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The role of cytogenetics and molecular diagnostics in the diagnosis of soft-tissue tumors

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Soft-tissue sarcomas are rare, comprising <1% of all cancer diagnoses. Yet the diversity of histological subtypes is impressive with >100 benign and malignant soft-tissue tumor entities defined. Not infrequently, these neoplasms exhibit overlapping clinicopathologic features posing significant challenges in rendering a definitive diagnosis and optimal therapy. Advances in cytogenetic and molecular science have led to the discovery of genetic events in soft-tissue tumors that have not only enriched our understanding of the underlying biology of these neoplasms but have also proven to be powerful diagnostic adjuncts and/or indicators of molecular targeted therapy. In particular, many soft-tissue tumors are characterized by recurrent chromosomal rearrangements that produce specific gene fusions. For pathologists, identification of these fusions as well as other characteristic mutational alterations aids in precise subclassification. This review will address known recurrent or tumor-specific genetic events in soft-tissue tumors and discuss the molecular approaches commonly used in clinical practice to identify them. Emphasis is placed on the role of molecular pathology in the management of soft-tissue tumors. Familiarity with these genetic events provides important ancillary testing for pathologists to include in their diagnostic armamentarium.
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Over the past half-century, a multitude of genomic technologies with increasing levels of resolution have contributed to recognition of important soft-tissue tumor morphologic–genetic associations. Indeed, much of the current classification system has been shaped by careful correlation of recurrent somatic alterations with discrete histopathologic subtypes.[1]

Cytogenetic changes constitute one of the earliest, and still one of the most influential, in typing soft-tissue tumors. Since the first description of the t(11;22)(q24;q12) translocation in Ewing sarcoma, cytogenetic discoveries have provided a catalog of chromosomal alterations specifying distinct mesenchymal tumor entities (http://cgap.nci.nih.gov/Chromosomes/Mitelman).[1–4] Cytogenetic data often guide other molecular studies in further defining the underlying genes and corresponding pathways involved. Of the 94 benign and malignant soft-tissue entities listed in the third edition of the *WHO Pathology & Genetics; Tumours of Soft Tissue and Bone,*[5] a characteristic cytogenetic abnormality was described for 10 (10/94; 11%) and both the cytogenetic and corresponding molecular findings for an additional 19 (19/94; 20%).[5] Progress witnessed in the most recent edition of this universal classification system of 117 soft-tissue tumor entries includes the definition of the underlying molecular events for 7 of the 10 tumors for which only cytogenetic changes were known previously such as chondroid lipoma, low-grade fibromyxoid sarcoma, and epithelioid hemangioendothelioma.[1] Overall, ~45% (53/117) of the entities listed in the fourth edition of the *WHO Classification of Tumours of Soft Tissue and Bone,*[1] including a few introduced since this publication, feature recurrent cytogenetic and/or molecular abnormalities.[1,6–10]

A considerable number of soft-tissue tumors are associated with recurrent chromosomal rearrangements, most commonly translocations. Isolation and sequencing of the involved translocation breakpoints has led to the identification of highly specific gene fusions that are involved in the causation of these tumors.[11] A host of molecular assays have been adopted into routine clinical practice for the detection of fusion genes as well as other genetic events of valuable diagnostic utility to include recurrent patterns of imbalance like gene amplification (eg amplification of *MDM2* in atypical lipomatous tumor) or specific activating or inactivating mutations of certain genes such as *KIT* in gastrointestinal

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stromal tumor or *SMARCB1* in malignant rhabdoid tumor respectively, among others.[12]

The following is focused on the molecular diagnostic tools available to the pathologist for the subclassification of specific soft-tissue tumor types and the recurrent aberrations frequently examined. The application of clinicohistopathologic criteria for capitalizing on soft-tissue tumor genetic features with inclusion of paradigms and pitfalls is also underscored.

Categories of genetic abnormalities in soft-tissue tumors

Broadly, genetic abnormalities in sarcomas have been divided into two major categories: (1) tumors exhibiting a relatively simple karyotype dominated by a recurrent structural abnormality, usually a defining translocation, or tumors featuring specific activating mutations within oncogenes or inactivating mutations within tumor suppressor genes, and (2) tumors with multiple, often complex chromosomal aberrations (Figure 1). General biological differences between these categories have been addressed previously.[13,14] The second category of multiple, often complex anomalies can be further subdivided into: (a) soft-tissue tumors demonstrating a reproducible pattern of genomic imbalances and/or involved chromosomal breakpoints. These aberrant patterns when viewed with other clinicohistopathologic features contribute to accurate nosology and (b) tumors with no specific pattern whereby the high degree of genomic complexity and instability (highlighted by large numbers of unidentifiable marker chromosomes, variable copy number changes, intertumor and intratumor mutational heterogeneity, and chromothripsis, among others) precludes the use of many routine clinical genetic approaches as a discriminating tool. There will be no further discussion of the latter group lacking a reproducible or recognizable pattern in the current review.

Functional consequences of soft-tissue tumor translocation events

The nonrandom, often reciprocal translocations or exchanges of chromosomal material in soft-tissue tumors lead to juxtapositioning of two genes, one from each translocation partner, resulting in the production of fusion genes encoding for abnormal, oncogenic proteins. Soft-tissue tumor translocations are often the sole karyotypic abnormality in tumors arising *de novo* and are presumed to be the initiating oncogenic event (driver mutation). Thirty (26%) of the 117 benign and malignant soft-tissue tumors listed in the current WHO classification are characterized by one or more fusion genes,[1,6–10] Table 1.

Functionally, the majority of soft-tissue tumor translocation-associated fusion genes encode for aberrant transcription factors that cause transcriptional deregulation; examples include Ewing sarcoma, synovial sarcoma, alveolar rhabdomyosarcoma, myxoid/round cell liposarcoma, and clear cell sarcoma, among others. Less commonly, deregulated kinase signaling is the consequence of the creation of chimeric tyrosine kinases (eg inflammatory myofibroblastic tumor, infantile fibrosarcoma) or chimeric autocrine growth factors (eg tenosynovial giant cell tumor (localized and diffuse types), dermatofibrosarcoma protuberans).

Genetic approaches commonly used in clinical practice for the detection of fusion genes, genomic imbalances, or missense mutations

Genetic approaches commonly used in clinical practice for the detection of fusion genes and/or genomic imbalances in soft-tissue tumors include conventional cytogenetic analysis, fluorescence *in situ* hybridization (FISH) and cytogenomic array techniques, reverse transcription PCR (RT-PCR), and sequencing. Each of these approaches bears its own set of advantages and limitations that may render it more or less suitable for the assessment of a given clinical specimen (well reviewed previously[12,15–17]).

Conventional Cytogenetic Analysis

Briefly, tissue submitted for cytogenetic analysis must be fresh (not frozen or fixed in formalin) because living, dividing cells are required.

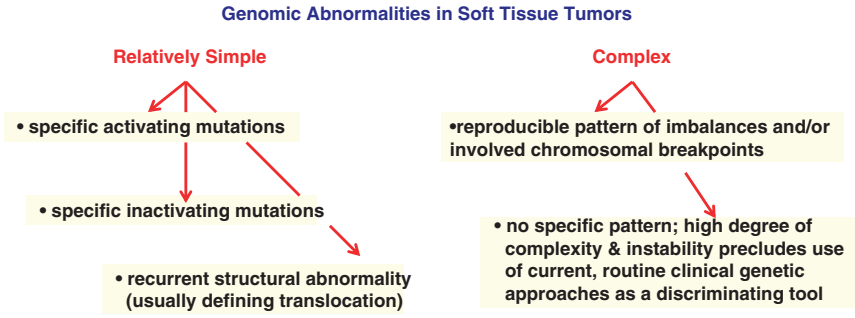

Figure 1 Schematic illustrating the categorization of genetic abnormalities in soft-tissue tumors.

Table 1 Characteristic and variant somatic chromosomal events and associated molecular abnormalities

Adipocytic tumors		
Benign	**Translocation or other**	**Fusion gene(s) or other**
Lipoma, conventional	12q15 rearrangements t(3;12)(q27–28;q13–15)	*HMGA2* *HMGA2-LPP*
	6p21 rearrangements	*HMGA1*
	Loss of 13q material, particularly 13q14	↓ *C13orf1* expression
Lipoblastoma	8q11-13 rearrangements Excess copies of chromosome 8	*PLAG1*
Myolipoma of Soft Tissue		*HMGA2*
Chondroid lipoma	t(11;16)(q13;p12-13)	*C11orf95-MKL2*
Spindle cell lipoma/pleomorphic lipoma	Monosomy 13 or loss of 13q material, particularly 13q14 Loss of 16q22-qter	Unknown
Hibernoma	11q13-21 rearrangements	*MEN1* and/or *AIP* homozygous or hemizygous loss
Intermediate (locally aggressive)	**Translocation or other**	**Fusion gene(s) or other**
Atypical lipomatous tumor/well-differentiated liposarcoma	Supernumerary ring or giant rod marker chromosome(s) containing amplified sequences of 12q14-15	*MDM2* amplification ± *CDK4* amplification and other frequently co-amplified genes *HMGA2, YEATS4, CPM, FRS2*
	± Co-amplified 1q21-25 sequences	*ATF6* and *DUSP12* amplification in some cases with 1q21-25 amplicon
Malignant	**Translocation or other**	**Fusion gene(s) or other**
Dedifferentiated liposarcoma	Supernumerary ring or long marker chromosome(s) containing amplified sequences of 12q14-15	*MDM2* amplification ± *CDK4* amplification and other frequently co-amplified genes *HMGA2, YEATS4, CPM, FRS2*
	± Co-amplified 1p32, 6q23 and 6q25 sequences	*JUN, ASK1* and *MAP3K7IP2* amplification as 1p32, 6q23 and 6q25 target genes, respectively
Myxoid/round cell liposarcoma	t(12;16)(q13;p11) t(12;22)(q13;q12)	*FUS-DDIT3* *EWSR1-DDIT3*
Fibroblastic/myofibroblastic tumors		
Benign	**Translocation or other**	**Fusion gene(s) or other**
Nodular fasciitis	t(17;22)(p13;q13.1)	*MYH9-USP6*
Fibroma of tendon sheath	t(2;11)(q31-32;q12)	Unknown (however, see desmoplastic fibroblastoma below)
Desmoplastic fibroblastoma	t(2;11)(q31;q12) t(11;17)(q12;p11.2)	Deregulated expression of FOSL1
Mammary-type myofibroblastoma	Partial monosomy 13q Partial monosomy 16q	Unknown
Soft tissue angiofibroma	t(5;8)(p15;q13)	*AHRR-NCOA2*
Cellular angiofibroma	Partial monosomy 13q Partial monosomy 16q	Unknown
Intermediate (locally aggressive)	**Translocation or other**	**Fusion gene(s) or other**
Palmar/plantar fibromatosis	+7, +8	Unknown
Desmoid-type fibromatosis	+8, +20 5q21-22 loss	Unknown *APC* inactivating mutations (germline; may be seen with or without gross chromosomal changes of 5q21-22) *CTNNB1* mutations in ~85% of sporadic lesions
Giant cell fibroblastoma	t(17;22)(q21.3;q13)	*COL1A1-PDGFB*

Table 1 (Continued)

Fibroblastic/myofibroblastic tumors		
Intermediate (rarely metastasizing)	**Translocation or other**	**Fusion gene(s) or other**
Dermatofibrosarcoma protuberans	t(17;22)(q21.3;q13) or r(17;22)	*COL1A1-PDGFB*
Extrapleural solitary fibrous tumor	12q13 rearrangements	*NAB2-STAT6*
Inflammatory myofibroblastic tumor	t(1;2)(q22;p23) t(2;19)(p23;p13) t(2;17)(p23;q23) t(2;2)(p23;q13) t(2;2)(p23;q35) t(2;11)(p23;p15) t(2;4)(p23;q21) inv(2)(p23q35) t(2;12)(p23;p11)	*TPM3-ALK* *TPM4-ALK* *CLTC-ALK* *RANBP2-ALK* *ATIC-ALK* *CARS-ALK* *SEC31A-ALK* *ATIC-ALK* *PPFIBP1–ALK*
Myxoinflammatory fibroblastic sarcoma	t(1;10)(p22;q24) with amplified 3p11-12	der/t(1;10)(p22;q24) involving *TGFBR3* and *MGEA5* without detectable chimeric fusion transcript & transcriptional upregulation of *FGF8* *VGLL3* amplification and overexpression
Congenital/infantile fibrosarcoma	t(12;15)(p13;q25)	*ETV6-NTRK3*
Malignant	**Translocation or other**	**Fusion gene(s) or other**
Low Grade Fibromyxoid Sarcoma, Hyalinizing Spindle Cell Tumor with Giant Rosettes	t(7;16)(q33;p11) t(11;16)(p13;p11)	*FUS-CREB3L2* *FUS-CREB3L1*
Sclerosing epithelioid fibrosarcoma	t(7;16)(q33;p11) – identified in LGFMS with SEF-like foci	*FUS* rearrangement has been detected in a minority of 'pure' SEF cases
So-called fibrohistiocytic tumors		
Benign	**Translocation or other**	**Fusion gene(s) or other**
Tenosynovial giant cell tumor, localized type	t(1;2)(p13.3;q37) or other rearrangements of 1p13.3	*CSF1-COL6A3* *CSF1* overexpression
Intermediate (locally aggressive)	**Translocation or other**	**Fusion gene(s) or other**
Tenosynovial giant cell tumor, diffuse type	t(1;2)(p13.3;q37) or other rearrangements of 1p13.3 Subset with +5 and/or +7 as sole anomaly	*CSF1-COL6A3* *CSF1* overexpression
Intermediate (rarely metastasizing)	**Translocation or other**	**Fusion gene(s) or other**
Giant cell tumor of soft tissue	Telomeric associations (tas)	
Smooth muscle tumors		
Benign	**Translocation or other**	**Fusion gene(s) or other**
Benign metastasizing leiomyoma	6p21 rearrangement 19q and 22q terminal deletions	*HMGA1*
Pericytic (perivascular) tumors		
Benign	**Translocation or other**	**Fusion gene(s) or other**
Pericytoma with t(7;12)	t(7;12)(p22;q13)	*ACTB-GLI1*

Table 1 (Continued)

Skeletal muscle tumors		
Benign	**Translocation or other**	**Fusion gene(s) or other**
Fetal rhabdomyoma		*PTCH1* loss of function mutations in syndromic lesions Hedgehog pathway activation in nonsyndromic lesions, mechanism unknown
Malignant	**Translocation or other**	**Fusion gene(s) or other**
Embryonal rhabdomyosarcoma	Loss or UPD of 11p15.5 +2, +8, +11, +12, +13, +20	*IGF2, H19, CDKN1C* and *HOTS*
Alveolar rhabdomyosarcoma	t(2;13)(q35;q14) t(1;13)(p36;q14) t(X;2)(q13;q35) t(2;2)(q35;p23) t(2;8)(q35;q13) t(8;13)(p12;q13)	*PAX3-FOXO1* *PAX7-FOXO1* *PAX3-FOXO4* *PAX3-NCOA1* *PAX3-NCOA2* *FOXO1-FGFR1*
Spindle cell rhabdomyosarcoma	8q13 rearrangements	*SRF-NCOA2* *TEAD1-NCOA2*
Vascular tumors of soft tissue		
Intermediate (rarely metastasizing)	**Translocation or other**	**Fusion gene(s) or other**
Pseudomyogenic hemangioendothelioma	t(7;19)(q22;q13)	Unknown
Malignant	**Translocation or other**	**Fusion gene(s) or other**
Epithelioid hemangioendothelioma	t(1;3)(p36;q25) t(X;11)(p11.2;q13)	*WWTR1-CAMTA1* *YAP1-TFE3*
Angiosarcoma of soft tissue		High-level amplification of *MYC* (8q24) is a consistent hallmark of radiation-induced, lymphedema-associated angiosarcoma
Chondro-osseous tumors		
Benign	**Translocation or other**	**Fusion gene(s) or other**
Soft tissue chondroma	12q13-15 rearrangements +5	*HMGA2*
Malignant	**Translocation or other**	**Fusion gene(s) or other**
Extraskeletal mesenchymal chondrosarcoma	inv(8)(q13q21)	*HEY1-NCOA2*
Gastrointestinal stromal tumors		
Gastrointestinal stromal tumor	 Monosomy or partial loss of 14 and/or 22 Deletions of 1p, 9p, 9q, 10, 11p, and 13q and gains/amplifications on 5p, 3q, 8q, and 17q are associated with malignant behavior	*KIT, PDGFRA* or *BRAF* mutations Unknown *CDKN2A/B* (9p21 loss)
Nerve sheath tumors		
Benign	**Translocation or other**	**Fusion gene(s) or other**
Schwannoma (including variants)	Monosomy or partial loss of 22	*NF2, SMARCB1*
Melanotic schwannoma	Amplification or deletion of 2p16 (with or without Carney complex)	*CNC2*

Table 1 (Continued)

Tumors of uncertain differentiation		
Benign	**Translocation or other**	**Fusion gene(s) or other**
Neurofibroma (including variants)	17q loss	*NF1*
Perineurioma	monosomy or partial loss of 22	*NF2*
Malignant	**Translocation or other**	**Fusion gene(s) or other**
Malignant peripheral nerve sheath tumor	17q loss 9p loss	*NF1* (germline and somatic) *CDKN2A*
Ectomesenchymoma	+2, +8, +11, +12, +13, +20	Unknown
Tumors of uncertain differentiation		
Benign	**Translocation or other**	**Fusion gene(s) or other**
Intramuscular myxoma		*GNAS* mutations (patients with or without fibrous dysplasia of bone)
Deep 'aggressive' angiomyxoma	12q13-15	*HMGA2*
Intermediate (locally aggressive)	**Translocation or other**	**Fusion gene(s) or other**
Hemosiderotic fibrolipomatous tumor	t(1;10)(p22;q24) with amplified 3p11-12	der/t(1;10) (p22;q24) involving *TGFBR3* and *MGEA5* without detectable chimeric fusion transcript & transcriptional upregulation of *FGF8* *VGLL3* amplification and overexpression
Intermediate (rarely metastasizing)	**Translocation or other**	**Fusion gene(s) or other**
Angiomatoid fibrous histiocytoma	t(2;22)(q33;q12) t(12;22)(q13;q12) t(12;16)(q13;p11)	*EWSR1-CREB1* *EWSR1-ATF1* *FUS-ATF1*
Ossifying fibromyxoid tumor	6p21 or monosomy 22 (more frequent in malignant form)	*PHF1* Unknown
Myoepithelioma/myoepithelial carcinoma/mixed tumor	t(1;22)(q23;q12) t(6;22)(p21;q12) t(19;22)(q13;q12) 16p11.2 rearrangement	*EWSR1-PBX1* *EWSR1-POU5F1* *EWSR1-ZNF444* *FUS-?*
Malignant	**Translocation or other**	**Fusion gene(s) or other**
Synovial sarcoma	t(X;18)(p11.2;q11.2) t(X;20)(p11.2;q13.3)	*SS18-SSX1* *SS18-SSX2* *SS18-SSX4* *SS18L1-SSX1*
Epithelioid sarcoma	22q11.2 anomalies +8q, often as i(8)(q10)	*SMARCB1*
Alveolar soft part sarcoma	der(17)t(X;17)(p11;q25)	*ASPSCR1-TFE3*
Clear cell sarcoma of soft tissue	t(12;22)(q13;q12) t(2;22)(q33;q12)	*EWSR1-ATF1* *EWSR1-CREB1*
Extraskeletal myxoid chondrosarcoma	t(9;22)(q22;q12) t(9;17)(q22;q11) t(9;15)(q22;q21) t(3;9)(q12;q22)	*EWSR1-NR4A3* *TAF15-NR4A3* *TCF12-NR4A3* *TFG-NR4A3*
Extraskeletal Ewing sarcoma	t(11;22)(q24;q12) t(21;22)(q22;q12) t(7;22)(q22;q12) t(17;22)(q21;q12) t(2;22)(q36;q12) t(16;21)(p11;q22) t(2;16)(q36;p11)	*EWSR1-FLI1* *EWSR1-ERG* *EWSR1-ETV1* *EWSR1-EIAF* *EWSR1-FEV* *FUS-ERG* *FUS-FEV*
Desmoplastic small round cell tumor	t(11;22)(p13;q12)	*EWSR1-WT1*

Table 1 (Continued)

Tumors of uncertain differentiation		
Benign	**Translocation or other**	**Fusion gene(s) or other**
Extrarenal rhabdoid tumor	22q11.2 anomalies	*SMARCB1*
PEComa	Deletion or loss of 16p	*TSC2*
Intimal sarcoma	Gain or amplification of 12q12–15 and 4q12	*CDK4*, *TSPAN31*, *MDM2*, *GLI* and *PDGFRA*, *KIT* , *CHIC2* respectively
Undifferentiated/unclassified sarcomas		
Malignant	**Translocation or other**	**Fusion gene(s) or other**
Primitive/undifferentiated round cell tumor or possible variants of Ewing sarcoma	inv(22)(q12q12) t(2;22)(q31;q12) t(20;22)(q13;q12) t(4;22)(q31;12) t(6;22)(p21;q12) t(4;19)(q35;q13) t(10;19)(q26.3;q13) inv(X)(p11.2p11.4)	*EWSR1-PATZ1* *EWSR1-SP3* *EWSR1- NFATC2* *EWSR1-SMARCA5* *EWSR1-POU5F1* *CIC-DUX4* *CIC-DUX4* *BCOR-CCNB3*

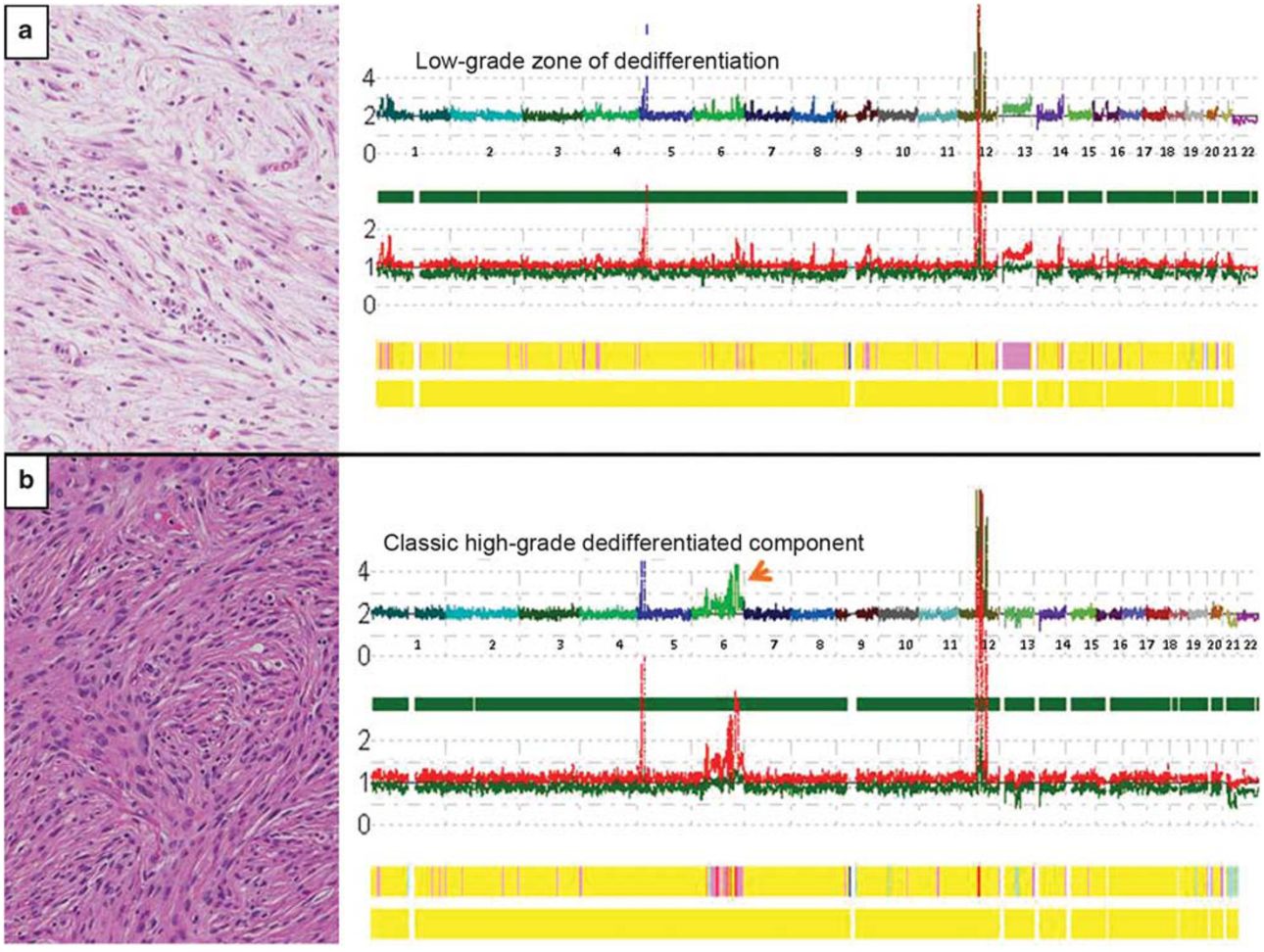

Figure 2 Dedifferentiated liposarcoma. (**a**) Low-grade dedifferentiation characterized by uniform spindle cells with mild nuclear atypia and a SNP profile with 5p14.1–p14.2 and discontinuous 12q13.3–q21.33 amplicons in a background of scattered gains of other chromosomal regions. (**b**) High-grade dedifffferentiated component resembling pleomorphic undifferentiated sarcoma with the corresponding SNP profile exhibiting acquisition of a 6q amplicon (including gene loci involved in the *JNK–MAPK* signaling pathway, orange arrow) and a more complex, discontinuous 12q14–15 driver amplicon involving the *MDM2* locus among other imbalances.

A mesenchymal tumor sample submitted for karyotyping should be representative of the neoplastic process and preferably be part of the specimen submitted for pathological study. Small biopsy specimens or fine-needle aspirates (<500 mg) can be analyzed successfully. On average, a short-term culture usually results in a sufficient number of mitoses within 6–10 days or fewer. A 24-h turnaround time or less however can be achieved by conducting a direct or same-day harvest whereby endemic dividing cells are arrested after a 1–12-h incubation in colchicine. A significant strength of cytogenetic analysis is that it provides a global assessment of both numerical and structural abnormalities in a single assay, including both primary and secondary anomalies. Moreover, in contrast to FISH or RT-PCR, knowledge of the anticipated anomaly or histological diagnosis is not necessary. Historically this technical approach, by revealing recurrent chromosomal translocations, has been responsible for the initial characterization of numerous soft-tissue tumors.

Molecular Cytogenetic Analysis

Standard chromosomal analysis is not considered a high-resolution technique. Routine karyotyping of soft-tissue tumors typically yields 350–550 bands per haploid set with each band representing $\sim 5–10 \times 10^6$ base pairs (bp) of DNA and potentially containing hundreds of genes at any one band.[18] In contrast, over the past 25 years molecular cytogenetic methods of increasingly higher resolution have been developed and incorporated into the management of soft-tissue tumors.[4] With this versatile technology, labeled nucleic acid sequences (probes) are hybridized to morphologically preserved metaphase chromosomes, interphase cells of fresh/frozen cytologic preparations or formalin-fixed, paraffin-embedded material (FFPE) (FISH or cytogenomic arrays (array comparative genomic hybridization (aCGH) or single-nucleotide polymorphism (SNP) arrays).

The overall resolution of interphase FISH is 50–100 kb. FISH testing with bicolor break-apart or dual fusion probe sets are most commonly employed for the detection of translocation events and locus-specific probes (coupled with a copy number control probe) are frequently used to evaluate for amplification or loss of an oncogene or tumor suppressor gene locus respectively in soft-tissue tumors. While there are a variety of quality-controlled DNA probes intended for clinical use manufactured commercially and sold as analyte-specific reagents, relatively few of these are designed specifically for the study of mesenchymal neoplasms. Specificity for a particular diagnostic entity is inconsistent as rearrangements of some loci are involved in only one tumor type and others are not. For example, rearrangement of the *SS18* locus, the hallmark of synovial sarcoma, is exclusive to this entity. In contrast, although the t(11;22)(q24;q12) is characteristic of Ewing sarcoma, rearrangement of *EWSR1* (22q12) is not confined to Ewing sarcoma but is also seen in most or in smaller subsets of desmoplastic small round cell tumor, clear cell sarcoma, extraskeletal myxoid chondrosarcoma, myxoid/round cell liposarcoma, and myoepithelial tumors of soft tissue, to name a few. To enhance diagnostic specificity, to provide testing for uncommon but clinically relevant abnormalities, or to aid in deciphering complex rearrangements, some laboratories also elect to custom-design probe sets for these types of clinical purposes for which commercial probes may not be available. These laboratory-developed probes are used exclusively in-house (not sold to other laboratories) and are not currently regulated by the US Food and Drug Administration; clinical laboratories using such probes must verify or establish, for each specific use of each probe, the performance specifications for applicable performance characteristics, eg accuracy, precision, analytical sensitivity and specificity.[19]

Global assessment of genomic imbalances and acquired uniparental disomy (copy neutral loss of heterozygosity (cnLOH)) can be achieved through SNP array analysis. This high-density technology contributes to tumor classification and diagnosis as well as aids in predicting the prognosis of some soft-tissue tumors.[20–23] For example, cytogenomic array studies (aCGH or SNP array) have identified recurrent patterns of copy number changes and/or cnLOH in embryonal rhabdomyosarcoma (+2, +7, +8, +11, +12, +13, +20, cnLOH 11p15.5) with acquisition of genomic amplification in lesions distinguished by the presence of anaplasia, benign metastasizing leiomyoma (loss of 1p, 13q, 19q, and 22q material), and dedifferentiated liposarcoma whereby gain of amplicons in 1p32 and 6q23–25 (containing genes involved in the c-jun NH_2-terminal kinase/mitogen-activated protein kinase pathway) parallels the progression from an atypical lipomatous tumor/well-differentiated liposarcoma to dedifferentiated liposarcoma, Figure 2.[24–30]

Sequencing Analysis

Second-generation sequencing also represents a comprehensive technology that through whole-genome, whole-exome and whole-transcriptome approaches, resolution at the nucleotide level is conveyed. A remarkable discovery tool, investigators are engaging high-throughput second-generation sequencing practices in soft-tissue sarcomas to identify novel chromosomal rearrangements such as the recently identified *BCOR–CCNB3* in undifferentiated small-cell sarcoma and *NAB–STAT6* in solitary fibrous tumor as well as copy number changes, and point mutations.[6–8,31–33] As a consequence, we are gaining a deeper understanding of the underlying mechanisms of sarcomagenesis, which

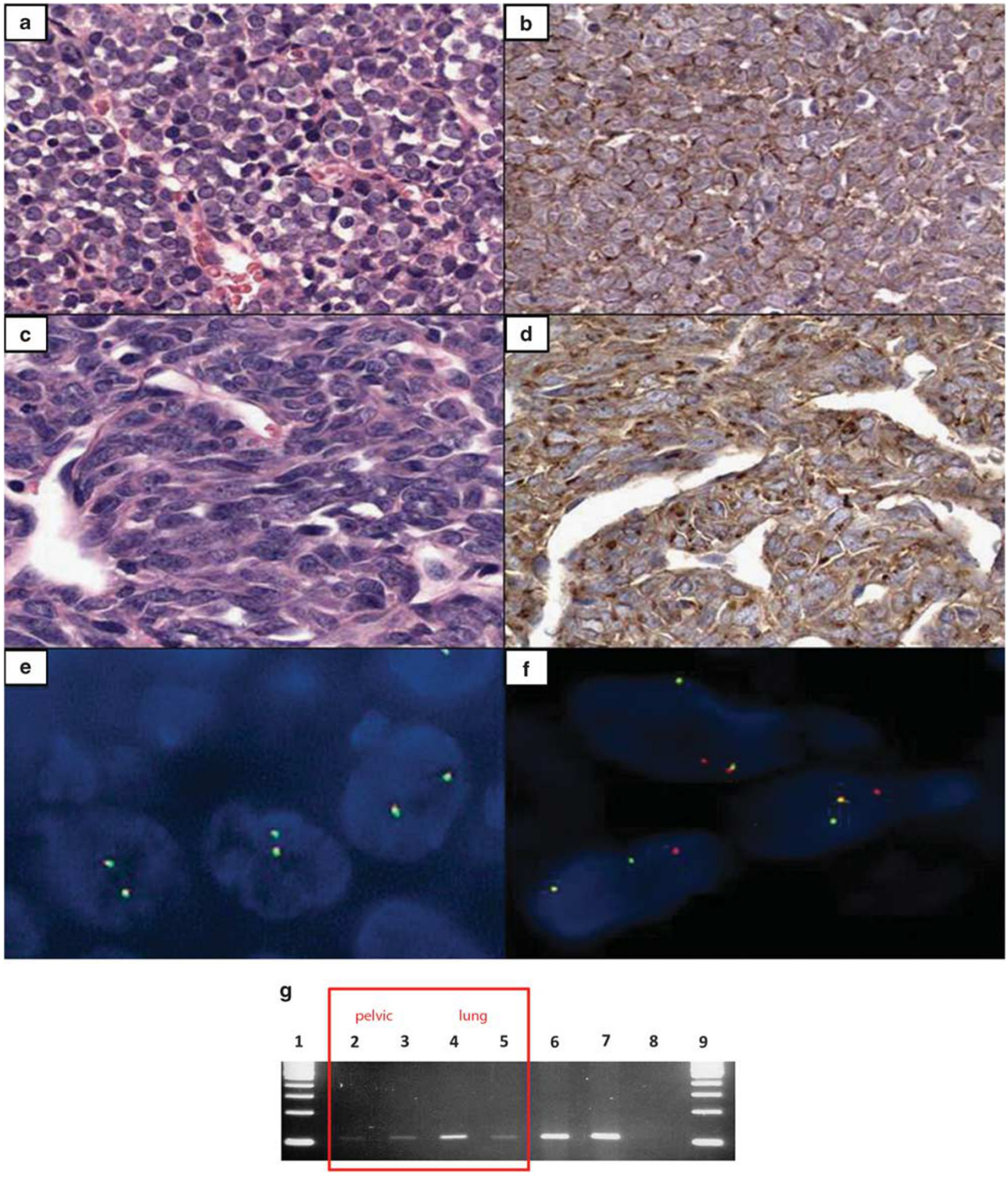

Figure 3 (**a**,**b**) Poorly differentiated synovial sarcoma arising in the pelvis with a small round cell morphologic appearance and CD99 immunohistochemical staining pattern mimicking a Ewing sarcoma. Initial FISH testing of this specimen further complicated the initial diagnosis as it was interpreted as positive for a rearrangement of the *EWSR1* locus. (**c**,**d**) Subsequent lung metastasis demonstrated a spindle-cell morphology with focal staghorn vascular pattern and non-specific CD99 immunostaining pattern. (**e**,**f**) Repeat FISH testing at another center revealed that both the initial pelvic lesion and the subsequent lung metastasis were negative for an *EWSR1* rearrangement (**e**) but were positive for an *SS18* rearrangement (**f**). (**g**) In addition, RT-PCR analysis demonstrated the presence of a *SS18–SSX1* fusion transcript in both the pelvic and lung lesions. (Parts **a–d** of this figure courtesy of Dr John Reith, University of Florida Health Science Center.)

in turn is enabling further advances in diagnosis and selection of therapy.

Targeted DNA sequencing approaches such as Sanger sequencing (dideoxynucleotide sequencing), pyrosequencing, and predesigned or custom-designed second-generation sequencing cancer panels are increasingly used for the identification of activating or inactivating missense mutations, deletions and insertions in oncogenes and tumor suppressor genes such as *KIT*, *PDGFRA*, *BRAF*, *SMARCB*, and *TP53* that may have a primary or secondary role in soft-tissue tumors and/or are used to direct therapy.[34–38] Sequencing can be performed on fresh or FFPE material if the DNA is of sufficient quality. Micro- or macrodissection may be required depending on the calculated percent neoplastic cellularity of the individual test specimen and the established analytical sensitivity of the technical approach.[39]

Reverse Transcription PCR

RT-PCR technique uses specific synthetic oligonucleotides or primers to amplify a section of a given cDNA (the DNA complement generated by reverse-transcribing the RNA of interest) in snap-frozen or FFPE pathology material.[40] Due to its simplicity, specificity, sensitivity and quick turn-around-time, RT-PCR is commonly used to detect tumor-specific chimeric or fusion genes created by chromosomal translocations such as the X;18 translocation (t(X;18)(p11.2;q11.2)) of synovial sarcoma. In addition to its value as a diagnostic adjunct, RT-PCR testing has also been advocated for the detection or monitoring of minimal residual or minimal disseminated disease for some soft-tissue tumors.[41–46] An important pitfall to be aware of is that uncommon or novel molecular or cytogenetic variant translocations may elude detection by RT-PCR or interphase FISH analysis because of primer or probe design.

Indications for molecular testing in sarcomas; capitalizing on genetic changes

Molecular testing has a direct, potentially decisive role in the examination of soft-tissue tumors. Fusion genes resulting from chromosomal rearrangements including translocations, inversions, deletions and insertional or tandem duplications represent excellent markers for tumor classification. Sarcomas with fusion genes do not usually show a benign or premalignant phase. Distinct advantages of testing for chromosomal translocations/fusion genes as a diagnostic aid are that these molecular aberrations are typically exhibited from the earliest disease presentation and persist in metastatic and previously treated lesions as well as in neoplasms as they become less differentiated. Moreover, identification of some fusion genes is important in directing therapy. For example, dermatofibrosarcoma protuberans is characterized by a 17;22 translocation involving the *COL1A1* and *PDGFB* genes, which results in the overproduction of fusion COL1A1–PDGF–BB ligand and consequent hyperactivation of PDGFRB, rendering these tumors responsive to targeted therapy with tyrosine kinase inhibitors such as imatinib mesylate.[47,48]

The aim of the following is to highlight indications for molecular testing in the management of soft-tissue tumors and the advantages of capitalizing on these methods when facing tumors of a confusing nature or challenging differential diagnosis. Certain case illustrations are included to serve as useful paradigms.

Small Round-Cell Tumors

The homogeneous light microscopic appearance of small round-cell neoplasms to include those of mesenchymal, epithelial, and lymphoreticular origin may cause diagnostic difficulties. Establishing an accurate diagnosis often requires studies beyond routine hematoxylin and eosin-stained sections. Immunohistochemical features may be helpful, but are sometimes not specific, may be simulated by different tumor types, or are absent in poorly differentiated tumors.[49–51] Critical to arriving at the correct diagnosis is not only an awareness of the diverse entities (eg Ewing sarcoma, rhabdomyosarcoma, mesenchymal chondrosarcoma, desmoplastic small round cell tumor, round cell liposarcoma, poorly differentiated synovial sarcoma, and neuroblastoma, among others) that may present as small round cell tumors but also the ancillary testing capable of narrowing or establishing the diagnosis with a command of its significance and limitations.

For example, poorly differentiated synovial sarcoma with a round cell pattern may mimic an extraskeletal Ewing sarcoma both histologically and immunohistochemically. Approximately two-thirds of synovial sarcomas are immunoreactive for CD99 and conversely, cytokeratin immunoreactivity may be seen in Ewing sarcoma.[52–54] The identification of specific rearrangements molecularly is often necessary for establishing the definitive diagnosis: identification of *SS18–SSX* fusions or *SS18*–rearrangement for synovial sarcoma as opposed to *EWSR1–FLI1* fusions or *EWSR1* or *FUS* variant rearrangements for Ewing sarcoma. Figure 3 illustrates a case scenario of a synovial sarcoma that was initially diagnosed as a Ewing sarcoma based not only on the clinicohistopathologic impression but also strongly influenced by an inaccurate FISH study interpreted as positive for a rearrangement of the *EWSR1* locus in 12% of the cells analyzed. A subsequent metastatic lung lesion demonstrated a spindle-cell morphology with a focal staghorn vascular pattern; molecular studies to include RT-PCR and FISH (also performed on the former primary tumor specimen) confirmed the diagnosis

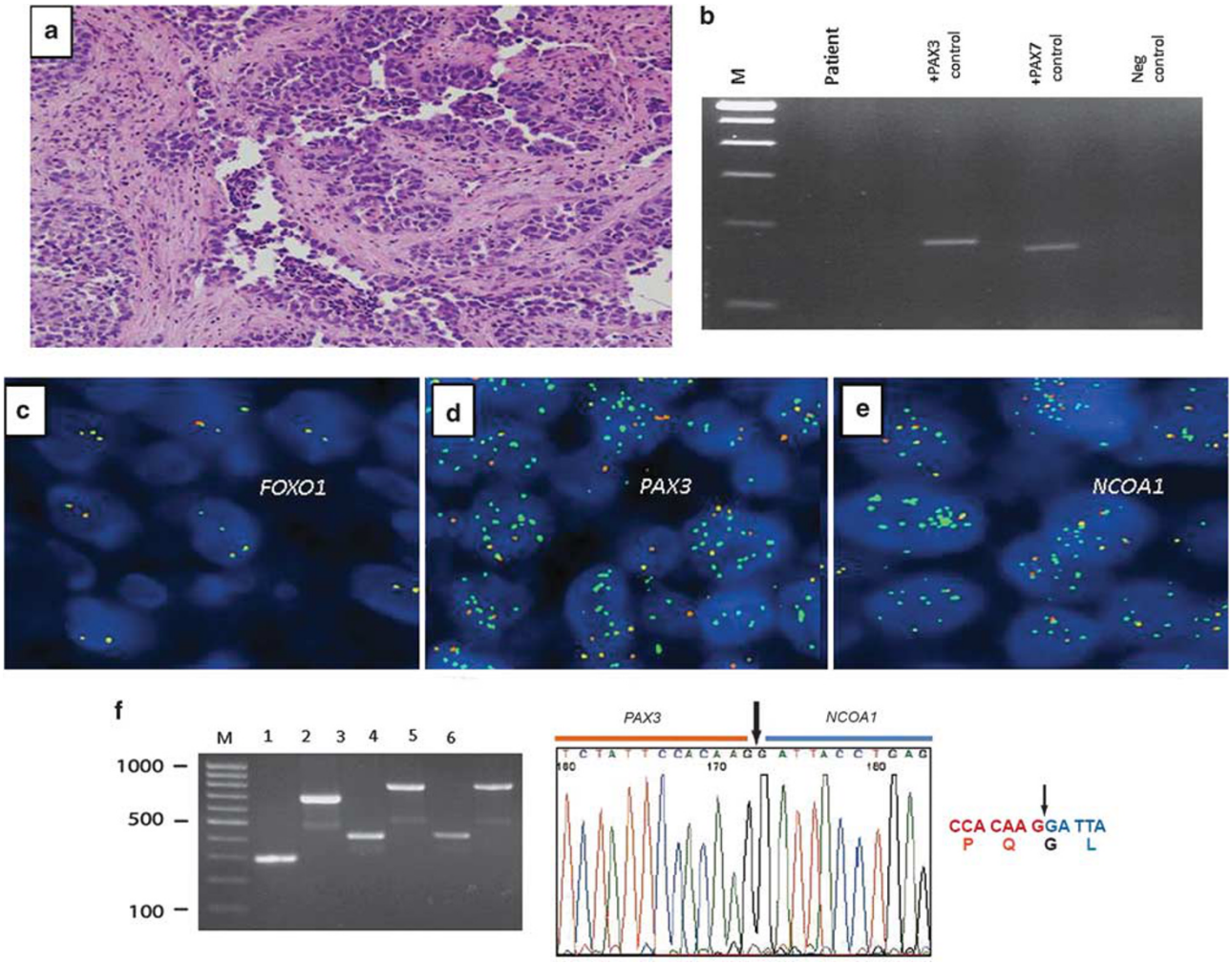

Figure 4 (**a**) Alveolar rhabdomyosarcoma histologic pattern with loss of cellular cohesion in a *PAX3–NCOA1* variant case. (**b**) RT-PCR studies were negative for a *PAX3*- or *PAX7–FOXO1* fusion transcript in this specimen. (**c**) FISH analysis for a rearrangement of the *FOXO1* locus is also negative for this case. (**d**) FISH analysis with a laboratory developed, custom-designed *PAX3* break-apart probe set demonstrates split of orange and green signals with amplification of the latter. (**e**) Following identification of the unique *PAX3* fusion transcript gene partner by rapid amplification of cDNA ends (RACE) approach, FISH analysis with a laboratory developed, custom-designed *NCOA1* break-apart probe set demonstrates split of orange and green signals with amplification of the latter. (**f**) *PAX3–NCOA1* fusion transcript variants also demonstrated by RT-PCR using gene-specific primers and sequence confirmation.

of synovial sarcoma. FISH interpretation can sometimes be difficult. Before reporting patient results, it is necessary for molecular laboratories to establish performance characteristics for normal reference ranges.[19,55] For example, reference ranges may differ between different types of preparations (eg cytologic touch preparation vs FFPE tissue section). Care should be taken in reporting results near the cutoff values. In general, it is wise to have available more than one genetic diagnostic modality, to be ready to confirm an equivocal, unexpected, or discrepant result by two independent techniques.[56]

Interestingly, there also exists a faction of primitive small round-cell sarcomas that exhibit features both similar to and distinct from Ewing sarcoma (Ewing-like), but have most recently been addressed in the fourth edition of *WHO Classification of Tumours of Soft Tissue and Bone*[1] as undifferentiated round cell sarcomas. Genetically, a subset of these tumors have shown rearrangements of *EWSR1* with a non-ETS gene partner such as *PATZ1*, *POU5F1*, *SMARCA5*, *NFATC2*, and *SP3* or in the case of *CIC–DUX4* characterized tumors, a subclass of the ETS family of genes is upregulated by the chimeric protein.[57–62] Notably, there is a strong likelihood that a diagnostic work-up for an undifferentiated small round-cell sarcoma (particularly in a pediatric patient) would include molecular testing for the Ewing sarcoma-associated rearrangements. If conducted, RT-PCR analysis for the principal Ewing sarcoma associated *EWSR1–FLI1* fusion transcript would be negative, however, FISH analysis for an *EWSR1* rearrangement would be positive in the *EWSR1*/non-ETS variant tumors

listed above. Additional molecular testing would be required to further distinguish these entities. Currently however, the treatment for most of these cases has been the same as for Ewing sarcoma.[1]

Spindle-Cell Sarcoma

Analogous to small round-cell tumors, the differential diagnosis of spindle-cell neoplasms occurring in the soft-tissue is diverse. Establishing a diagnosis of fibrosarcoma, leiomyosarcoma, malignant peripheral nerve sheath tumor, monophasic synovial sarcoma, and spindle-cell carcinoma to name a few may pose unique challenges depending on variables such as biopsy size, immunostaining, anatomic location, and clinical presentation. For precise classification, genetic studies may be required when standard pathologic examination is unable to differentiate between some of these conditions.

In the pediatric population, the morphologic appearance of congenital/infantile fibrosarcoma may be virtually indistinguishable from other spindle-cell neoplasms that may occur during childhood, such as the 'adult-form' of fibrosarcoma, infantile fibromatosis (lipofibromatosis), and infantile myofibromatosis/myofibroma. These issues can be problematic due to differences in clinical behavior and management of these disorders. For example, some cases of infantile myofibromatosis/myofibroma exhibit a prominent cellular fascicular pattern with hyperchromatic nuclei and high mitotic rate resembling infantile fibrosarcoma and conversely, some infantile fibrosarcomas possess a biphasic pattern with foci resembling infantile myofibromatosis, including whorls of primitive spindle cells and perivascular/intravascular projections of myofibroblastic nodules.[63] Molecular diagnostic testing for the *ETV6–NTRK3* gene fusion that arises as a result of the t(12;15)(p13;q25) is a reliable and sensitive assay for the diagnosis of infantile fibrosarcoma and may be superior to conventional cytogenetic analysis because the 12;15 translocation is morphologically subtle as the regions

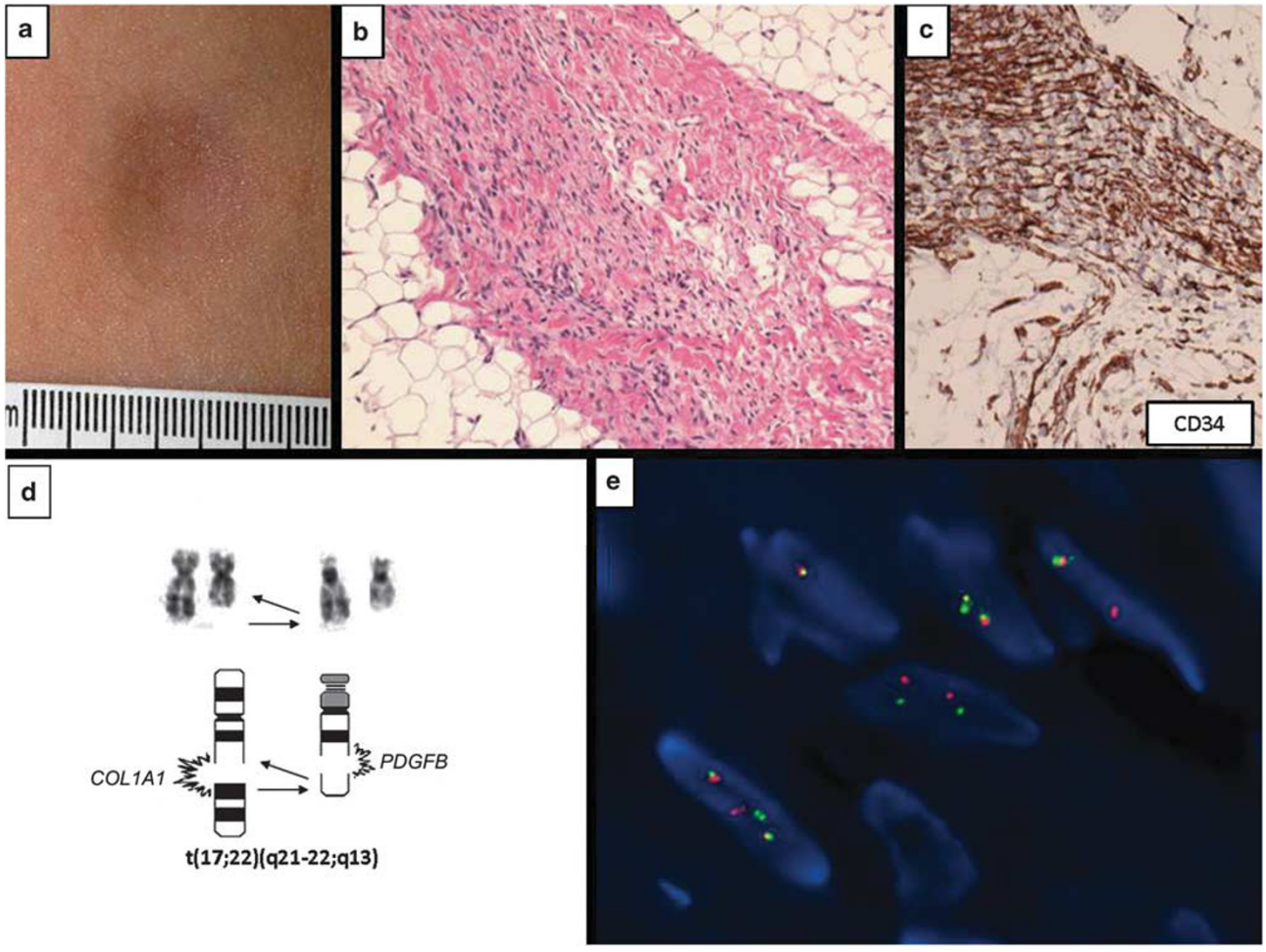

Figure 5 (**a**) Example of a depressed plaque characterizing the clinical presentation of this DFSP arising in a child with ADA–SCID (courtesy of Drs Fabio Candotti and Robert Sokolic, National Institutes of Health). (**b**) DFSP invading the subcutanous tissue. (**c**) CD34 immunoreactivity. (**d**) Partial karyotype and schematic illustrating the 17;22 translocation of DFSP. (**e**) FISH analysis with a custom-designed, dual color, dual fusion probe set spanning the *COL1A1* and *PDGFB* loci. Juxtaposed red/green (or yellow) signals represent the *COL1A1–PDGFB* fusion.

exchanged between chromosomes 12 and 15 are similar in size and banding characteristics.[64]

Another opportunity for a diagnostic misinterpretation is exemplified in the differential diagnosis of primary intrathoracic or pleural monophasic synovial sarcoma; these tumors must be discriminated from solitary fibrous tumor, sarcomatous malignant mesothelioma, smooth muscle tumor, malignant peripheral nerve sheath tumor, thymoma, sarcomatoid carcinoma, and pleuropulmonary blastoma.[65–67] Differences in histologic and immunohistochemical features among these entities may not be sufficient to arrive at a definitive diagnosis in all cases. Moreover, a limited sample size, as in the evaluation of any tumor, may preclude or restrict some of the ancillary testing desired. Increasingly, pathologists must weigh the advantages of conducting a battery of immunostains with the risk of exhausting the tissue sample source *vs* reserving a nominal number of unstained slides[1–3] for molecular testing that may prove essential for accurate classification or treatment design. Certainly the field has witnessed a rise in the diagnosis of synovial sarcoma in this rare anatomic site benefiting from an increased awareness and the diagnostic capabilities afforded by molecular technology.[67]

Rhabdomyosarcoma Subtype

Rhabdomyosarcomas are heterogeneous, clinically aggressive tumors that show varying degrees of skeletal muscle differentiation.[68] Embryonal rhabdomyosarcoma (ERMS) and alveolar rhabdomyosarcoma (ARMS) comprise the two main histologic subtypes. Morphologic evaluation alone is often insufficient to make the distinction between ARMS and ERMS as some ARMSs lack the alveolar architecture ('solid variant') and ERMS can be densely cellular and poorly differentiated.[69,70] Yet, this distinction is clinically critical in assigning patients appropriate-risk therapeutic regimens. The current risk stratification scheme used by the Children's Oncology Group (COG) excludes ARMS from the low-risk stratum regardless of other clinical features.[71]

Thus, a valuable diagnostic adjunct in ARMS is the identification of translocations t(2;13)(q35;q14) and t(1;13)(p36;q14), and the associated *PAX3–FOXO1* and *PAX7–FOXO1* fusion transcripts, respectively. Recognition of these specific translocations is also prognostically important as *PAX–FOXO1* fusion status imparts an unfavorable outcome for children with rhabdomyosarcoma. Specifically, a recent COG report of event-free (EFS) and overall survival (OS) at 5 years correlated with histopathologic subtype and *PAX–FOXO1* status in 434 D9803 study enrollees showed that fusion negative ARMS (ARMSs lacking a detectable *PAX3-* or *PAX7-FOXO1* fusion and representing ~18% of all ARMSs) had an outcome similar to ERMS and superior EFS compared with ARMS with either *PAX3-* or *PAX7–FOXO1* fusions, when given therapy designed for children with intermediate-risk RMS.[68,69] In other words, the presence or absence of a *PAX–FOXO1* fusion gene in ARMS confers distinct biological properties, despite a similar histological appearance. It was concluded that these findings support incorporation of *PAX–FOXO1* fusion status into risk stratification and treatment allocation for rhabdomyosarcoma patients.[71]

Of interest, rare *PAX3* and *FOXO1* variant translocations have also been uncovered in a small subset of rhabdomyosarcomas (some previously classified as fusion-negative ARMS) by RT-PCR, gene expression profiling, FISH positional cloning and RACE (rapid amplification of cDNA ends), or SNP array methodologies, Figure 4.[72–75] Owing to low prevalence, the clinical behavior of these rare fusion variant positive rhabdomyosarcomas is unknown, although the fusion protein variant PAX3–NCOA1 rhabdomyosarcoma has been shown to exhibit a gene expression signature akin to ARMS (transactivation properties similar to PAX3–FOXO1).[73] Consequently, it is plausible that identification of unusual variant translocations in rhabdomyosarcoma will also be important in risk stratification and management of this disease.

Confirmation of Lesions with an Unusual Clinicopathologic Presentation (Uncommon age, Rare Anatomic Location or Atypical Histopathologic Features)

Descriptions of unusual or atypical clinical or histopathological presentations for nearly every mesenchymal tumor type exist in isolated case reports or small series. Molecular diagnostic testing is particularly helpful in confirming the diagnosis in these types of extraordinary cases and for certain diagnoses, it has expanded the recognized spectrum of presentations.

Although it is beyond the scope of this review to provide a comprehensive account of all unusual soft-tissue tumor presentations, the following represent a few interesting paradigms. Adamantinoma-like Ewing sarcoma was initially considered a morphologic variant of adamantinoma, but subsequently was shown to harbor the t(11;22)(q24;q12) *EWSR1–FLI1* fusion and accepted as a rare variant of Ewing sarcoma arising in bone or soft-tissue.[53,76–78] The histopathologic diagnosis of alveolar rhabdomyosarcoma equated with a fairly uniform age incidence (10–25 years), and has been confirmed in patients up to 76 years of age by molecular detection of *PAX–FOXO1* fusions permitting assessment of possible fusion gene clinical correlates in this unique older patient population.[79,80] The recent description of adenosine deaminase-deficient severe combined immunodeficiency (ADA–SCID) predisposing to a childhood presentation of dermatofibrosarcoma protuberans (DFSP) has revealed several atypical features in the association of these two rare conditions: nearly all

patients present with multiple DFSP lesions in the preprotuberant morpheaform plaque stage with absence of a classic storiform histologic pattern, Figure 5.[81] Affirmation of the diagnosis of DFSP by genetic analysis proved very helpful in this previously unrecognized union.

Unanticipated Therapeutic Response or Direction of Treatment Strategy

It goes without saying that a misdiagnosis of any pathologic entity may lead to inappropriate treatment and incorrect assessment of the prognosis. One of the most common challenges in diagnostic soft-tissue pathology is the distinction between lipoma and atypical lipomatous tumor/well-differentiated liposarcoma (ALT/WDL). This challenge is intensified when the atypical hyperchromatic or pleomorphic cells of the expanded fibrous septae characterizing ALT/WDL are scarce or the atypia is cytologically subtle. Discrimination is important not only because ALT/WDL is more likely to locally recur than a lipoma but more importantly because of the potential for ALT/WDL to dedifferentiate into a high-grade sarcoma, particularly for lesions arising in the retroperitoneum. A related pitfall in the examination of well-differentiated liposarcomas with a sclerosing pattern is that surgical sampling exclusive to nonlipogenic areas may lead to an erroneous conclusion that the tumor is not a liposarcoma jeopardizing appropriate patient care.[17,82] The cytogenetic and molecular genetic characteristics of lipoma and ALT/WDL are distinct permitting a definitive diagnosis Table 1.[1]

Owing to the extensive morphologic and immunohistochemical overlap between clear cell sarcoma and conventional melanoma, detection of the t(12;22)(q13;q12) and its associated fusion gene *EWSR1–ATF1* (or the related variant t(2;22)(q34;q12) and resultant *EWSR1–CREB1* fusion) are of crucial value in establishing the diagnosis of soft-tissue clear cell sarcoma unequivocally.[83–86] In this regard, the utility of molecular confirmation in establishing the diagnosis of clear cell sarcoma of soft parts in rare primary sites such as cutaneous or skeletal or peculiar metastatic locations like ovary and breast has been emphasized.[83,87–91] The *BRAF* gene, encoding for a serine/threonine protein kinase of the MAP kinase/ERK-signaling pathway, is mutated in ~50% of melanomas.[92,93] The use of selective inhibitors against metastatic or nonresectable melanomas harboring BRAF c.1799 T>A (p.V600E) mutations can produce impressive therapeutic responses underscoring the importance of performing clinical mutational analysis.[92,93] Clear cell sarcoma of soft-tissue was initially thought to lack activating *BRAF* mutations, however, rare confirmed *EWSR1* rearranged clear cell sarcomas have recently been reported to contain *BRAF* mutations.[35,89,94,95]

Loss of Immunophenotype or Dedifferentiation

When a soft-tissue tumor is poorly differentiated or has undergone dedifferentiation, identification of diagnostic morphological features is difficult. Often, key or defining immunohistochemical and ultrastructural attributes are lost and arriving at a definitive diagnosis is compromised. In contrast, primary cytogenetic changes and associated molecular events such as the 11;22 translocation/*EWSR1–FLI1* fusion of Ewing sarcoma are retained as a given tumor becomes less differentiated, providing a diagnostic advantage in these settings.[96]

Liposarcomas represent the single most common group of soft-tissue sarcomas. Dedifferentiated liposarcoma is a distinct subtype of liposarcoma, showing abrupt or gradual transition into a nonlipogenic sarcoma of variable histologic grade, either in the primary tumor or in a recurrent tumor from a well-differentiated liposarcoma.[1] The extent of the dedifferentiated component may vary from minor to overwhelmingly dominant. Liposarcomas with high-grade dedifferentiation may be difficult to distinguish from a high-grade pleomorphic sarcoma or other poorly differentiated sarcomas (especially with small biopsies) and those with low-grade dedifferentiation should not be confused with well-differentiated spindle-cell liposarcoma. Areas of dedifferentiation may resemble myxofibrosarcoma, solitary fibrous tumor, fibrosarcoma, and gastrointestinal stromal tumor; heterologous differentiation of rhabdomyosarcomatous, osteosarcomatous, and leiomyosarcomatous elements might also be present.[97] Cytogenetically, supernumerary ring chromosomes and/or giant rod-shaped marker chromosomes composed at least in part of chromosome 12 material are characteristically observed in both ALT/WDL and dedifferentiated liposarcoma.[98] FISH and cytogenomic profiling studies have demonstrated that the ring/marker chromosomes in both histopathologic subtypes contain amplified 12q13–15 material, including the *MDM2* gene that is considered the primary driver gene of the 12q amplicon. Dedifferentiated liposarcoma differs by the acquisition of complex secondary chromosomal changes representing co-amplifications of other regions/genes such as 1p32 (*JUN*), 6q23 (*ASK1*), and 6q25 (*MAP3K7IP2*).[25,28–30] For clinical purposes, molecular demonstration of *MDM2* (+*CDK4*) amplification is recommended when the diagnosis of ALT/WDL or dedifferentiated liposarcoma is not possible based on clinicohistopathologic information alone.

CD34 immunoreactivity is useful in narrowing the differential diagnosis of dermatofibrosarcoma protuberans. Importantly, however, loss of this immunophenotypic marker occurs frequently in DFSPs containing areas indistinguishable from fibrosarcoma or undifferentiated pleomorphic sarcoma.[99] Another potential diagnostic complication is that variable sampling may lead to representation of the

transformed element exclusively or not at all in the examined material because these areas may occupy nearly the entire tumor or may occupy only small foci. Patients with this DFSP variant, termed 'fibrosarcomatous DFSP', are at risk for metastatic disease.[100,101] Identification of the characteristic fusion gene that is maintained in the high-grade component, *COL1A1–PDGFB*, may be required for definitive diagnosis. A recent observation of fibrosarcomatous DFSP arising in the deep soft-tissue of the thorax suggests that it may be a worthwhile exercise to search for the DFSP-associated *COL1A1–PDGFB* in fibrosacoma-like tumors irrespective of their location.[102] Notably, cytogenetic/molecular studies are also required to predict the clinical response to imatinib mesylate (PDGF receptor tyrosine kinase antagonist), an agent that may be employed in cases of local advanced or metastatic disease when surgery is insufficient.[103,104]

Conclusion

Soft-tissue tumors form a diverse and complex group that shows a wide range of differentiation. Morphologic assessment of a soft-tissue tumor is frequently challenging and can be complicated when the expected range of immunohistochemical markers or ultrastructural aspects are absent. The identification of mesenchymal tumor-associated gene fusions corresponding to chromosomal rearrangements, genomic imbalances to include recurring patterns of loss and/or gain of specific chromosomal regions or gene loci, and activating or inactivating mutations of select oncogenes or tumor suppressor genes have contributed significantly to a comprehensive classification of soft-tissue tumors based on clinicopathologic and genomic abnormalities. Embracing the use of the various molecular methodologies with their differing strengths and weaknesses in the formulation of a diagnosis improves accuracy considerably as well as provides or predicts key features of tumor behavior such as progression and response to therapeutics.

Of final note, although genetic profiling of soft-tissue tumors has revealed an impressive number of associated abnormalities to date, the progress of molecular pathology in this arena is expanding at a rate faster than ever before. Sophisticated technological advances in the sequencing of cancer genomes together with developing bioinformatic models are revealing new alterations that are central to sarcomagenesis and promise an exciting future of refined personalized care of patients with soft-tissue tumors.

Disclosure/conflict of interest

The author declares no conflict of interest.

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