

Title: Review of the medical literature and assessment of current utilization patterns regarding the use two common fluorescence in situ hybridization assays in the diagnosis of dermatofibrosarcoma protuberans and clear cell sarcoma

Short Running Title: Literature review & utilization of 2 FISH assays

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Abstract (205 words): Background

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Dermatofibrosarcoma protuberans (DFSP) is a tumor of intermediate malignancy, which in selected circumstances can pose difficulty in diagnosis. Clear cell sarcoma (CCS), is a very rare aggressive soft tissue sarcoma that can histologically be difficult to distinguish from melanoma.

Methods

The current literature on t(17;22) COL1A1-PDGFB fluorescence in situ hybridization (FISH) assay in DFSP was reviewed. Also reviewed was the current literature on dual color break-apart EWSR1 FISH assay in CCS. Finally, the current utilization patterns of these tests was assessed in attendees of the American Society of Dermatopathology annual meeting (Chicago, 2016).

Results

The literature indicates that (17;22) COL1A1-PDGFB FISH assay has limited value for classic DFSP where the diagnosis can be established by routine morphology and immunohistochemistry. Given the high specificity of the EWSR1 FISH assay and significant complexity in the diagnosis of CCS, this ancillary study is helpful in distinguishing CCS from melanoma.

Conclusions

In attendees, t(17;22) COL1A1-PDGFB FISH testing for classic cases of DFSP is appropriately not being used by respondents. However, the literature sustains that it is useful in selected circumstances in which a definitive diagnosis is challenging. The majority of respondents are

utilizing the EWSR1 FISH assay to distinguish CSS from melanoma as is supported by the literature.

Introduction:

Appropriate use criteria (AUC) combine the best scientific evidence available with the collective judgment of experts to yield a statement of the appropriateness of performing a particular ancillary test in specific clinical scenarios encountered in everyday practice. In 2015, the American Society of Dermatopathology (ASDP) created the AUC Task Force to help guide dermatopathologists in their use of ancillary tests. Four subgroups were established and each group chose two to three ancillary studies for which to develop AUC. This review provides a synopsis of the best scientific evidence (literature review) for the ancillary studies chosen by the "Soft Tissue" subgroup: t(17;22) *COL1A1-PDGFB* fluorescence *in situ* hybridization (FISH) for the diagnosis of dermatofibrosarcoma protuberans (DFSP) and *EWSR1* breakapart FISH in differentiating melanocytic tumors from clear cell sarcoma (CCS). In addition, a summary of the current clinical practice from a group of attendees at the 53rd Annual Meeting of the ASDP (Chicago, 2016) is presented.

t(17;22) *COL1A1-PDGFB* FISH for diagnosis of DFSP

DFSP is a distinctive slow-growing dermal and subcutaneous tumor of intermediate malignancy. Patients are typically in their early or middle adulthood. Tumors have a predilection for the trunk, proximal extremities, and head/neck region (1). Grossly, the tumor appears as a well-

circumscribed gray-white nodule involving the dermis and subcutis. Microscopically, despite its apparent gross circumscription, the tumor diffusely infiltrates the dermis and subcutis. DFSP is composed of uniform monomorphic spindle cells arranged in a distinctive storiform or cartwheel pattern. There is little nuclear pleomorphism and no significant mitotic activity (2). By immunohistochemistry DFSP typically expresses CD34 and is negative for factor XIIIa and S100 protein.

The most common and challenging differential diagnoses for DFSP are represented by the deep and cellular variants of dermatofibroma. In contrast to cellular dermatofibroma, DFSP is characterized by a larger size, infiltrative pattern within the subcutis and uniform morphology while typically lacking secondary elements such as giant cells, xanthoma cells or inflammatory cells. Another common differential diagnosis is the diffuse variants of neurofibroma. In this case, the lower cellularity and positivity for S100 stain seen in neurofibroma allows for the differentiation from DFSP (3). Variations from the classic histology are seen in pigmented DFSP (Bednar tumor) and DFSP with areas of fibrosarcomatous change. Uncommon histologic variants include DFSP with myxoid changes, which may cause confusion with a myxoid liposarcoma, DFSP with areas of undifferentiated pleomorphic sarcoma, DFSP resembling a vascular tumor, DFSP with myoid nodules, and the sclerotic variant of DFSP. (3-7)

DFSP is a tumor of intermediate malignancy with low metastatic potential, but locally aggressive behavior. Historically, recurrence rates are reported to be as high as 50%; however, more recent studies show an overall recurrence rate of 7.3% (8, 9). The presence of fibrosarcomatous areas indicates a more aggressive behavior with higher potential for metastasis (10). Wide local excision is the treatment of choice.

Cytogenetically, DFSP is characterized by a balanced or unbalanced $t(17;22)(q22;q13)$ translocation or a supernumerary ring chromosome, resulting in the fusion of exon 2 of *PDGFB* gene encoding the platelet-derived growth factor beta with various exons (from 6 to 47) of *COL1A1* gene encoding the alpha chain type 1 collagen (11-14). Interestingly, the same rearrangement was demonstrated in giant cell fibroblastoma (GCF), a tumor developing in children that is now considered to represent a juvenile form of DFSP (15). The ring chromosome is more common in adult cases of DFSP while the linear translocation tends to occur in children and is prevalent in GCF (14, 15). The translocation deletes exon 1 of *PDGFB* and puts the gene under control of the *COL1A1* gene promoter, compromising the physiological regulation of this factor. This results in overexpression of PDGFB, which enables downstream signaling through the PDGFB receptor and MAP-kinase pathway (13). In addition, this alteration renders the tumor sensitive to imatinib mesylate which is now used for the treatment of inoperable or metastatic disease. (16-18)

The t(17;22) translocation is specific for DFSP; therefore, its detection can potentially be used as an ancillary diagnostic tool in cases with unusual histology or atypical clinical presentation.

Another potential use is to identify cases that are susceptible to imatinib therapy. Several methods can be employed to detect the translocation including conventional cytogenetics, dual fusion *COL1A1/PDGFB* FISH, *PDGFB* or *COL1A1* break-apart FISH, reverse-transcriptase PCR (RT-PCR) with primers flanking the translocation breakpoint, and Next Generation Sequencing (NGS). This review focuses on FISH methods for the detection on the genomic rearrangements characteristic for DFSP. The dual fusion FISH test uses two distinctly labeled probes, usually red and green, which span the *COL1A1* gene on chromosome 17q21.33 and *PDGFB* gene on chromosome 22q13.1. The presence of a rearrangement resulting in a fusion is manifested by the occurrence of a yellow signal. A normal cell shows 2 red and 2 green signals per nucleus. A reciprocal balanced translocation shows 2 yellow fusion signals, 1 red, and 1 green signal per nucleus. Of note, in addition to the expected pattern associated with a reciprocal t(17;22) translocation (2 yellow fusion signals, 1 red, and 1 green signal), a significant number of DFSP cases show atypical patterns characterized by numerous yellow fusion signals (3 to 10) and extra copies of both green (*PDGFB* – 2 to 8) and red (*COL1A1* – 3 to 10) signals (Figure 1). This is caused by the presence of a ring chromosome containing multiple copies of the rearranged genomic material (19). The *PDGFB* and *COL1A1* break-apart FISH employs dual color probes (red and green) which flank the *PDGFB* and *COL1A1* gene, respectively. A normal cell shows 2 yellow fusion signals. The presence of a rearrangement is manifested in a split of

the red and green signal. A probe is considered to be split when the distance between the red and green signal is two times the size of a hybridization signal. Similar to the fusion probe, in addition to the expected pattern associated with balanced t(17;22) translocation (1 yellow fusion signal, one red, and one green signal), a significant number of DFSP cases show atypical patterns which include one or multiple copies (from 2 to 5) of the 5' telomeric region of *COL1A1* gene or 3' centromeric segment of *PDGFB* gene indicating unbalanced rearrangements (15, 20).

***EWSR1* breakapart FISH in differentiating melanocytic tumors from CCS**

CCS (aka malignant melanoma of soft parts) was initially described by Enzinger in 1965 and is a very rare aggressive soft tissue sarcoma showing neuroectodermal and melanocytic differentiation (21, 22). Although it shares clinical, histologic, immunohistochemical, molecular, and ultrastructural features with melanoma; CCS is considered to be a distinct entity that is separate from cutaneous melanoma. (23-25). Distinction between these two entities is crucial as the treatment modality and prognosis are different. CCS typically occurs in adolescents and young adults (third to fourth decades of life) with a slight female predominance and preferentially arises in the deep soft tissue of tendons, aponeuroses, and fascial structures of the distal extremities. It is associated with a high propensity for multiple local recurrences with late metastases and a high death rate (26-28). It is one of the few sarcomas with a high propensity for lymph node metastases, which are present in up to 50% of cases (29). Histologically typical cases of CCS are characterized by a nested or fascicular growth pattern of fusiform and/or

epithelioid cells with clear to finely granular cytoplasm and prominent nucleoli (30). Delicate fibrous septa encase the cellular aggregates and in two thirds of cases multinucleated cells are observed (31). Immunohistochemically, virtually all CCSs express S100-protein diffusely and most are also positive for Melan-A, HMB45 and MiTF similar to cutaneous melanomas (30). Melanin pigment can be detected either by H&E or with appropriate histochemical stains (e.g. Fontana-Masson) and melanosomes can be seen in varying stages of development by electron microscopy (32). *BRAF/NRAS* mutations, which are present in approximately 50-60% of melanomas, have also been rarely detected in CCSs (33, 34). Superficial cutaneous examples are well documented in the literature (31, 35, 36). In these instances, the tumor is dermal based with potential subcutaneous extension. In addition, rare cases with a junctional component mimicking melanoma in situ have also been reported (37, 38). Consequently, CCS can be confused with cutaneous spindle melanoma or metastatic melanoma with significant prognostic and predictive repercussions for the patient.

CCS has a characteristic translocation that most commonly fuses *EWSR1* on chromosome 22 with activating transcription factor-1 (*ATF1*) gene on chromosome 12 t(12;22)(q13;q12) resulting in four fusion transcripts (39). Less commonly *EWSR1* is fused with *CREB1* on chromosome 2 t(2;22)(q34;q12). The chimeric protein functions as a potent constitutive activator and mimics the action of melanocyte stimulating hormone by binding to and constitutively activating the promoter for *MITF*, the melanocyte master transcription factor (40). Many

methods for diagnosis are based on the aforementioned molecular characteristics, including classic cytogenetics, RT-PCR, FISH and NGS. Although *EWSR1* is a promiscuous gene associated with a number of sarcomas, carcinomas and very recently reported in a subset of malignant mesotheliomas (41) and in a group of acral fibroblastic spindle cell neoplasms (42), its rearrangement has never been reported in cutaneous melanomas. Consequently, a dual-color break-apart *EWSR1* FISH probe, which is commercially available and allows for detection in formalin-fixed paraffin-embedded tissues, can potentially serve as a very useful ancillary tool to support the diagnosis of CCS in challenging cases. It could also conceivably be used to distinguish CCS from benign melanocytic proliferations that mimic melanoma such as cellular blue nevus.

The commercially available probe spans the known common breakpoints in the *EWSR1* (introns 7-10). A probe specific for the 3' (telomeric) side of *EWSR1* is labeled one color (e.g. green) and the other probe specific for the 5' (centromeric) side is labeled a different color (e.g. orange). Subsequently 50-200 tumor cell nuclei are evaluated with fluorescence microscopy. Cells with a chromosomal rearrangement have two discrete colors distanced from each other indicating a translocation involving one *EWSR1* allele while, the second allele is intact with two colors (Figure 2). The interpretation of intact and split signals follows generally accepted guidelines that are used for all commercially available break-apart FISH assays in clinical laboratories. This requires the space between two signals to be greater than one signal width in order to be

considered split signal. Depending on the laboratory a result is considered positive when more than 10-20% of the tumor nuclei have evidence of the rearrangement. Nuclear truncation by the processing and overlapping cells can potentially lead to false positives; therefore, only tumor cells with all four signals are analyzed.

A literature review to identify the current scientific evidence behind the use of *COL1A1-PDGFB* FISH for the diagnosis of DFSP as well as dual-color break-apart *EWSR1* FISH for the diagnosis of CCS was performed. Next the scientific evidence for each was enumerated and summarized. Finally, we utilized an audience response system during Short Course I “Best Practices” at the 51st annual meeting of the American Society of Dermatopathology in Chicago, IL to assess the current utilization patterns of the tests in attendees.

Material and methods

Literature review

t(17;22) *COL1A1-PDGFB* FISH for diagnosis of DFSP (Table 1)

A search for journal articles written in English was performed in PubMed using keywords “Dermatofibrosarcoma” combined with either “FISH”, “fluorescence in situ”, “translocation”, “fusion”, “*COL1A1*” or “*PDGFB*” and a date range from 2000 to present. A total of 596 articles were obtained. Titles and abstracts were reviewed and overlapping studies were filtered out.

Articles with relevant data about the use of FISH for detection of chromosomes 17 and 22 rearrangements in DFSP were included. Case series of greater than three were included if no other evidence was available. Few case reports discussing unusual variants of DFSP were also included.

We identified 22 papers which evaluated the presence of *COL1A1-PDGFB* rearrangement in DFSP, summarized in Table 1 (4-7, 15, 18, 19, 43-55). Among the selected studies, half of them were retrospective case series (15, 19, 44, 48, 50-52, 54-57), 2 were prospective studies (45, 46), 2 were phase II imatinib trials (18, 47) and 7 were case reports (4-7, 43, 49, 53). The case reports were included because they described less common variants of DFSP such as pigmented DFSP (Bednar tumor) (43), DFSP in a patient with Cowden Syndrome (53), DFSP with pleomorphic sarcomatous transformation (7), DFSP with labyrinthine plexiform and braided pattern high-grade fibrosarcoma (5, 6), and a vascular variant of DFSP (4). Overall, 853 samples belonging to 830 patients were included in this meta-analysis. As expected, most tumors were located on the trunk (43.06%) and extremities (39.38%) with less frequent distribution on the head/neck (12.02%), groin (1.22%), and axilla (0.12%). Median age across studies varied between 24.5 and 53 years and genders were equally represented (males: 49.8%, females: 50.2%). The distribution of primary tumors, local recurrences and metastatic tumors among the cases included in this meta-analysis was 83.9%, 3.17% and 1.64%, respectively. In 11.25% of cases this data was not available. The overall distribution of diagnoses was as follows: classic DFSP – 457

cases (77.45%), DFSP with fibrosarcomatous transformation – 76 cases (12.88%), pigmented DFSP (Bednar tumor) – 7 cases (1.18%), DFSP with GCF component – 9 cases (1.52%), pure GCF – 10 cases (1.69%), DFSP with pleomorphic sarcomatous transformation – 5 cases (0.84%), myxoid DFSP – 5 cases (0.84%), DFSP with labyrinthine plexiform high-grade fibrosarcoma – 2 cases (0.33%), DFSP with vascular pattern – 2 cases (0.3%), sclerotic DFSP – 8 cases (1.35%), DFSP with myoid nodules – 4 cases (0.6%), DFSP mimicking cellular dermatofibroma – 2 cases (0.33), and atrophic DFSP and DFSP with round cell component, – 1 case each (0.16%). Data regarding CD34 immunohistochemical staining was available in 11 studies. The frequency of CD34 positive cases ranged between 80% and 100% with most studies reporting >90% positivity.

A total of 13 studies used dual fusion FISH (4-7, 43, 45-48, 50, 52, 54, 55), 4 used *PDGFB* break-apart FISH (15, 18, 44, 53), one study used *COL1A1* break-apart FISH (20), 2 studies used both dual fusion and *PDGFB* break-apart FISH (49, 57), 2 studies used both dual fusion FISH and RT-PCR (19, 56), and one study used *PDGFB* break-apart FISH and RT-PCR (51). In all but 2 studies (20 studies) a relatively certain diagnosis of DFSP or variants thereof could be made based on histology and CD34 staining. In the remaining 2 studies, the authors separated cases with a certain diagnosis from those with a probable or possible diagnosis of DFSP (45, 46). Overall, a total number of 582 cases with a relatively certain diagnosis of DFSP were identified

and out of these, dual fusion FISH and *PDGFB* break-apart FISH were successfully performed in 441 and 120 cases, respectively.

The overall sensitivity of the dual fusion FISH test defined as percentage of FISH positive cases out of total DFSP cases was 94.33%, ranging in various studies from 86% to 100%. For the evaluation of sensitivity, only cases with a definitive diagnosis of DFSP based on histology and CD34 expression were considered (441 cases) and cases with probable or possible diagnosis were excluded. A total of 25 cases (5.6%) with a certain diagnosis of DFSP were negative for FISH. One reason could be represented by the low number of translocated cells that in some tumors are reported to be as low as 2% and thus can be easily overlooked (19). Another cause could be the presence of alternative rearrangements such as t(5;8) (58). A total of 4 studies on dual fusion FISH included normal controls allowing for determination of test specificity defined as percentage of FISH negative cases per total negative control cases (19, 50, 52, 56). Normal skin, postsurgical scar tissue, and dermatofibroma were used for normal controls. One study included colon and lung carcinoma as normal controls (56) and another used dermal dendrocyte hamartoma, a mimic of DFSP (50). In all studies, the dual fusion FISH test performed with a specificity of 100%. Data regarding the rate of test failure for dual fusion FISH could be derived from 10 studies (4-6, 19, 43, 45-47, 49, 52), which showed an overall test failure frequency of 8.12%. In most cases, the reason for test failure was determined to be inappropriate fixation or use of a fixative other than formalin (46).

From the 6 studies evaluating the *PDGFB* break-apart FISH test, the overall sensitivity was 95% (range 91% to 100%). There was no data available to determine the specificity of the *PDGFB* break-apart FISH test. The overall failure rate for this test was 3.22%. Only one study evaluated the *COL1A1* break-apart probe in DFSP with a sensitivity of 100% (20). In 3 studies, RT-PCR was performed in parallel with FISH (19, 51, 56). The overall sensitivity for RT-PCR was 72.53% and the incidence of failed tests was 17.69%.

In a prospective study by Karanian et al., 448 consecutive tumors suspected to be DFSP were subjected to FISH testing using dual fusion FISH (46). All tumors were subclassified as certain (200 cases), probable (122 cases) and possible DFSP (126 cases). A tumor was classified as probable DFSP when DFSP was the most likely diagnosis, but another diagnosis such as cellular dermatofibroma was also considered. A tumor was classified as possible DFSP when the first considered diagnosis was not DFSP. The percentage of FISH positive cases in the cohort of certain DFSP cases was 96%, similar to the sensitivity of the dual fusion FISH test in other studies. However, the percentage of FISH positivity dropped to 91% and 19% in the cohorts of cases with probable and possible DFSP diagnosis, respectively. In the cohort of cases with a probable DFSP diagnosis, the negative FISH test resulted in reclassification of 7% of cases from DFSP to another diagnosis. In the cohort of cases with a possible DFSP diagnosis, the positive FISH test resulted in reclassification of 19% of cases from undifferentiated sarcoma,

myxofibrosarcoma or benign soft tissue tumors into classic DFSP or DFSP variants. In another similar prospective study by Italiano et al., 50 cases of DFSP, classified as certain (27 cases – 54%), probable (7 cases – 14%) and possible (16 cases – 32%) were subjected to FISH testing (45). Criteria for diagnosis were similar to those in the study by Karanian et al.: certain – when DFSP was the only possible diagnosis, probable – when DFSP was the most likely diagnosis, and possible – when there were other equally likely diagnoses. While FISH was positive in all cases with a certain diagnosis, only 86% and 56% of the probable and possible cases respectively were FISH positive. As a result of molecular studies, 3 cases initially classified as benign were reclassified as DFSP and treated with wide local excision and 2 undifferentiated sarcomas were reclassified as DFSP and responded to imatinib therapy.

Two phase II imatinib trials in DFSP correlating the response to therapy with presence of *COL1A1-PDGFB* fusion were identified. In a study by McArthur et al., 10 cases of DFSP were treated with imatinib. Of these, 8 cases were locally advanced cases and 2 cases were metastatic cases. FISH was positive in 9 cases, all of which demonstrated either total (4 cases) or partial (5 cases) response to therapy. One case was negative by FISH and showed no response to imatinib (18). In a study by Kerob et al., 21 out of 25 DFSP patients with *COL1A1-PDGFB* rearrangement 9 (38%) achieved complete or partial response, while none of the 2 patients without the translocation responded to imatinib therapy (47).

Dual color break-apart *EWSR1* FISH in differentiating melanocytic tumors from CCS (Table 2)

Due to the rarity of CCS, the date range was not limited when performing the literature search for dual-color break-apart *EWSR1* FISH for differentiating melanocytic tumors from CCS. The translocation was first identified in 1990. A search for journal articles written in English was performed in PubMed and only case reports with scientifically sound evidence of molecular testing were included. Articles addressing visceral CCS (gastrointestinal, pulmonary, and renal) were excluded.

We identified 18 relevant articles, all retrospective studies, that are summarized in Table 2 (30, 31, 33, 34, 36, 40, 59-70). The overall number of patients was 234, which included 236 samples analyzed. As expected, a significant proportion of cases were from acral sites (46.67%, 91/195) and the majority of patients (83%) were less than 50 years of age (171/206). Only a few cases reported the sarcoma having a component “mimicking junctional nests”; one case remarked on a junctional component (37). Most series used melanoma cases or melanoma cell lines as negative controls with the exception of one study that compared many different types of sarcoma (24). The overall sensitivity of dual-color break-apart *EWSR1* FISH was 88.89% and the specificity 97.91%; the test failed for various reasons in 6.33% of cases. The sensitivity of the dual fusion test was 60%, whereas its specificity was 100%. There was no data available regarding the percentage of failed tests. The sensitivity for RT-PCR was 91.61% and the specificity was 100%; RT-PCR failed in 22.22% of cases.

In one of the first series after the discovery of the translocation, where FISH or RT-PCR was not performed, conventional karyotypes were positive for t(12;22)(p11.2;p11.2) in one case and t(12;22)(q13;q13) in another case out of five in total (58). Two other cases displayed chromosome 22 abnormalities without a definitive translocation identified. This series exemplifies the low yield or negative predictive value (NPV) of conventional cytogenetic karyotyping as well as the difficulties in ascertaining precise location of chromosomal rearrangements. This study also demonstrated that none of the CCS cases displayed microsatellite instability (MSI). One CCS case did have loss of heterozygosity of 9p21, raising the question if the lesion should be better classified as a melanocytic/spitzoid tumor.

Another study described 2 purely cutaneous cases (31). Six cases were entirely dermal, whereas the other six showed invasion of the subcutis. In six cases, the nests bordered the epidermis mimicking junctional nests of melanocytes although “true nests” were not identified. Falconieri et al. also reported three cases of dermal CCS with minimal extension to the subcutis all of which were confirmed by *EWSR1* FISH (36).

In one other large study from a tertiary center, FISH and RT-PCR results on a variety of sarcoma cases, including CCS, were reviewed (64). The study highlights the difficulties that arise when the methods of tissue fixation and processing of referral blocks are not certain resulting in higher RT-PCR failure rates.

Song et al. subjected 18 cases with malignant melanoma diagnosis from non-cutaneous, deeply located sites and unknown primary sites to break-apart *EWSR1* FISH (65). They identified two patients with *EWSR1* gene rearrangement with a mean of 67.5% positive cells per sample re-classifying them as CCS. The cases were subsequently validated by RT-PCR identifying the presence of type I (*EWSR1* exon8-*ATF1* exon 4) fusion transcripts. Retrospective analysis revealed that the masses were located in the foot and buttock.

In a retrospective study of 52 patients with CCS, Hocar et al. identified 1 of 22 tested cases with a *BRAF* mutation and 1 of 22 tested cases with a *NRAS* mutation (33); both cases were confirmed by RT-PCR. Park et al. also described two cases of CCS, one dermal and one subcutaneous, which were confirmed by FISH and RT-PCR (34). *BRAF* mutation was detected in the dermal type and *KIT* mutation in the subcutaneous one raising interesting questions regarding treatment options.

Lastly Ito et al. reported an exceedingly rare case of CCS in the penis (68) whereas Feasel et al. described three cases in the head and neck expanding the anatomic distribution (69).

Survey of current utilization

During the 51st annual meeting of the ASDP in Chicago, IL, an audience response system surveyed attendees of short course I “Best Practices” regarding their current utilization of t(17;22) *COL1A1-PDGFB* dual fusion FISH for the diagnosis of DFSP and *EWSR1* break-apart FISH in differentiating melanocytic tumors from CCS. The audience was polled prior to the presentation of the literature overview for their overall current utilization and after for the utilization of the specific ancillary studies in a case setting presentation.

Comments and Conclusions:

Current utilization

An audience response system was used to identify the current utilization patterns of ordering FISH for DFSP and CCS. Basic demographic information of the participants revealed that the majority of respondents are in an academic practice setting (47%). There was a relatively even distribution of experience in dermatopathology with 36% in training, 23% practicing less than 5 years, 22% practicing 5-10 years, and 19% practicing more than 15 years. The majority of respondents were from the United States (93%) with the highest representation from the Central region (36%). There were between 81 and 105 unique participants that responded to the various queries for the soft tissue portion of the presentation. The majority of responders (38%) send FISH testing out to an academic (27%) or a commercial (11%) referral laboratory. Only 35% of participants have FISH available in-house at their academic (22%) or commercial (5%)

laboratory. Of note, 37% send their entire case for an outside consultation if it needs or may need FISH; meaning if they do not perform the FISH in house and they also do not order the test in isolation. The majority of participants (51%) are diagnosing DFSP in their practice approximately 1 to 2 times in one year. Of note, the audience responses showed that participants were more likely to order FISH for distinguishing melanocytic lesions from CCS. 69% of respondents would order FISH with an additional 17% ordering FISH after contacting the referring clinician and obtaining clinician / insurance / patient approval. Conversely, only 21% would order FISH for DFSP with an additional 16% of respondents ordering the study after contacting the referring clinician and getting clinician / insurance / patient approval.

t(17;22) *COL1A1-PDGFB* FISH for the diagnosis of DFSP

The reviewed evidence demonstrates that FISH is a sensitive and specific diagnostic test for DFSP. The sensitivity of the dual fusion and *PDGFB* break-apart FISH appears to be similar (94% and 95%, respectively). The sensitivity of the *COL1A1* break-apart probe is likely in the same range; however, only one study explicitly mentioning this probe was identified. The specificity of the dual fusion FISH test was 100%. No data was found about the specificity of the break-apart FISH tests. The overall percentage of failed tests was about 8% for the dual fusion FISH probe and 3% for the break-apart *PDGFB* FISH test. The lower rate of failed tests for the break-apart versus dual fusion FISH could be related to the lower complexity of the former FISH test with only 2 probes flanking a gene versus the latter with at least 4 probes flanking 2 genes

which increases the likelihood that one or more probes will fail hybridization. However, the difference could also be related to differences in study format. In the series of studies on break-apart FISH, only one study explicitly reported the failure rate versus 4 studies reporting this information in the dual fusion FISH cohort. As expected, RT-PCR showed a lowered sensitivity (73%) and higher rate of failed tests (18%) compared to FISH, likely due to the challenges in obtaining good quality RNA from formalin-fixed paraffin embedded material.

The data suggests that FISH testing has limited value in classic DFSP as the overwhelming majority would be positive for *COL1A1-PDGFB* rearrangements if the test is performed. This is reflected in the current practice as the majority of responders surveyed during the ASDP short course would not order FISH testing to support a diagnosis of classic DFSP. However, FISH testing is useful in circumstances where a definitive diagnosis cannot be made based on histology and CD34 staining. Benign tumors such as deep or cellular dermatofibromas can be confused with DFSP, especially in limited biopsies and in this instance a negative FISH test can support the correct diagnosis and prevent over- or under-treatment. Unusual variants of DFSP may mimic other sarcomas such as fibrosarcoma, undifferentiated pleomorphic sarcoma or myxofibrosarcoma and in these instances FISH testing can be instrumental in accurate classification. Finally, there is data supporting the use of FISH testing to predict response to treatment with imatinib. In summary, FISH testing (either dual fusion or break-apart), when used judiciously, can be a valuable tool in correctly diagnosing and managing DFSP.

***EWSR1* break-apart FISH in differentiating melanocytic tumors from CCS**

The review of the literature indicates that the fusion (either *EWSR1-ATF1* or *EWSR1-CREB1*) is present in the majority of cases of CCS, whereas no melanomas were identified to harbor these translocations. Given the high sensitivity and specificity of the dual-color break-apart FISH test in this clinical scenario and the significant consequences of a misdiagnosis the literature supports the use of dual-color break-apart *EWSR1* FISH to differentiate CCS from melanoma or other melanocytic neoplasms. This is reflective of the current practice of respondents attending the short course as the majority (69%) would order the test to support the diagnosis with an additional 17% doing so after contacting the referring clinician.

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LEGEND

Figure 1: Dual fusion interphase FISH conducted on a DFSP with a custom probe set spanning the *COL1A1* (Spectrum Orange) and the *PDGFB* (Spectrum Green) loci demonstrates multiple juxtaposed orange/green (yellow) signals indicative of multiple copies of *COL1A1/PDGFB* fusion (200X, courtesy of Julia Bridge, M.D.).

Figure 2: Dual-color, break-apart interphase FISH in clear cell sarcoma. One signal is fused (red and green=yellow) indicating an intact *EWSR1* (22q12) allele whereas the other signal is split indicating the presence of *EWSR1* gene rearrangement (200X, courtesy of Julia Bridge, M.D.).

Supporting Information:

Additional supporting Information may be found in the online version. Supplement 1 (S1) and Supplement 2 (S2).

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Table 1: DFSP literature review summary

Summary of Cited Articles

Total # articles: 23
Number of patients/samples 830/853

FISH Dual Fusion

Sensitivity 416/441 (94.33%)
Specificity 41/41 (100%)
% Failed test 61/751 (8.12%)

FISH Breakapart

Sensitivity 114/120 (95%)
Specificity N/A
% Failed test 4/124 (3.22%)

RT-PCR

Sensitivity 104/143 (72.72%)
Specificity N/A
% Failed test 23/130 (17.69%)

Clinical

M 409/821 (49.81%)
F 412/821 (50.19%)
Extremities 321/815 (39.38%)
Trunk 351/815 (43.06%)
H&N 98/815 (12.02%)
Groin 10/815 (1.22%)
Axilla 1/815 (0.12%)
Other/Unknown 34/815 (4.17%)

Table 2: CCS literature review summary

Summary of Cited Articlced

Total # articles: 18

Number of patients/samples: 234/236

FISH Dual Fusion

Sensitivity 60%

Specificity 100%

% Failed test N/A

FISH Breakapart

Sensitivity 88.89%

Specificity 97.91%

%Failed test 6.33%

RT-PCR

Sensitivity 91.61%

Specificity 100%

% Failed test 22.22%

Clinical

Acral 91/195 (46.67%)

< 50 years old 171/206 (83%)

Other findings

- Most series used melanoma cases or melanoma cell lines as negative controls for tests (except one study which compared many different types of sarcoma)
- Few cases reported with areas “mimicking junctional nests”, one case with reported junctional component
- BRAF mutation + in 4.55% cases, NRAS mutation + in 4.55% cases
- One study looked at deep tumors previously called melanoma and found *EWSR1* rearrangements in 2/18 cases (11.11%)

Title: Review of the medical literature and assessment of current utilization patterns regarding the use two common fluorescence in situ hybridization assays in the diagnosis of dermatofibrosarcoma protuberans and clear cell sarcoma

Short Running Title: Literature review & utilization of 2 FISH assays

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