *SYT* is adjoined to *SSX4*, also located on chromosome Xp11. Recently a novel translocation, a t(X;20) involving an *SYT* homolog on chromosome 20 (*SS18L1*), has been described resulting in a *SS18L1*/*SSX1* fusion gene.[19] Though not absolute, there is an association of the *SYT*/*SSX2* fusion gene with monophasic synovial sarcomas, whereas most biphasic synovial sarcomas contain the *SYT*/*SSX1* fusion gene. The t(X;18) translocation is not found as a nonrandom translocation in any other sarcoma including MPNST.[3,20] Hence, *SYT* FISH is highly specific and also a highly sensitive test for synovial sarcoma.[13,16] Our study confirmed these findings, with 96% of monophasic synovial sarcomas harboring rearrangement of *SYT*, whereas all of the MPNSTs in the study were negative. Of note, all of the poorly differentiated synovial sarcomas in our study (3 of 3) harbored rearrangement of *SYT*.

Malignant Peripheral Nerve Sheath Tumor

Malignant peripheral nerve sheath tumor is a spindle cell sarcoma with a complex karyotype, often with a near triploid chromosomal complement. Up to 25% to 50% of MPNST are associated with neurofibromatosis 1, the most common inherited cancer syndrome. Biallelic loss of *NF1* is important in the pathogenesis of both sporadic and neurofibromatosis 1-associated MPNSTs in that this is thought to be the initiating event in the development of the precursor neurofibromas. Loss of p53 is involved in progression from neurofibroma to MPNST. MPNSTs do not harbor the t(X;18) translocation.[13,16,20] Although no positive FISH test can support the diagnosis of MPNST, the absence of rearrangement of the *SYT* gene can be helpful in supporting the diagnosis of MPNST when an adjacent neurofibroma or nerve is not present.

Leiomyosarcoma

Leiomyosarcoma is another spindle cell sarcoma with a complex karyotype.[16] The diagnosis is usually suggested by the histologic appearance, and unlike MPNST and synovial sarcoma, it is usually supported by a relatively robust immunohistochemical panel including smooth muscle actin, desmin, and h-caldesmon. Thus, FISH generally does not play an important role in the diagnosis of leiomyosarcoma.

Fibrosarcomatous Dermatofibrosarcoma Protuberans

Fibrosarcoma arising in dermatofibrosarcoma protuberans (DFSP) is considered a form of tumor progression and enters the differential diagnosis of superficial spindle cell sarcomas. It is usually characterized by a transition from storiforming areas (DFSP) to a fascicular spindle cell sarcoma (the fibrosarcomatous component). The

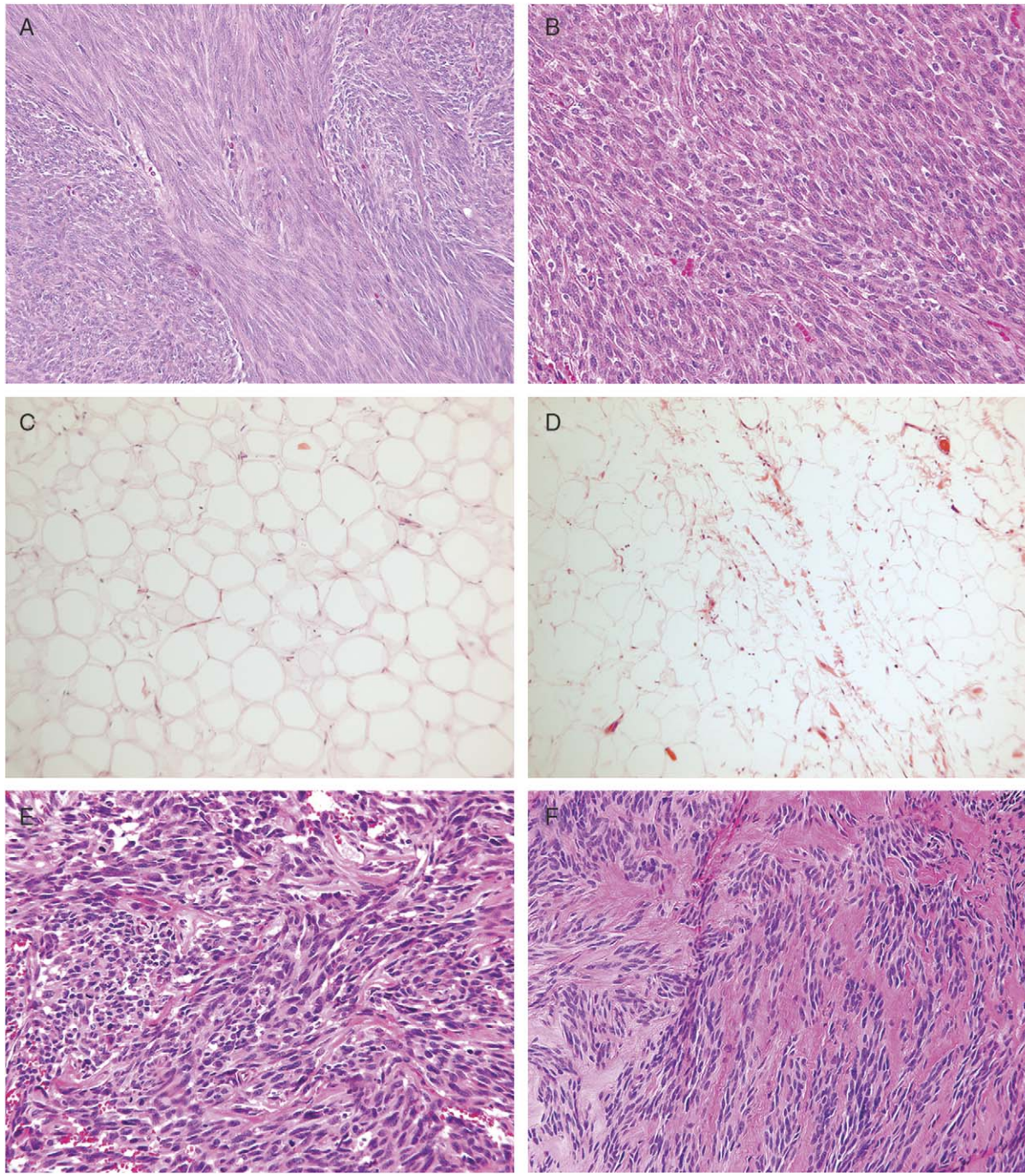

FIGURE 2. Some of the more common morphologic patterns in which FISH is used. A and B, Spindle cell sarcomas: synovial sarcoma (A) and malignant peripheral nerve sheath tumor (B). C and D, Well-differentiated lipomatous neoplasms: a retroperitoneal lipoma (negative for amplification of the MDM2 gene region by FISH) (C) and atypical lipomatous tumor/well-differentiated liposarcoma (cytologic atypia can be focal at times) (D). E and F, Malignant melanocytic neoplasms: malignant melanoma (E) and clear cell sarcoma (F).

fibrosarcoma component is often accompanied by loss of expression of CD34. The ring/marker chromosomes found in DFSP result in a t(17;22) translocation between the *COL1A1* promoter on chromosome 17 and the platelet derived growth factor B on chromosome 22.[13,16] FISH probes have been developed for the platelet derived growth factor B locus. However, as distinction from the above mentioned spindle cell sarcomas can usually be made upon recognition of the precursor DFSP, FISH does not usually play a role in the diagnosis of fibrosarcomatous DFSP except in cases which are unusual clinically or histologically.

LOW GRADE MYXOID NEOPLASMS

We also found FISH to be useful in the diagnosis of low-grade myxoid neoplasms, the next most frequent morphologic category in which FISH was used. The differential diagnosis in this morphologic category includes entities such as low-grade fibromyxoid sarcoma (LGFMS, Evans tumor), myxoma, myxofibrosarcoma (MFS), and myxoid liposarcoma (Table 2). These diagnoses share in common a myxoid stroma, the lack of a characteristic immunohistochemical signature, and (with the exception of MFS) minimal to no cytologic atypia. Thus, molecular studies are helpful in distinguishing between many of these entities.

Low-grade Fibromyxoid Sarcoma (Hyalinizing Spindle Cell Tumor With Giant Rosettes)

LGFMS is a deceptively bland appearing sarcoma, which has a risk for local recurrence and metastasis. No immunohistochemical marker is robustly positive in this entity, although epithelial membrane antigen is reported to be at least focally positive in most cases.[21] Thus, diagnosis of this sarcoma is dependent upon recognition of its histologic features and verification of its characteristic cytogenetic alterations. The most common cytogenetic finding is the presence of a t(7;16)(q33;p11) translocation involving *FUS* (earlier termed TLS) on chromosome 16 and *CREB3L2* on chromosome 7. Hyalinizing spindle cell tumor (HSCT) with giant rosettes is a neoplasm with a similar appearance and immunohistochemical profile but characterized by the presence of large collagen rosettes. Given the overlapping morphologic features and the identification of the t(7;16) in the tumor, HSCT is considered to be a variant of LGFMS.[22]

One study reported the presence of a *FUS*/*CREB3L2* gene fusion in 22 of 23 (96%) cases of LGFMS.[23] The same study described a novel translocation t(11;16) involving *FUS* and *CREB3L1* (11p11).[23] We found 91% of low-grade fibromyxoid sarcomas to harbor a rearrangement of *FUS* at our institution. These translocations join what seems to be a strong FUS promoter to the DNA-binding domain of CREB3L2/1.[16] Importantly, MFS and perineurioma, 2 entities commonly in the differential diagnosis with LGFMS, do not harbor rearrangements of *FUS*. One potential pitfall of *FUS* FISH, however, is confusion with myxoid liposarcoma, which does harbor a t(12;16)(q13;p11) translocation, and thus a rearrangement of *FUS*. Although histologic features can distinguish between these 2 entities, additional testing in the form of FISH for *DDIT3* may also be helpful (see below).

Myxoid/Round Cell Liposarcoma

Myxoid/round cell liposarcoma is characterized by a t(12;16)(q13:p11) translocation in up to 90% to 95% of cases, resulting in a *DDIT3*/*FUS* fusion gene (*DDIT3* was formerly known as *CHOP*). In this setting, *FUS* encodes for a RNA-binding protein similar to the *EWSR1* protein whereas *DDIT3* is a transcription factor involved in adipocyte differentiation. Given the functional similarity between *FUS* and *EWSR1*, it is not surprising that a small number of myxoid/round cell liposarcomas contain a t(12;22)(q13;q12) translocation resulting in a *DDIT3*/*EWSR1* fusion gene. In contrast with *FUS* (which is also rearranged in LGFMS, discussed above), rearrangements of *DDIT3* have been seen only in myxoid/round cell liposarcoma.[13,16] As expected, all myxoid liposarcomas contained a rearrangement of *DDIT3* in our study, indicating the presence of the t(12;16) and t(12;22) seen in myxoid/round cell liposarcoma.

Several important conclusions can be drawn from the above observations. When evaluating for myxoid/round cell liposarcoma, the most efficient approach includes a single FISH test for *DDIT3*, which detects both the t(12;16) and t(12;22) translocations in myxoid/round cell liposarcoma. Conversely, FISH for *FUS* is fraught with several potential pitfalls including possible confusion with LGFMS (which also contains a rearrangement of FUS), and the possibility of obtaining a false-negative result, because a minority of myxoid/round cell liposarcomas harbor a t(12;22) rather than the t(12;16). Round cell liposarcoma can assume a small round blue cell morphology, and as discussed above, may harbor a rearrangement of the EWSR1 gene, thus resembling ES/PNET. Immunohistochemistry for CD99 (positive in ES/PNET and negative in myxoid/round cell liposarcoma) is useful in this regard.

Myxofibrosarcoma

Myxofibrosarcoma (myxoid malignant fibrous histiocytoma) is characterized by a highly complex karyotype, often with triploid or tetraploid alterations and ring chromosomes.[13] No consistent cytogenetic findings, however, have been identified in MFS. Distinction from myxoid liposarcoma and LGFMS can usually be made by presence of a greater degree of atypia found in MFS than the other entities and recognition of its typical clinical presentation (elderly patient, extremities, subcutaneous location); thus most cases of MFS do not require utilization of FISH. However, FISH can play a role in ruling out neoplasms with overlapping clinical and histologic features in low-grade MFSs, which have only minimal cytologic atypia (eg, *FUS* FISH in evaluating for superficial low-grade fibromyxoid sarcoma). In our study, no examples of MFS harbored rearrangements of *FUS* or *DDIT3*, arguing against the differential diagnoses of low-grade fibromyxoid sarcoma (*FUS* FISH) and myxoid/round cell liposarcoma (*DDIT3* FISH).

Myxoma

Myxomas not infrequently enter the differential diagnosis of the above mentioned sarcomas. Although they have been associated with mutations in *PRKAR1-α* (Carney complex) and mutations in *GNAS1* (Mazabraud syndrome), no translocations are associated with these neoplasms.[16] The role of FISH in the diagnosis of myxoma is restricted to exclude the above mentioned myxoid sarcomas with characteristic gene rearrangements, usually when the entire neoplasm cannot be visualized (eg, on a biopsy). None of the examples of myxoma in our study contained rearrangements of *FUS*.

ADIPOCYTIC NEOPLASMS

FISH was also useful in the appraisal of adipocytic neoplasms, the next most common category in which FISH was used (Table 2). The most frequently used study was MDM2 FISH, which was most commonly used to distinguish ALTs (WDLPS), which are characterized by amplification of the MDM2 gene region, from lipomas, which do not contain amplification of MDM2 (Figs. 2C, D).[2,24–26] The lipomas evaluated by FISH were either deep-seated (eg, intramuscular lipomas) or those that either contained prominent fibrous septa (so-called fibrolipomas)

or a prominent myxoid component. Although less specific in this setting, MDM2 FISH was also used to provide support for the diagnosis of dedifferentiated liposarcoma when the well-differentiated component was not easily identifiable.

Lipomas

Conventional lipomas harbor simple but abnormal karyotypes, most commonly involving rearrangement of chromosome 12q13 to 15 (but not amplification, as found in ALT) usually involving the chromatin remodeling gene *HMGA2*. Although rearrangement of *HMGA2* can be identified by FISH, these probes are not widely available. The role of FISH in the diagnosis of lipomas is generally limited to FISH for the MDM2 gene region, with the chief differential diagnosis of ALT/WDLPS in mind (see below). MDM2 FISH was negative for amplification in all lipomas in our study, echoing what has been found in the literature.

Atypical Lipomatous Tumor/Well-differentiated Liposarcoma

Cytogenetically, atypical lipomatous tumor/well-differentiated liposarcoma is characterized by the presence of giant marker/ring chromosomes, which harbor amplification of the MDM2 gene region (12q13 to 15). The region, in addition to containing the *MDM2* gene is the site of several other genes (*SAS*, *HMGA2*, and *CDK4*). FISH probes, which span the MDM2 gene region, have been developed at our institution. The most common differential diagnosis is between ALT and lipoma. Clinical features, which support the diagnosis of ALT over a lipoma, include a large size and deep-seated location. However, deep-seated lipomas do occur (particularly in the extremities) and cytologic atypia in ALTs may be focal, despite extensive sampling of the neoplasm. In these settings, amplification of *MDM2* is very helpful as it is amplified in essentially all the examples of ALT and is not amplified in lipomas[2]; thus it is a very sensitive and specific probe. All examples of ALT/WDLPS in our study were characterized by amplification of the MDM2 gene region.

Dedifferentiated Liposarcoma

ALT/WDLPS may undergo tumor progression to a dedifferentiated liposarcoma, either in the primary tumor, or in the setting of repeated recurrences. Although in the process of tumor progression dedifferentiated liposarcoma may sustain additional genetic alterations, amplification of *MDM2*, believed to be an early genetic event, is still maintained.[27] Due to the somewhat better prognosis for high-grade dedifferentiated liposarcoma in comparison with other pleomorphic, high-grade sarcomas, distinction between these 2 entities does have clinical import. Importantly, use of *MDM2* FISH in this setting is complicated by the fact that up to 40% of pleomorphic sarcomas show amplification of the MDM2 gene region,[2] thereby significantly decreasing the specificity of the test in this differential diagnosis. However, as amplification of *MDM2* is found in essentially all dedifferentiated liposarcomas, it is very sensitive. Thus, a negative FISH result can be informative in this setting, as this strongly argues against the diagnostic possibility of dedifferentiated liposarcoma. In 1 case in our study, this test was used to favor the diagnosis of pleomorphic liposarcoma over the diagnosis of dedifferentiated liposarcoma, when it was determined that amplification of *MDM2* was absent.

Pleomorphic Liposarcoma

Pleomorphic liposarcoma is a pleomorphic sarcoma with at least focal adipocytic differentiation in the form of lipoblasts/pleomorphic lipoblasts. Not surprisingly, this tumor is characterized by a complex cytogenetic profile. Because of the overlap in their anatomic distribution (retroperitoneum and extremities)[13] and overlapping histologic features, high-grade dedifferentiated liposarcoma often enters the differential diagnosis of pleomorphic liposarcoma, particularly when a WDLPS is not identifiable to support the diagnosis of dedifferentiated liposarcoma. *MDM2* FISH can play a role in distinguishing between these 2 entities, however, it is bound by the same restrictions present when evaluating other pleomorphic sarcomas (see Dedifferentiated Liposarcoma).

Myxoid/Round Cell Liposarcoma

Although myxoid/round cell liposarcoma is a sarcoma with adipocytic differentiation, it more commonly enters the myxoid neoplasm differential diagnosis, and is discussed above.

MELANOCYTIC NEOPLASMS

The last major class of neoplasms for which FISH was used was the melanocytic neoplasms which included the differential diagnosis of malignant melanoma and clear cell sarcoma (malignant melanoma of soft parts). Malignant melanoma and clear cell sarcoma are notable for their similar histologic appearances (Figs. 2E, F) including a nested/packed growth pattern, spindled to ovoid cells with vesicular nuclei and prominent nucleoli, and an identical immunohistochemical profile including immunoreactivity for S100 protein and the melanocyte-specific markers [HMB-45, MelanA (Mart1), tyrosinase, and microphthalmia transcription factor]. Factors favoring clear cell sarcoma include a neoplasm occurring within the classic clinical setting (younger patient, acral/aponeurotic location) and a deep-seated location. Neoplasms containing a greater degree of pleomorphism are more consistent with melanoma. Because of the aforementioned overlap in their histologic and immunohistochemical features, molecular studies play an important role in differentiating between the 2 entities.

Clear Cell Sarcoma

Clear cell sarcoma is a sarcoma with melanocytic differentiation, which predominantly occurs in a subcutaneous or deep-seated location; they rarely involve the dermis. As mentioned above, the differential diagnosis includes primary or metastatic melanoma which can have an overlapping histologic and immunohistochemical profile. Clear cell sarcoma is characterized by a t(12;22)(q13;q12) translocation which results in fusion of *EWSR1* to the transcription factor *ATF1*. Functionally, *ATF1*/*EWSR1* is known to bind to the promoter of the microphthalmia-associated transcription factor, which plays an important role in melanocytic differentiation. The same *EWSR1* FISH probes used in other above mentioned entities can also be used to detect the t(12;22) translocation present in clear cell sarcoma. The translocation can be identified in almost all cases of clear cell sarcoma, whereas no cases of melanoma have been found to harbor this translocation.[13,28,29] We found that 88% of clear cell sarcomas contained a rearrangement of *EWSR1*, whereas none of the melanomas contained a rearrangement

of this gene, making it a highly sensitive and specific test for clear cell sarcoma (Table 2). Hence, *EWSR1* FISH is a highly sensitive and specific test for clear cell sarcoma.

Of note, a variant of clear cell sarcoma that exists within the gastrointestinal tract has also been identified which harbors the t(12;22) translocation and also a novel t(2;22)(q32;q12) translocation resulting in a *EWSR1/CREB1* fusion gene. Interestingly, this same translocation is the most common translocation found in angiomatoid fibrous histiocytoma. The *EWSR1/ATF1* fusion gene (described above) found in somatic clear cell sarcoma has also been found in some angiomatoid fibrous histiocytomas. These observations are interesting because they represent rare examples of where identical nonrandom translocations can be present in disparate entities.[30] *EWSR1* FISH can be performed to distinguish these gastrointestinal clear cell sarcomas from metastatic melanoma as well.[16]

Malignant Melanoma

Malignant melanoma does not contain consistent nonrandom translocations which are amenable to evaluation by FISH. As mentioned above, we found that none of the melanomas in our study harbored rearrangements of *EWSR1*. However, FISH can be useful in selected cases where the differential diagnosis of clear cell sarcoma is considered, as mentioned above.

GENERAL COMMENTS ABOUT THE USE OF FISH IN THE EVALUATION OF MESENCHYMAL NEOPLASMS

FISH is very useful in the diagnosis of soft tissue neoplasms, which harbor consistent molecular alterations including nonrandom translocations and amplification of gene regions. Approximately 9% of the soft tissue consults at our institution (at the time of this study) used FISH. The following summarizes our overall experience with FISH as well and specific observations which we would like to highlight:

- FISH is used to distinguish between entities with similar histologic appearances, and oftentimes overlapping immunohistochemical profiles.
  The most common morphologic/immunohistochemical categories in which FISH was used include: high-grade round cell sarcomas, nonmyogenic spindle cell sarcomas, low-grade myxoid neoplasms, adipocytic neoplasms, and melanocytic neoplasms, in descending order of frequency used.
- For many entities, FISH has a high degree of sensitivity and specificity.
  Entities such as ES/PNET and synovial sarcoma had rearrangements by FISH documented in 96% of cases. For some genes, it was very specific as well; for example, while almost all examples of synovial sarcoma contained a rearrangement of *SYT*, no MPNSTs had a rearrangement of that gene.
- The distinctions between these diagnostic entities that FISH affords often have clinical import.
  Different chemotherapeutic regimens are given for the high grade round cells sarcomas such as ES/PNET and ARMS. Determination of whether a well-differentiated lipomatous neoplasm of the extremity is an ALT or a lipoma by MDM2 FISH is helpful in determining whether a reexcision is necessary (for ALT). FISH for *EWSR1* can help distinguish between a primary sarcoma (clear cell sarcoma) and metastatic melanoma.
- Advantages over conventional cytogenetics.
  The main advantage of FISH is that nondividing (interphase) nuclei can be evaluated, making it unnecessary to evaluate the neoplastic cells in culture. This, in turn allows retrospective analysis of formalin-fixed, paraffin embedded tissue, the most common form of archived tissue. The technique confers an advantage in small biopsies, where it may not be possible to submit tissue for cytogenetics, but FISH can still be performed, sometimes as few as 40 to 100 cells. At an analytic level, it can be used to evaluate for the presence of cryptic translocations, which are often difficult to resolve with G-banded karyotyping.
- Advantages over RT-PCR.
  For a given translocation with multiple breakpoints, multiple PCR primers are necessary to evaluate for all the possible fusion transcripts. Even then, it is still possible that a subset of translocations with variant breakpoints might not be detectable. This stands in contrast with FISH, where most commercially available FISH probes span most known translocation breakpoints. Therefore 1 set of FISH probes (instead of the multiple PCR primers and PCR reactions) can identify essentially all known breakpoints of a given translocation, resulting in increased sensitivity.
  In addition to advantages in evaluating multiple break points, FISH is also helpful in situations where 1 translocation partner is largely conserved (present in nearly all the possible fusion genes) but the second translocation partner varies. Examples of this include ES/PNET (with the various translocation partners adjoined to *EWSR1*) and myxoid/round cell liposarcoma [*DDIT3* is rearranged in both t(12;16) and t(12;22)]. This avoids the situation where multiple primer sets are necessary to evaluate for all of the possible fusion products derived from the variant translocation partner or partners.
- Versatility of FISH probes.
  Soft tissue neoplasms often have the same gene locus involved in translocations found in different entities. Therefore, the same set of probes can also be used for a gene rearranged in multiple diagnostic entities (see Fig. 1). For example, *EWSR1* is rearranged in ES/PNET, desmoplastic round cell tumor, EMC, some myxoid/round cell liposarcomas, clear cell sarcoma, and angiomatoid fibrous histiocytoma.
  Care must be taken, however, in the interpretation of the FISH results in this setting as careful correlation of a positive FISH result should be undertaken with the morphology, immunophenotype, and clinical features of the neoplasm.
- Limitations of FISH.
  Use of FISH requires some speculation on the part of the pathologist about what type of underlying genetic abnormalities may be present and thus a

gene rearrangement can obviously only be detected if it is considered as a possibility. Conventional cytogenetics, in contrast, allows for a screening of the entire karyotype with essentially no assumptions made a priori. Furthermore, some rearrangements, including insertions, may be difficult to detect by FISH. In this setting, confirmatory testing with RT-PCR may be helpful. FISH studies cannot detect smaller genetic alterations such as point mutations, which can be detected by PCR analysis or direct sequencing.

Although FISH cannot be used as a stand-alone technique in the diagnosis of soft tissue neoplasms, it is an important adjunct to these above mentioned techniques, and is very useful in evaluating the majority of soft tissue neoplasms in which molecular analysis is desired.

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