The Current State of Molecular Pathology in Diagnosing Sarcomas

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INTRODUCTION
Soft tissue sarcomas (STS) and bone sarcomas are rare malignancies of mesenchymal origin that account for less than 1% of all adult solid tumors.1-3 Sarcomas provide a particular diagnostic dilemma, not only due to their rarity, but also due to their wide diversity, with greater than 50 histological subtypes currently recognized.4,5 This heterogeneity in classification is accompanied by a broad spectrum of biologic behavior, from locally aggressive but non-metastasizing tumors (e.g. desmoid fibromatosis) to tumors that metastasize infrequently or behave relatively indolently in the metastatic state (e.g. solitary fibrous tumor and alveolar soft part sarcoma, respectively), to those which are highly aggressive and rapidly metastasizing (e.g. synovial sarcoma or Ewing sarcoma). While many tumors show differentiation towards specific mesenchymal phenotypes, as in the case of liposarcoma, leiomyosarcoma, or rhabdomyosarcoma, up to 25% of tumors are unclassifiable and do not resemble normal tissues.4 Moreover, benign soft tissue neoplasms are much more frequent than malignant tumors, probably at least a 100-fold more common, and may be confused with sarcomas in some situations.

Pathology is the medical specialty that provides the underlying scientific foundation for the practice of medicine. A pathologist is a physician who specializes in the diagnosis and characterization of disease by scientifically-based laboratory methods. Pathology is typically subdivided into clinical and anatomic pathology. On the one hand, clinical pathology handles all fluid tests and bloodwork, including diagnostic testing and blood banking, as well as microbiological studies, while on the other, anatomic pathology handles tissue-based tests, including cytologic aspirates, biopsies, and surgical resection specimens. It is the pathologist who is ultimately responsible for rendering a diagnosis based on the available tissue.

Because the anatomic pathologist's analysis of tissue from a patient's tumor is absolutely critical in sarcoma treatment, sarcoma pathologists are specially trained in the diagnosis of these rare tumors. Correct identification of the specific sarcoma subtype is important because treatment protocols differ for various subtypes of sarcoma.

The pathologist's report contains the diagnosis (the identification of the particular subtype of sarcoma), as well as information about the size, shape, and appearance of a specimen, and information about the completeness of resection for surgical specimens.
Molecular pathology is a growing field within pathology, which uses the genetic alterations identified within specific tumor types to provide additional diagnostic, prognostic and therapeutic information. Pathologists often practice as consultant physicians who develop and apply their knowledge of tissue and laboratory analyses to assist in the diagnosis and treatment of patients. As physicians and scientists, pathologists specializing in sarcoma employ clinical studies, models of disease, and basic science research to advance the understanding and treatment of sarcoma.

A correct diagnosis is critical in separating benign tumors from malignancies, for predicting the behavioral patterns of malignant tumors, and for determining appropriate therapeutic options. Unfortunately, because of the morphologic similarities between some benign and malignant tumors, and between different subtypes of sarcomas, diagnosis may prove challenging.

How a Pathologist Makes a Diagnosis and Characterizes a Tumor: Tissue specimens are usually received in the pathology laboratory as small needle core biopsies, or as large resection specimens resulting from a surgical procedure. The first step in diagnosis is gross examination of the specimen and/or correlation with radiographic imaging and clinical findings. Diagnostic information may be gleaned from the color, consistency and growth patterns of the gross tumor specimen. After the tumor is sectioned (cut into slices), tissue sections (smaller slivers of tissue) are fixed in formalin and embedded in paraffin blocks. Thin (4-5 micrometer) slices are then cut and put onto glass slides. These sections are normally stained with hematoxylin and eosin (H&E) in order to visualize the tissue architecture and cellular features. Pathologists are trained to recognize the different patterns of cell shape and growth pattern, staining characteristics, and extracellular connective tissue production characteristic of specific diseases and tumors.

For a variety of reasons, including the increased use of small biopsies for diagnosis, as well as the overlap in appearances of many tumor types, definitive diagnoses cannot always be made on H&E stained sections alone. In these instances, a pathologist will determine the most likely diseases the lesion could be (the differential diagnosis). Ancillary testing is then used to confirm the most likely diagnosis and exclude other potential mimics. The first line of these ancillary tests are immunohistochemical stains, in which antibodies are used to probe tissue sections for expression of specific proteins. Often a panel must be used, which is specifically tailored to rule in or out entities in the differential diagnosis.

Molecular alterations are being increasingly used for diagnostic purposes as well. Because no one diagnostic technique is entirely specific, all findings are interpreted in both the context of the morphologic features of the tumor on H&E together with the clinical setting. In most cases, the combination of data from all available sources is enough to make a definitive diagnosis. However, on occasion, tumors which are poorly differentiated (showing no specific features of any tissue or tumor type), may not stain for immunohistochemical markers or have specific molecular alterations. In cases such as these, a diagnosis of unclassified sarcoma or tumor may be made.

Morphologically, sarcomas may be divided up into broad subcategories of spindle-cell tumors, small round cell tumors, pleomorphic tumors, and epithelioid tumors. Within each category, there are varying degrees of morphologic and clinical overlap, to the extent that, for example, the differential diagnosis of a small round cell tumor may include Ewing sarcoma, alveolar rhabdomyosarcoma, desmoplastic small round cell tumor (DSRCT), lymphoma, some carcinomas, or even variants of synovial sarcoma or extraskeletal myxoid
chondrosarcoma, among others. In such instances, the first line tool of diagnosis is immunohistochemical stains.\textsuperscript{6,7} However, there may be considerable overlap in expression of these markers – for example, CD99 (Mic2), commonly used as a marker of Ewing sarcoma, may also be positive in lymphoma, synovial sarcoma, DSRCT and rhabdomyosarcoma, while desmin, a common marker of muscle differentiation, is seen in both rhabdomyosarcoma and DSRCT. Moreover, misinterpretation of stains may also pose difficulty, and may be due to nonspecific staining patterns, or staining of admixed non-neoplastic cells such as intratumoral dendritic cells or entrapped, degenerated skeletal muscle fibers. In fact, the difficulty of accurately diagnosing sarcoma is a well-known problem, and one recent study reported that almost 25% of cases had a significantly discordant diagnosis between referring institutions and a sarcoma referral center; in over half of these instances (16% of total cases) this discordance was clinically significant, impacting therapeutic decisions or prognosis.\textsuperscript{8} Older studies report an even worse 61% concordance rate on diagnosis of histologic type.\textsuperscript{9} These challenges are compounded by the clinical trends of smaller core needle biopsies being used for initial diagnosis, which may cause difficulties due to morphologic heterogeneity within a tumor, whereby malignant tumors may mimic benign tumors, other sarcomas with different clinical behavior, or even non-sarcomas (e.g., carcinoma or melanoma).

Fortunately, molecular alterations within tumors are being increasingly recognized and used as specific diagnostic markers. In fact, between 20-30% of sarcomas are currently estimated to harbor specific chromosomal abnormalities, which may assist in diagnosis and offer potential targets for future therapies.\textsuperscript{10,11} This review will provide an update on known molecular alterations in benign and malignant soft tissue mesenchymal tumors, with emphasis on those with diagnostic utility, and discuss the techniques used for molecular testing. This review will focus predominately on diagnostic techniques for sarcomas and locally aggressive neoplasms such as desmoid-type fibromatosis. However, known alterations in benign tumors will be briefly discussed, as these tumors may enter the differential diagnosis of more aggressive tumors. Thus broad familiarity with specific alterations in all mesenchymal tumors may therefore prove diagnostically relevant.

\textbf{Clinical and Research Molecular Pathology:} At the current time, many of the techniques discussed in this review, including GEA and CGH and next generation sequencing, are primarily research tools with only limited clinical applications (mostly outside of the sarcoma field). Other techniques, such as conventional karyotyping, are standard at most large institutions within the US. The use of FISH and RT-PCR for sarcoma diagnosis varies widely by institutions, due to the rarity of these tumors. Focused single nucleotide assays for demonstrating “hot spot” point mutations (such as Sequenom® and SNaPshot®) have come online in some clinically approved labs for testing of certain carcinomas and are applicable for some sarcomas, though presently, their use is not widespread but is growing. The purpose of this review is to introduce the reader to clinical and research techniques and the general scenarios in which they may be useful.
MOLECULAR ALTERATIONS IN SARCOMAS

Sarcomas may be roughly categorized by having one of three types of molecular alterations:

1. a relatively simple karyotype with a defining translocation or amplification of a particular locus
2. a simple or complex karyotype with a specific oncogenic mutation
3. a complex karyotype with multiple chromosomal rearrangements, duplications, and deletions

Approximately 20% of sarcomas belong to the first category, and it is in this group in which molecular diagnostics first proved to be useful. The category of sarcomas with simple karyotypes includes Ewing sarcoma, myxoid liposarcoma, dermatofibrosarcoma protuberans (DFSP), well-differentiated liposarcoma, and synovial sarcoma, among others. Tumors in which specific mutations drive sarcomagenesis include gastrointestinal stromal tumor (GIST) with mutations in KIT or PDGFRB being required for tumorigenesis, and desmoid type fibromatosis, which usually contain mutations in the CTNNB1 gene locus encoding beta-catenin. The third class comprises the majority of sarcomas and includes leiomyosarcoma, undifferentiated pleomorphic sarcoma (UPS)/malignant fibrous histiocytoma (MFH), and angiosarcoma.

1. SARCOMAS WITH RELATIVELY SIMPLE KARYOTYPES

Many sarcomas with simple karyotypes have been found to be associated with specific chromosomal translocations (Figure 1).

These translocations result in the fusion of portions of two different genes into a chimeric or fusion gene. There are several different fusion types currently recognized in sarcoma:

- those resulting in chimeric transcriptional regulators
- those resulting in overexpression of a growth factor
- those which alter signaling by changing cellular localization or activity of a signaling protein

It is thought that, in order for specific recurrent translocations to occur, a number of criteria must be met. At a basic level, this means that involved loci must be in physical proximity, and DNA breaks must be present. It has been suggested that some sequences are more prone to breakage than others, and in part this may depend on cellular stress (including genotoxic stress, oxidative stress, and replication stress) and transcriptional activity. Proximity of gene loci may be influenced by coordinated transcriptional regulation which brings specific portions of chromosomes into one regulatory unit. Taken together,
these findings imply that the frequent identification of specific fusion partners in sarcoma may not be an entirely random event, but is influenced by both cell type and prior transcriptional and replication activity within pre-neoplastic cells.

**Figure 1. Translocation.** Chromosomal translocation occurs when a portion of one (or more) chromosomes is transferred to a different chromosome. Translocations in sarcoma are usually balanced, that is two chromosomes each swap a portion of their DNA for that of the other chromosome, and no material is lost or gained (although, sometimes one derivative may be lost after the fusion process). This exchange creates derivative chromosomes consisting of the fused portions of the involved native chromosomes. The example illustrated here depicts the chromosomal translocation seen in DFSP between chromosome 17 and 22. It is thought that translocation occurs as a result of DNA damage or possibly to errors in normal recombination.

**TRANSLOCATIONS RESULTING IN NOVEL CHIMERIC TRANSCRIPTION FACTORS**

The archetype for this class of translocation is Ewing sarcoma, in which the EWSR1-FLI1 fusion product combines the transcriptional regulatory domain of EWSR1 with the DNA-binding domain of FLI1 to create a more potently active transcription factor which may alter expression of genes involved in growth or differentiation. Of note, almost half of the oncogenic fusion genes identified in sarcoma belong to the “FET” gene family of transcriptional regulators, which includes FUS/TLS, EWSR1, and TAF1168. In addition to Ewing sarcoma, FET fusion genes are also seen in DSRCT, clear cell sarcoma, angiomatoid fibrous histiocytoma, extraskeletal myxoid chondrosarcoma, myxoid liposarcoma, and myoepithelial tumors, among others.

**TRANSLOCATIONS RESULTING IN INCREASED GENE EXPRESSION**

The prototypical example of this translocation type is the fusion of the COL1A1 promoter with the PDGFB coding sequence in dermatofibrosarcoma protuberans (DFSP), which leads to expression of the growth factor PDGF-β at the constant, constitutive level of collagen, thus allowing neoplastic cells to grow in an unregulated fashion. Similar events may occur in more benign mesenchymal tumors such as tenosynovial giant cell tumor with colony stimulating factor under control of the COL6A3 collagen promoter, or nodular fasciitis with
USP6 (encoding a factor involved in growth factor signaling) under the control of myosin heavy chain promoter.25

**TRANSLOCATIONS ALTERING SIGNALING CHARACTERISTICS OF FUSION PARTNERS**

This category is exemplified by inflammatory myofibroblastic tumor, in which the chimeric transgene encodes a functional ALK receptor tyrosine kinase fused to an intracellular or intranuclear protein, thus altering the subcellular localization of ALK from the cell membrane and allowing unregulated receptor dimerization with subsequent constitutive activation of proliferation and survival pathways.26-28

It has been postulated that the frequency of translocations identified within tumors increases with the number of cases investigated.29 This finding certainly holds true in sarcoma, and the past few years have seen an explosion of newly identified translocations (Table 1).2, 24, 30-42

Translocations have been identified in benign mesenchymal neoplasms such as nodular fasciitis,25 and aneurysmal bone cyst,36, 43 intermediate malignancies such as epithelioid hemangioendothelioma,32 and in sarcomas such as mesenchymal chondrosarcoma40 and EWSR1-negative undifferentiated small round blue cell tumors,38 with these latter three being very recent discoveries. With each new addition to these ranks, the potential power of molecular diagnosis is increased. One caveat, however, is that up to 10% or so (depending on the histologic subtype) of cases with morphologic features compatible with translocation sarcomas, are not found to have the characteristic translocation.35 While some of these negative findings may result from lack of sensitivity of the testing methods employed, another explanation may be the presence of rare, alternate fusion partners or variant translocations yet to be identified. For instance, in Ewing sarcoma, approximately 85% of cases are found to have the canonical EWSR1-FLI1 fusion, and 10% to have EWSR1-ERG. Of the remaining cases, more rare fusion partners include FEV, E1AF, and ETV1, which are in the same ETS family of transcription factors as FLI1 and ERG, and have each been reported in a handful of cases.44 In addition, variant translocations utilizing FUS as the fusion partner with ERG or FEV have been identified in <1% of cases. FUS is in the same family of FET transcriptional regulators as EWSR1;44 therefore, this translocation is consistent with a Ewing family tumor. Recently, a recurrent translocation apparently unrelated to the FET family has been described in 68% of EWSR1-negative primitive small round blue cell tumors, involving the CIC and DUX4 gene loci.38 It is not certain if these cases represent Ewing sarcoma with variant translocation, or a different entity entirely. Thus, the true proportion of cases lacking a characteristic rearrangement and their biological significance compared to their translocation-positive counterparts is not
yet understood. Given the distinct characteristics of most of the translocation-associated sarcomas in terms of cellular differentiation and tumor biology, it seems plausible that cases lacking detectable translocations could harbor molecular derangements that activate the same downstream pathways through alternative means.

**CHROMOSOMAL AMPLIFICATION**

Another form of relatively simple chromosomal abnormality is amplification of a particular chromosomal locus. This finding is typified by well-differentiated liposarcoma / atypical lipomatous tumor and dedifferentiated liposarcoma, as well as by low grade intra-osseous and parosteal osteosarcoma. These sarcomas demonstrate an amplification of chromosome 12, in the region of q14~15, which contains a number of potentially oncogenic gene loci, including MDM2 and CDK4. On conventional karyotyping, this amplification is seen in the form of giant marker chromosomes or ring chromosomes composed exclusively of repeated material from chromosome 12 (Figure 2). Amplification results in overexpression of involved gene loci, and subsequent dysregulation of cellular proliferation.

**Figure 2. Chromosomal Amplification.** Chromosomal amplification occurs when a small portion of one chromosome is abnormally duplicated many times. The mechanisms for this amplification are not well understood, but may be due to dysregulated response to DNA damage. In sarcoma, the most frequent amplified region is chromosome 12q14-15, and the abnormally duplicated regions tend to occur as multiple repeats within giant marker chromosomes or ring chromosomes (arrow). (Image courtesy of Dr. L. Abruzzo)
DIAGNOSTIC AND PROGNOSTIC UTILITY OF TRANSLOCATIONS IN SARCOMA

In many cases, specific chromosomal translocations are considered to be unique to a particular subtype of sarcoma. For instance, FUS-DDIT3 translocation has only been reported in myxoid/round cell liposarcoma. Accordingly, positive molecular test for DDIT3 rearrangement is confirmatory of a diagnosis of myxoid/round cell liposarcoma. On the other hand, FUS is known to be rearranged in a number of other sarcomas, and therefore rearrangement of this locus is not specific to myxoid liposarcoma. In other instances, identical translocations may be shared by different sarcomas and even carcinomas, including the EWSR1-ATF1 fusion encountered in both angiomatoid fibrous histiocytoma and clear cell sarcoma, as well as in hyalinizing clear cell carcinoma of the salivary gland. Likewise, ASPSCR1-TFE3 characterizes alveolar soft part sarcoma and translocation-associated renal cell carcinoma, while ETV6-NTRK3 is found in congenital/infantile fibrosarcoma, congenital mesoblastic nephroma and secretory breast carcinoma. Moreover, identical translocations can be seen in both sarcoma and leukemia, as with the FUS-ERG fusion which is rarely found in Ewing sarcoma and approximately 1% of all acute myeloid leukemias. Thus, the same fusion events can be seen in mesenchymal, epithelial and lymphoid neoplasms.

Because of the shared nature of some translocations or translocation partners, and the fact that these may be seen in tumors of different lineages with radically different treatment modalities and outcomes, molecular tests for chromosomal rearrangements cannot be used as a standalone diagnostic test, but must be integrated with clinical, morphologic and immunohistochemical findings.

In addition to being helpful in diagnosis, specific translocations are being investigated as prognostic or therapeutic markers. Earlier studies reported better outcomes for PAX7-FOXO1A fusion compared to PAX3-FOXO1A in alveolar rhabdomyosarcoma. EWSR1-FLI1 in Ewing sarcoma and SS18-SSX1 in synovial sarcoma. However, more recent studies have not found such an association, possibly due to improved therapeutic regimes in the case of Ewing sarcoma, and confounding factors such as histologic type, grade and staging in synovial sarcoma. Nevertheless, in alveolar rhabdomyosarcoma, translocation-negative tumors are known to behave similarly to the embryonal subtype of rhabdomyosarcoma, and have better outcomes than translocation positive tumors, despite histologic appearances. Thus, it appears that differences between fusion variants may be minimal, given the conflicting study results, while absence or presence of any translocation may be more helpful for prognosis.

2. SARCOMAS WITH SPECIFIC ONCOGENIC MUTATIONS

Only a few mesenchymal tumors have been identified in which distinct mutations both drive tumorigenesis and are of diagnostic utility (Table 2).
As mentioned above, these include GIST, in which activating mutations in the KIT growth factor receptor are found in about 85% of cases, or in PDGFRA in an additional 10%, and sporadic desmoid–type fibromatosis, a locally aggressive malignancy in which proliferation of myofibroblastic cells is driven by mutations in CTNNB1 that stabilize the protein and allow constitutive activation in about 85% of cases. Desmoid fibromatosis may also be associated with familial adenomatous polyposis (FAP), which is caused by inactivating germline mutations in APC, a negative regulator of beta-catenin. In these patients, APC mutations 3’ of codon 1444 have been reported to predict desmoid development in extra-abdominal locations. The ability to detect mutational status in fibromatosis is especially important when assessing recurrent tumor, and even margin status, as a reactive fibrous proliferation such as scar may be histologically and immunohistochemically indistinguishable from tumor. However, if a primary tumor is known to harbor CTNNB1 mutation, then recurrence may be verified by tests for an identical mutation – which normal, reactive tissue would not have. Nuclear β-catenin expression may also be identified by immunohistochemical staining, and a positive stain is suggestive of the presence of fibromatosis, however, nuclear β-catenin may very focal or equivocal in some cases, and may also be seen to some degree in activated myofibroblasts in reactive scar tissue. Thus, while immunohistochemistry can have utility, mutational analysis, if positive, can be more definitive. Mutation status may also carry prognostic significance, as in the case of GIST, where certain mutations in KIT respond better to imatinib therapy, while in fibromatosis, some evidence suggests that certain mutations carry a higher risk for local recurrence. Thus, tests for these mutations may convey both diagnostic and prognostic information. For more information on the role of mutational status in GIST therapy and prognosis see Prognosis in GIST.

### 3. Sarcomas with Complex Karyotypes

The majority of sarcomas are composed of high grade spindle or pleomorphic cells, as typified by leiomyosarcoma and UPS/MFH. These tumors demonstrate few consistent cytogenetic abnormalities. Rather, they are remarkable for their highly complex genomes. Complex karyotype sarcomas are characterized by genomic instability with multiple chromosomal duplications, deletions and complex rearrangements, often with production of marker chromosomes made up of genetic material from multiple, indeterminate chromosomes (Figure 3).
Figure 3. Complex Cytogenetic Alterations. Complex cytogenetic alterations include losses and gains of multiple chromosomes, as well as complex, non-balanced translocations, deletions and duplications of portions of chromosomes. These are thought to arise from failure of DNA damage repair mechanisms as well as from errors in cell division. This karyotype shows a hypertriploid clone, with additional complex gains and losses and multiple translocations including, among others, 6;15, X;17, and 2;12. (Image courtesy of Dr. L. Abruzzo)

It is postulated that this genomic instability may be due to loss of telomeres, which allows repeated chromosome breakage and fusion during mitosis (Figure 4) and has also been associated with P53 mutation, which is frequently seen in karyotypically complex sarcomas.

A number of additional mutations in oncogenes such as RAS or tumor suppressor genes including RB1 may be present, but do not have diagnostic utility. In such cases, diagnosis is typically made on a morphologic, clinical and immunohistochemical basis. Unfortunately, in a subset of cases, tumors do not demonstrate significant expression of markers of differentiation commonly assessed by immunohistochemistry, and are therefore grouped into the UPS/MFH classification. Recent studies using high throughput molecular techniques such as comparative genomic hybridization (CGH) and gene expression arrays (GEA) (discussed below) have shown that, in fact, many of these UPS cases can be reclassified as pleomorphic versions of known sarcoma types. Others believe that at least a subset of UPS arise de novo from mesenchymal stem cells arrested at an pre- or undifferentiated stage rather than devolving from a more differentiated sarcoma. Techniques to better diagnose these tumors and predict response to therapy are still being investigated.
Figure 4. Telomeres and genomic instability. Telomeres represent the end regions of chromosomes, and are composed of extended repeats of the nucleotide sequence TTAGGG. These repeats, together with associated DNA-binding proteins, form a protective cap for the chromosome. This cap prevents DNA-repair machinery from mistaking the chromosome end as damaged (broken) DNA. Telomeres shorten successively during cell division, unless replaced by the action of telomerase enzyme. They may also break spontaneously, especially in conditions of cellular stress. In normal cells, shortening of telomeres eventually signals a cell that it may no longer divide, causing the cell to enter a terminal stage of cellular senescence. In cancer, due to dysregulation of cell division, short telomeres do not induce senescence and tumor cells continue to proliferate. The lack of a protective cap on the chromosome allows DNA sticky ends to adhere to other chromosomes or to a sister chromatid during mitosis. The inter- or intra-chromosomal bridges thus formed, then break, transferring a portion of one chromosome or chromatid to the other. This cycle may then repeat until translocation caps the broken end with a telomere from another chromosome.

THE MOLECULAR DIAGNOSTIC TOOLBOX

Pathologists and clinicians have an ever-expanding array of molecular tests at their disposal for use in the diagnosis and prognosis of sarcoma. These range from the tried-and-true method of conventional karyotype analysis to, increasingly, such cutting edge techniques as multiplex single-nucleotide primer extension analysis and deep exome sequencing, as well as research technologies that are currently moving toward clinical applications such as CGH and GEA, which have been previously discussed in “Gene Profiling Studies on Sarcomas” and “Gene Profiling: Unlocking the Inner Workings of Sarcoma Cells.”

At the present time, in most institutions, the use of these techniques in clinical diagnosis is limited to conventional cytogenetics, FISH, and PCR (including RT- and Q-PCR). Focused single-nucleotide multiplexed assays based on mass spectrometry or other detection methods are coming online. At some
institutions, these technologies already form part of the clinical testing repertoire for diagnostic and prognostic purposes and are applicable for some mutations in sarcoma as well. Array technologies, including CGH, CEA and next generation sequencing, remain expensive and unwieldy for routine clinical usage in many situations. Nevertheless, they are included in the discussion due to their current value as research tools and their emerging value in clinical diagnostic and prognostic studies.

CONVENTIONAL CYTOGENETICS: THE HISTORICAL GOLD STANDARD FOR SIMPLE CHROMOSOMAL REARRANGEMENTS

Classic cytogenetics and karyotype analysis has long been the gold standard for detection of translocations, as all chromosomes may be visualized.

**Karyotyping** is a method of evaluating chromosome number (ploidy) and structure. Fresh tumor cells are taken at the time of biopsy or resection and grown in culture. Cells are harvested in mid-mitosis (metaphase), when the chromosomes are in their condensed form prior to cell division. In order to visualize differences between chromosomes, an enzymatic digestion is employed followed by staining (most frequently with Giemsa stain). The digestion and staining result in a pattern of light and dark bands (G-bands) which are unique to each chromosome pair and therefore allow identification of number of chromosomes as well as translocations between different chromosomes.

However, the technique does have a number of important limitations. Karyotype analysis requires fresh tissue to grow, as chromosomes can only be analyzed during the metaphase to early pro-phase stage of mitosis when they are condensed. In addition, skilled interpretation is needed, as alterations may be subtle. Conventional cytogenetics has a high failure rate, due in part to the difficulty of culturing tumor cells. Often there is no growth of tumor cells, or overgrowth of normal tissue cells such as fibroblasts. In addition, the type of chromosomal rearrangement determines the ability of karyotype analysis to detect an alteration. Conventional karyotypic banding is at best able to detect genomic alterations on a 1.5 megabase scale (2000-band resolution), and generally, the average resolution is around 7-10 megabases (300 band resolution). Cryptic translocations and insertions in which a small portion of one chromosome are inserted into another, and single gene deletions may be impossible to detect. Complex translocations involving multiple chromosomes may also create difficulties in determining which specific regions of the chromosomes of interest are rearranged. Finally, complex karyotypes, in which there are multiple copies of many chromosomes, as well as unrecognizable "marker" chromosomes which cannot be correlated with known normal chromosomes, may be difficult to interpret.

**SPECTRAL KARYOTYPING**

Spectral karyotyping (SKY) is another method that may be used to more precisely identify the components of complex karyotypes. In this technique and others using similar principles, fluorescent tagged, chromosome-specific probes are used to “paint” each
chromosomal pair in a unique color with appropriate pseudocolor assignment. Thus, insertions and complex rearrangements that may be too subtle to detect or identify the source chromosome of on conventional karyotype analysis can be more precisely determined. In practice, SKY is rarely used as the technique is technically difficult and interpretation may be time consuming. Moreover, in complex karyotypes, multiple rearrangements cause difficulty with interpretation and breakpoints cannot always be identified. However, application of SKY or other chromogenically enhanced karyotyping technique can suggest alternations that can then be confirmed by other techniques such as Fluorescence In Situ Hybridization (FISH).

**FLUORESCENCE IN SITU HYBRIDIZATION (FISH)**

Because of the requirement for fresh tissue in karyotyping, and the labor and skill-intensive nature of the technique, as well as the poor growth of some sarcoma types in culture, it has been variably to infrequently utilized for sarcoma diagnosis outside of specialized centers. Even in this latter context, alternative means applicable to fixed tissue samples are becoming the predominant modes of genetic assessment. Fortunately, other more rapid detection methods for chromosomal rearrangements have become available. One of the most popular techniques is fluorescence in situ hybridization (FISH). The biggest advantage of FISH is that it may be performed on standard formalin-fixed paraffin-embedded sections, frozen tissue sections, or cytology specimens and smear preparations. While metaphase chromosomes are not required, FISH can be utilized on the metaphase spreads utilized for traditional karyotype analysis to better define breakpoint region.75 Additionally, for FISH performed on tissue sections, it is possible to more clearly determine areas of tumor, and specifically examine nuclei in these areas, whereas in conventional cytogenetics, this information is lost during the tissue culture step.

There are three basic types of probe sets used in FISH: break-apart, fusion, and locus/centromeric probe sets.76 Break-apart probes are those in which DNA probes bind to opposite ends of one gene or chromosomal region. One probe is labeled with a red/orange fluorescent chromophore, while the other is labeled in green. In an intact (normal) chromosome, the signals are close together and the resulting overlapping signal appears as a yellow dot due to spectral overlap of the colocalizing probes or as closely juxtaposed red and green signals. In a rearranged locus, one signal remains on the parent chromosome, while the second signal has been translocated to a distant location, usually on a different chromosome, but occasionally on the same chromosome. In this abnormal situation, the red and green signals are separated from each other and are visualized as distinct separate dots (Figure 5).
This method is especially useful for screening for the more common translocation partners known to be involved in sarcomagenesis. For example, in the case of synovial sarcoma, SS18 (SYT) located on chromosome 18, may recombine with SSX1, SSX2, or rarely, SSX4 on the X chromosome. Performing FISH for each of the SSX family members would not be efficient or cost-effective; however, as SS18 is the translocation partner in all instances, a positive FISH result for rearrangement of SS18 is diagnostic of synovial sarcoma. The limitation of the break-apart probe is that no information is gleaned regarding the second locus involved in the rearrangement, which may have additional diagnostic information.

The second type of common FISH probe is the fusion probe. This probe requires foreknowledge of what both translocation partners are expected to be; for example, in desmoplastic round cell tumor, probes corresponding to EWSR1 and WT1 could be used. As with break-apart probes, usually one red and one green chromophore are used, with one corresponding to the 5’ (upstream) fusion partner and the other corresponding to the 3’ (downstream) partner. A positive result consists of 3 signals, a fused yellow signal, as well as one red and one green signal from each of the uninvolved alleles. A negative result is 4 split signals (2 each red and green). Chromosomal aneuploidy can complicate these results due to changes in chromosomal copy number. This test is more specific than the break-apart probe, but is not as sensitive, as a negative result does not preclude the presence of a different fusion partner. In clinical practice, fusion probes are seldom used in sarcoma.

A third type of FISH technique can be used to quantify amplification or deletion of a gene locus on a specific chromosome. Here one probe corresponds to the gene locus in question,
while a second probe labels the centromere of the chromosome the gene locus is on. For instance, in well-differentiated liposarcoma, one probe targets the 12q14–15 region, which contains multiple gene loci found amplified in well-differentiated liposarcoma, including MDM2 and CDK4, among others, while the second probe recognizes the centromere of chromosome 12. In a normal cell, the ratio between target and centromere probes is 1-2:1 (depending if the cell is undergoing DNA replication and mitosis). In the amplified state, the ratio is greatly increased. In sarcoma, this test is mainly used to identify the presence of high level 12q15 amplification characteristic of well-differentiated liposarcoma/atypical lipomatous tumor and dedifferentiated liposarcoma, (Figure 6) although this probe may also detect low level duplication as well as aneuploidy which can be distinguished by comparison with centromere 12 probe enumeration.

Another diagnostic situation in which similar probe is useful is in the detection of high level amplification of MYC (8q24), which is seen in radiation-induced angiosarcoma, but not in radiation-induced atypical vascular proliferations, or primary (de novo) angiosarcoma and may therefore be used to distinguish between them.39,78

Some limitations of FISH include the requirement for adequate tissue fixation, as well as the frequent failure in decalcified tissue due to extensive DNA damage. Moreover, with the more commonly used break-apart probes, only one of the possible fusion partners is identified. Thus, the test may not be specific to one particular sarcoma- as is the case with EWSR1 rearrangements, which may be seen in a number of sarcoma types (Figure 7).13
EWSR1 and FUS are among the most commonly rearranged loci in sarcoma, and may partner with a variety of fusion partners. These chimeric genes may be characteristic of a specific type of sarcoma, or may be seen in several mesenchymal, epithelial, or lymphoid tumors. The diverse nature of these rearrangements complicates the use of both FISH and RT-PCR based techniques in diagnoses, as the former is inadequately specific, and the latter, insufficiently sensitive for full diagnostic accuracy.

Thus, as discussed earlier, FISH studies must always be interpreted within the context of histologic features, clinical information and immunohistochemical findings to avoid pitfalls and yield the correct diagnosis. Furthermore, in some cases, the specific breakpoints of each gene locus may be important for therapy or prognosis, and this information is difficult to resolve by FISH.

Another caveat of FISH performed on paraffin sections is that the results are non-quantitative—that is, one cannot accurately quantify the number of copies of a particular locus in a single nucleus, due to sectioning artifact, though signal quantification of 100 to 200 or more nuclei provides a statistical remedy that avoids misinterpretation possible with more limited sampling. FISH may also be performed on disaggregated nuclei from thick tissue sections, however, this method results in a loss of tissue architecture and risks contamination by normal cells (false negative results) if sections are not carefully chosen. An advantage of this technique, however, is that intact nuclei are scored, which allows for quantitation of copy number when relevant. For instance some loci may be considered amplified at low level when the ratio of probe signals for the gene locus to centromeric signals is >2.2, but high level amplification requires a ratio of >2.5 (and in practice, 12q15 amplification in liposarcoma is usually at least 10 to 25-fold). Differences between low and high level amplification can have diagnostic, prognostic or therapeutic significance. In practice, however, these assessments are readily performed in standard (4-5 um) sections.

**Polymerase-Chain Reaction-Based Methods**

PCR-based methods offer an alternative to FISH and conventional karyotyping. PCR may be performed from genomic DNA or from mRNA transcripts (reverse transcriptase, (RT-PCR)). PCR is a flexible technique that may be used in a variety of assays to detect specific
translocations, identify chromosomal breakpoints, as well to assess for specific point mutations, deletions, and even epigenetic silencing of genes via promoter methylation. Moreover, PCR-based techniques are amenable to multiplexed reactions, in which multiple assays for different genetic alterations may be performed simultaneously in the same reaction.

One benefit of PCR-based detection methods is that PCR amplifies target sequences, allowing for improved detection when very little DNA or RNA is present in the sample to be assessed. Thus even if only a very small percentage of tumor cells are present in a sample, PCR can allow for detection of this tumor DNA (Figure 8).

![Figure 8. Polymerase Chain Reaction.](image)

Figure 8. Polymerase Chain Reaction. PCR is a technique for amplifying specific DNA sequences utilizing thermal cycling and DNA Polymerase enzyme. Short primers are designed which correspond to sequences at opposite ends of complementary DNA strands framing the region of interest. Heating causes double stranded DNA to melt into single strands. Upon cooling, primers bind to template sequences. Beginning at the primer, the polymerase synthesizes DNA complementary to the template. After one cycle of PCR, the initial double stranded template has been replicated into 2 pieces of double stranded DNA. After 2 cycles, there are 4 strands, etc, thereby resulting in doubling of product with each additional cycle.

However, because of this sensitivity, care must be exercised to prevent cross- and carry-over contamination from other specimens being analyzed. In order to enrich tumor cells in a sample, clinical labs can microdissect paraffin sections to remove non-neoplastic tissue or necrotic or hemorrhagic area. Microdissection may improve PCR yields particularly when tumor comprises <10% of the tissue, or when extensive hemorrhage is present on the section, as heme degradation products may interfere with the PCR reaction. However, in most cases, especially in when tumor comprises >80% of the tissue, microdissection is not required. Also tumor enrichment through microdissection is not really necessary when
examining for fusion transcripts such as EWSR1-FLI1 by RT-PCR since this event does not occur and thus is not detected does in non-tumor issue. When looking for mutations in genomic DNA, tumor purity is more critical as usually only one of the two gene alleles is mutated and thus any inclusion of additional non-tumor DNA can dilute the sample and make it harder to detect within the background of the non-mutated gene. Careful microdissection can alleviate this problem.

**DETECTION OF CHROMOSOMAL TRANSLOCATION VIA RT-PCR**

In chromosomal translocations resulting in functional fusion products, breakpoints usually occur within non-coding intronic sequences. Introns may be very long and the precise breakpoint may not be identical from one case to the next. However, the coding exons flanking the breakpoint are usually consistent. Thus RT-PCR on mRNA, which has undergone splicing, and therefore lacks introns, is an ideal detection method for fusion transcripts. Primers are chosen so that the resulting PCR product will straddle the region of the breakpoint, thus, one primer corresponds to one fusion partner, while the second corresponds to the other fusion partner. In sarcomas with multiple possible translocation partners, only the most common variants are usually assessed. For example, for Ewing sarcoma, RT-PCR for both EWSR1-FLI1 and EWSR1-ERG may be performed simultaneously, as together these translocations account for >95% of fusions in Ewing sarcoma, but less common fusion partners will not be tested.44

After amplification, PCR products can be subjected to Sanger sequencing, and the results compared to a database of human genomic sequence to ensure that the amplicon was in fact specific for the expected fusion transcript.

One caveat in the use of RT-PCR is that RNA is often degraded in formalin-fixed paraffin embedded tissues, and care must be taken to choose primers that are predicted to be no more than 300 base pairs apart, and preferably, closer to 150 base pairs in the fusion transcript. In cases with poor RNA quality, there may not be enough intact transcripts to yield an amplicon. Moreover, a negative result with RT-PCR does not preclude a diagnosis of translocation sarcoma – it merely means the specific fusion product assayed for is not present; alternate fusion partners or variant breakpoints involving different exons may still be present. Thus, RT-PCR has the ability to be very specific, but is not necessarily a sensitive test in sarcomas with multiple possible fusion variations.
**FISH vs. RT-PCR for Translocation Identification – Which to Use?**

There are no absolute guidelines in clinical practice as to when to use FISH versus RT-PCR for demonstration of a characteristic translocation event. As discussed above, both techniques have certain strengths and weaknesses that govern their applications, and neither is 100% sensitive or specific. The turn-around times are theoretically similar, but RT-PCR is more sensitive for translocation in a dilute sample—for instance when assessing early relapse of Ewing sarcoma or alveolar rhabdomyosarcoma on bone marrow biopsy. It should be noted, however, that these techniques do not have proven clinical efficacy when disease cannot be demonstrated using conventional light microscopy perhaps supplemented with immunohistochemistry in sarcoma. FISH may be preferable on older archival material, whereas RT-PCR may fail due to poor RNA preservation. In primary diagnosis, choosing between FISH and RT-PCR may simply be a matter of convenience, or may depend on the differential diagnosis. For instance, FISH may be more useful as a confirmatory test for clinically classic Ewing sarcoma, due to the multiple possible EWSR1 fusion partners, but when attempting to differentiate Ewing sarcoma from desmoplastic small round cell tumor, RT-PCR for the most common fusion partners of both is more helpful. In other instances, such as extraskeletal myxoid chondrosarcoma, RT-PCR for NR4A3-EWSR1 is not widely available, but FISH for EWSR1 is, and thus, though not the most sensitive (since non-EWSR1 variants exist) or specific, this is the test that will be performed. Therefore, when ordering a molecular diagnostic test for sarcoma, it is important to take into account the purpose for which the test is being used, and to be aware that a negative result does not necessarily exclude a diagnosis. Finally, a negative result by FISH does not preclude a positive RT-PCR study, and vice-versa, and some labs may perform both tests if initial results are equivocal or negative and suspicion for a particular diagnosis is elevated.
**PCR for Mutational Analysis**

PCR may be also performed on genomic DNA in order to identify specific point mutations. Due to DNA fragmentation as well as to cost issues, it can be difficult to sequence an entire gene for all possible oncogenic mutations using conventional techniques. Accordingly, most diagnostic laboratories focus on specific known mutational hotspots. Primers are designed to flank a region where the most common “hotspot” mutations are located. After amplification, the resulting product is sequenced in order to assess for the presence of mutations. For instance, in fibromatosis, primers for a region in exon 3 of CTNNB1 flanking codons 41 and 45 may be used to detect mutations in either one of these two sites.

**Real Time PCR**

A newer technique is real-time (quantitative) PCR, which may be used to detect mutations in genomic DNA (Q-PCR) or translocations in cDNA reverse transcribed from mRNA (QRT-PCR), or to assess expression levels of certain gene transcripts (QRT-PCR). In this technique, PCR is usually performed using primers labeled with fluorescent chromophores and a suppressor molecule. Upon elongation by polymerase, the suppressor is cleaved from the end of the primer allowing the fluorescent signal to be seen. After each cycle, the PCR machine records the fluorescent intensity, thereby providing a quantitation of copy numbers of the tagged amplicon. Alternatively, sequence specific probes labeled with a chromophore and a quencher may be used rather than labeled primers, as in the Taqman® system. These probes are included in the Q-PCR reaction and are designed to anneal to the region amplified by PCR primers. As new DNA is synthesized from the annealed primers, the bound probe is degraded, releasing the chromophore from the linked quencher and allowing fluorescence. In this way, for instance, a point-mutation specific probe could be used in Q-PCR to differentiate between the amplification of normal vs. mutant sequence, thus obviating the need for post-amplification Sanger sequencing. Alternatively, in QRT-PCR, simultaneous reactions may be performed for alternate fusion products in the same sample, utilizing multiple chromophores, which could then preclude a need for confirmatory Sanger sequencing. In practice, however, many will choose to confirm all of their amplicons by sequencing. Real time PCR may be performed in duplex (or multiplex) with one primer set corresponding to the sequence of interest (for instance, the EWSR1-ERG fusion point) and a second primer set corresponding to a ubiquitously expressed housekeeper gene, utilizing a contrasting chromophore. In this way, the relative abundance of the product of interest can be measured compared to overall DNA content/quality. Quantitation of tumor transcripts may be important in assessing disease relapse (eg. Ewings or rhabdomyosarcoma on bone marrow biopsy), as well as in the research setting, where QRT-PCR may be used to measure gene expression of specific target genes in response to certain therapies to assess their effectiveness.

Compared to traditional PCR, real time PCR offers increased sensitivity, faster results, and the ability to quantitate the frequency of the sequence of interest, though the accuracy of
this depends on accurate assessment of the ratio of tumor to normal nuclei in the analyte sample.80

**MULTIPLEXED PCR-BASED METHODS**

Although not currently in use for the diagnosis of sarcoma, these methods are gaining widespread acceptance for the detection of activating oncogenic mutations which represent therapeutic targets or prognostic biomarkers. Currently, multiplexed PCR methods are used to screen tumors for a wide array of potentially therapeutically targetable mutations simultaneously, and clinical applications of this technology are expanding. As the role of point mutations in many sarcomas is poorly understood, the use of this technology in sarcoma diagnosis will depend upon further advances in the field. For instance, multiplexed techniques could someday be used to identify specific, targetable receptor tyrosine kinase mutations in high grade sarcomas, or to efficiently screen for resistance mutations in GIST.

One technique for examining point mutations in multiple different loci in a single reaction is the single nucleotide extension assay, of which the SNapshot® system is one such variant. In this method a primer/probe is designed which corresponds to the sequence immediately adjacent to a known mutational hotspot. Instead of standard PCR, which allows for multiple base extension, here the reaction uses fluorescently-labeled ddNTPS, which terminate the reaction after a single base is added. Capillary electrophoresis is then used to sort the products by size, while the fluorescently labeled nucleotide informs as to whether this locus is wild-type or mutated. The power of this assay is that, merely by using primers of different lengths which separate during the electrophoresis step, multiple different mutation sites may be tested simultaneously. Moreover, SNapshot®-type assays are able to detect mutations, even when tumor DNA comprises less than 10% of total DNA.81,82

The Sequenom® platform is another method for detecting single nucleotide polymorphisms or mutations. In this system, mass spectrometry is used to directly identify the nucleotides present, rather than relying on fluorescent labeling. Similarly to the SNapshot® system discussed above, a single-nucleotide primer extension assay is performed. The allele-specific extension fragments are then purified and analyzed by mass spectrometry.83 These techniques offer the ability to multiplex and look for many different mutations, but they are relatively focused and will only detect the very specific mutations they are designed to detect and are not ideal for detecting translocations or large deletions and insertions. Moreover, multiplex techniques can be limited in their ability to amplify multiple loci simultaneously, as optimal conditions for each reaction may vary too widely to be effective in the same reaction; this method is thus not applicable to all mutation sets of interest. Parallel amplifications in small aliquot chambers using a variety of available technology platforms can circumvent these concerns.
HIGH THROUGHPUT “NEXT GENERATION” SEQUENCING

Newer methods of sequencing are generally based on the idea of sequencing many small pieces of DNA in parallel. These methods are used for whole genome sequencing as well as exome sequencing. As the vast majority of genomic DNA is composed of non-coding sequences and introns whose roles in disease pathogenesis is still being investigated, most current studies focus on the known protein-coding sequences that comprise the exome. These coding sequences account for about 1% of the human genome, but are estimated to contain about 85% of the pathogenic mutations; thus sequencing the exome is more efficient and much cheaper than whole genome sequencing. Currently, next generation sequencing is primarily a research tool, however the relative cost saving compared to traditional sequencing methods means that many labs are in the process of validating these technologies for clinical molecular diagnostics. The techniques for these next generation sequencing platforms vary widely and are beyond the scope of this article. Technology hurdles remaining in this arena include ensuring that the fidelity of the sequencing results are appropriate for clinical use and the application of appropriate computational power and informatic expertise for robust interpretations of the voluminous data. In the near future, these technologies will probably be used for efficient, multiplexed determination of specific sets of relevant mutations rather than examination of the whole genome, but the latter will likely have increased clinical applicability as research into this area grows.

ARRAY BASED TECHNIQUES

Array based techniques include array comparative genomic hybridization (CGH) and gene expression analysis (GEA). Both technologies rely on immobilized probe sets consisting of thousands of oligonucleotides arrayed on a chip. These probes then hybridize to fluorescently-labeled nucleic acids in the analyte sample to provide quantitation of the copy numbers of DNA or mRNA in a particular sample. Both CGH and GEA are currently research tools that have been used primarily to elucidate the classification and pathogenesis of sarcoma, but may someday become part of the clinical repertoire of personalized medicine. They have current clinical applications in other tumor types.

COMPARATIVE GENOMIC HYBRIDIZATION

The basis for CGH is the theory that tumors of specific histologic subtype, overall, possess similar patterns of genetic copy number changes (gains and losses). These similarities are often difficult to identify in complex-karyotype sarcomas by conventional cytogenetics, due to the sheer complexity and low resolution of conventional G-band karyotype spreads. However, CGH offers a much higher resolution look at genomic copy number changes. CGH may also be performed on routinely processed formalin-fixed paraffin-embedded (FFPE) tissues, offering another advantage over conventional cytogenetics. In array CGH, tumor DNA and DNA from non-neoplastic tissues are labeled with fluorescent tags and mixed together. This solution is then hybridized to an array containing defined DNA probes.
corresponding to genomic regions of interest. Image analysis detects whether the DNA bound to a probe is labeled with a tag indicating tumor DNA (copy number gain in tumor) or normal (copy number loss in tumor), or if there is neutral signal (indicating balanced copy number).\textsuperscript{85} Accordingly, conserved increases or losses of copy number between sarcomas of the same type can be identified. In fact, studies with CGH have been used to argue that, based on patterns of copy number changes, many UPS/MFH are in fact, related to leiomyosarcoma, myxofibrosarcoma or dedifferentiated liposarcoma.\textsuperscript{69,70,86}

Limitations of CGH include the inability to detect balanced translocations (because there is no change in overall copy number of the involved loci), and the inability to provide information as to ploidy (because copy numbers appear balanced in a DNA preparation if uniformly increased). Array CGH is preferable to the initial technique of hybridization to metaphase chromosomes, because array technology offers better resolution and throughput.\textsuperscript{85}

**Gene Expression Arrays**

Gene expression arrays have been previously described in "Gene Profiling: Unlocking the Inner Workings of Sarcoma Cells" and "Gene Profiling Studies on Sarcomas" and will only be briefly discussed here. Gene expression arrays are similar to array CGH in that immobilized oligonucleotide probes on a chip are used to assess amount of nucleic acid in a tumor compared to normal. In this instance, however, it is mRNA (cDNA) that is assayed, under the assumption that levels of mRNA reflect variation in protein expression. This test is frequently used in the research setting to identify potential prognostic or therapeutic biomarkers. In fact, one use of gene expression profiling for prognostic purposes was recently published as the CINSARC study. This study combined both genomic profiling and gene expression signature with histologic data to select for genes highly expressed in cases with high grade histology and/or chromosomal instability. Low or high expression of this panel of genes divided tumors into low and high risk groups, respectively. In a validation cohort, this test was found to outperform conventional FNCLCC sarcoma grading system in terms of risk assessment.\textsuperscript{87,88}

Both CGH and GEA may be ultimately rendered obsolete by the advent of next generation sequencing, which can be used with genomic DNA, exomic DNA or cDNA, thereby allowing direct measurement of DNA or mRNA content as well as mutation status. However, at this time, full scale genomic sequencing is of limited application, given the cost, time and limited return.

**Summary**

Soft tissue and bone sarcomas are a diverse group of neoplasms whose pathogenesis and behavior are increasingly recognized to be dependent on specific genetic and epigenetic alterations. Translocations and mutations limited to a few or to specific subtypes of
alterations. Translocations and mutations limited to a few or to specific subtypes of sarcomas can be identified by modern molecular testing performed in clinical laboratories, thereby increasing our ability to recognize and appropriately treat these tumors. Rapid advances in molecular diagnostics are driven both by specific research into genetic alterations responsible for sarcomagenesis, as well as by improvements in technology, with cheaper and faster technologies offering the ability to investigate more deeply and accurately into the genome. Together, these advances have led to immense progress in understanding the biology of sarcomas and will continue to further improvements in diagnosis and therapy of these rare but devastating malignancies.

GLOSSARY OF TERMS

Amplicon: An amplicon is a piece of DNA that is formed as the product of natural or artificial amplification processes.
Amplification: refers to the process of making of multiple identical copies (replicates) of a specific DNA sequence.
Benign: Term used to refer to neoplasms (tumors), that do not metastasize or infiltrate adjacent tissues, and which usually do not recur if completely removed.
Chimeric gene: Abnormal gene resulting from the fusion of portions of the protein coding sequences of two different normal genes.
Diagnostic marker: Protein or nucleotide sequence whose presence or absence may be correlated with particular tissues or tumor types.
Differential diagnosis: Systematic listing of the most probable or possible diagnoses for an entity or condition, when multiple diagnoses are possible. The differential diagnosis is then used to tailor specific ancillary testing to rule in or rule out the entities under consideration.
DNA Replication and DNA synthesis: DNA replication is a biological process that occurs in all living organisms and copies their DNA. DNA replication can also be performed artificially, outside a cell (in vitro) and is referred to as DNA synthesis.
Epigenetics: The study of changes in gene expression that are not due to changes in underlying DNA sequence. These changes are usually due to modification of DNA or DNA-associated proteins by the addition or subtraction of acetyl- or methyl- groups. This change the way regulatory factors interact with DNA and results in silencing of certain genes or upregulation of others.
FISH: FISH (fluorescence in situ hybridization) is a technique used to detect and localize the presence or absence of specific DNA sequences on chromosomes.
Fusion gene: Abnormal gene resulting from the fusion of portions of two different normal gene sequences. Fusion genes may result from the combination of non-coding sequences of one gene (e.g. promoter region) to coding sequences of another, or from the combination of portions of the protein coding sequences of two genes (see chimeric gene above).
**Heterogeneity and Homogeneity**: These terms are used to describe the uniformity or lack of uniformity in things. Homogeneous things are similar in composition or character; things are heterogeneous if they lack uniformity in composition or character.

**Histologic**: Characteristics identified by the examination of tissue specimens under a microscope. Learn more in this video.

**Immunohistochemistry**: Method of assessing expression patterns of proteins, using antibodies specific to the protein of interest. Because expression of certain proteins is tissue or tumor type specific, this technique can be used to classify tumors.

**Indolent**: A term used to describe a disease or condition that is relatively slow to develop or progress. Often used in sarcoma to refer to malignant tumors which are very slow growing, or which have prolonged survival times.

**Karyotype**: A pictoral description of chromosomes in the nucleus of a cell; the karyotype includes information about chromosome number and any observable structural alterations within each chromosome.

**Lesion**: An area of abnormal tissue.

**Malignant**: A term used to describe a severe and progressively worsening disease, e.g., a cancer in which abnormal cells divide without control and can invade nearby tissues, or metastasize to other body sites (see benign).

**Mesenchymal cells**: Cells that comprise and produce connective tissue, including: fat, muscle, cartilage, bone, blood vessels, fascia, and other fibrous tissues.

**Microdissection**: This term refers to a variety of techniques in which a microscope is used to aid in the dissection of specific cells or tissues. In the practical diagnostic application, this may refer to a procedure by which areas of tumor are identified on a hematoxylin and eosin stained tissue section and marked on the slide. This mark is then used to guide the dissection of unstained paraffin sections to enrich for tumor before DNA or RNA extraction for molecular diagnostic procedures.

**Molecular alteration**: Any change in the DNA sequence or expression that is not a normal variant. This includes point mutations, amplifications, and translocations, as well as promoter silencing, among other possible changes.

**Molecular diagnostics**: Diagnostic tests and methods used to identify a disease or the predisposition for a disease by analyzing DNA and/or RNA.

**Morphologic similarities**: This term refers to the specific appearance of cells within a tumor, which may share features with tumors of different differentiation. For instance, ‘small round blue cell tumor’ is a morphologic descriptor of a variety of tumors (including some lymphomas, carcinomas, and sarcomas), that show distinct differentiation toward specific tissue types and behave differently, but may look very similar on H&E sections. (Tissue sections are normally stained with hematoxylin and eosin (H&E) in order to visualize the tissue architecture and cellular features.)

**Neoplasms**: Abnormal proliferations of cells, resulting from underlying alterations in the DNA of the cells. Neoplasms may be benign or malignant.

**Nonspecific staining patterns**: Term referring to immunohistochemical results. A nonspecific stain may result from the antibody binding to a protein other than the specific one of interest, staining in the wrong cellular compartment (e.g. cytoplasm for a nuclear factor),
or may refer to weak, focal or patchy expression of proteins which may be seen in many different tumors. For example, immunohistochemical staining for the marker MyoD1 is used to identify skeletal muscle differentiation, however, expression of MyoD1 is only diagnostic when seen in the cell nucleus; cytoplasmic staining is considered nonspecific as this pattern may be seen in cells which do not show muscle differentiation. Another example is smooth muscle actin (SMA), which is strongly expressed in smooth muscle (along with another more specific marker, desmin)) – however, many sarcomas may express weak or focal SMA, without showing true smooth muscle differentiation.

**Oncogenic mutation**: An alteration in the DNA which results in mutation of a gene such that the product of the altered gene contributes to the uncontrolled proliferation or survival of a tumor cell.

**Pathogenesis**: A term used to describe: (1) the mechanism by which the disease occurs; (2) the origin and development of a disease.

**PCR, RT-PCR, Q-PCR**: Polymerase chain reaction (PCR) is a technique to amplify (see above) a single or a few copies of a piece of DNA to generate large numbers of copies of a particular DNA sequence. Real-time polymerase chain reaction (RT-PCR), also called quantitative real time polymerase chain reaction (Q-PCR), is a laboratory technique based on the PCR, which is used to amplify and simultaneously quantify a targeted DNA molecule. The ability to quantify the level at which a particular gene is expressed within a cell, tissue or organism can give important scientific information. NOTE: This should not be confused with reversion transcription PCR (also referred to as RT-PCR) where RNA is reverse transcribed to DNA so that it can be amplified (RNA cannot be directly amplified since thymine is substituted by uracil).

**Phenotypes**: Term which generically refers to observable characteristics or traits; this may refer to an entire organism or to a specific tumor or even cell type, and may include descriptors of morphology, development, biochemical or physiological properties. Phenotypes result from the expression of genes as well as the influence of environmental factors and the interactions between the two.

**Primer**: A primer is a short strand of nucleic acids that serves as a starting point for DNA synthesis. This term is particularly used when discussing PCR. There primers are designed to amplify specific portio of DNA. (see DNA Replication above).

**Staining of admixed non-neoplastic cells**: A pitfall in the interpretation of immunohistochemistry, this occurs when tumors infiltrate adjacent tissue, such as muscle or fat, or when reactive cells, such as inflammatory cells and dendritic cells, infiltrate tumor. Staining of these cells for specific markers of differentiation or proliferation may lead to misdiagnosis of tumor if not correctly identified as non-neoplastic bystanders.

**Subcellular localization**: Where in the cell a factor is found. Cellular compartments include: nucleus, cytoplasm, and cell membrane and a variety of organelles such as Golgi apparatus, ribosomes, etc.

**Transcriptional Regulator**: Protein or complex of proteins that plays a role in controlling gene expression, through a variety of mechanisms. Transcriptional regulators may promote increased gene expression or may act to shut down or decrease gene expression through their interactions with DNA and other DNA-associated factors.
**Tumor**: Generic term for any tissue mass. Tumors may be neoplastic, or may result from reactive processes (including edema or swelling). In pathology, tumor usually refers to a neoplastic process.

**Tumorigenesis**: The process by which normal cells become neoplastic cells, characterized by changes on cellular and genetic level which result in uncontrolled cell division. Tumorigenesis is also called oncogenesis. The terms carcinogenesis or sarcomagenesis may also be used when referring specifically to tumors of epithelial or mesenchymal differentiation.
Table 1: Chromosomal Translocations/Amplifications in Selected Mesenchymal Neoplasms

<table>
<thead>
<tr>
<th>Tumor Type</th>
<th>Translocation</th>
<th>Gene(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ewing sarcoma; Primitive neuroectodermal tumor (PNET)</td>
<td>t(11;12)(q24;q12)</td>
<td>EWSR1-FLI1</td>
</tr>
<tr>
<td></td>
<td>t(21;22)(q22;q12)</td>
<td>EWSR1-ERG</td>
</tr>
<tr>
<td></td>
<td>t(7;22)(p22;q12)</td>
<td>EWSR1-ETV1</td>
</tr>
<tr>
<td></td>
<td>t(2;22)(q33;q12)</td>
<td>EWSR1-FEV</td>
</tr>
<tr>
<td></td>
<td>t(17;22)(q12;q12)</td>
<td>EWSR1-E1AF</td>
</tr>
<tr>
<td></td>
<td>inv(22)(q21;12)</td>
<td>EWSR1-ZSG</td>
</tr>
<tr>
<td></td>
<td>t(16;21)(p11;q22)</td>
<td>FUS-ERG</td>
</tr>
<tr>
<td>Angiomatoid fibrous histiocytoma</td>
<td>t(12;22)(q13;q12)</td>
<td>EWSR1-ATF1</td>
</tr>
<tr>
<td></td>
<td>t(2;22)(q33;q12)</td>
<td>EWSR1-CREB1</td>
</tr>
<tr>
<td></td>
<td>t(12;16)(q13;p11)</td>
<td>FUS-ATF1</td>
</tr>
<tr>
<td>Clear-cell sarcoma</td>
<td>t(12;22)(q13;q12)</td>
<td>EWSR1-ATF1</td>
</tr>
<tr>
<td></td>
<td>t(2;22)(q33;q12)</td>
<td>EWSR1-CREB1</td>
</tr>
<tr>
<td>Low-grade fibromyxoid sarcoma</td>
<td>t(7;16)(q33;p11)</td>
<td>FUS-CREB3L2</td>
</tr>
<tr>
<td></td>
<td>t(11;16)(p11;p11)</td>
<td>FUS-CREB3L1</td>
</tr>
<tr>
<td>Desmoplastic small round-cell tumor</td>
<td>t(11;22)(p13;q12)</td>
<td>EWSR1-WT1</td>
</tr>
<tr>
<td>Extraskeletal myxoid chondrosarcoma</td>
<td>t(9;22)(q22;q12)</td>
<td>EWSR1-NR4A3</td>
</tr>
<tr>
<td></td>
<td>t(9;17)(q22;q11)</td>
<td>TAF2N-NR4A3</td>
</tr>
<tr>
<td></td>
<td>t(9;15)(q22;q21)</td>
<td>TCF12-NR4A3</td>
</tr>
<tr>
<td></td>
<td>t(3;9)(q11;q22)</td>
<td>TFG-NR4A3</td>
</tr>
<tr>
<td></td>
<td>Rearrangements of 9q22</td>
<td>NR4A3</td>
</tr>
<tr>
<td>Myoepithelial tumor of soft tissue and bone</td>
<td>t(6;22)(p21;q12)</td>
<td>EWSR1-POU5F1</td>
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<tr>
<td></td>
<td>t(19;22)(q13;q12)</td>
<td>EWSR1-ZNF444</td>
</tr>
<tr>
<td></td>
<td>t(1;22)(q23;q12)</td>
<td>EWSR1-PBX1</td>
</tr>
<tr>
<td>Myxoid/round-cell liposarcoma</td>
<td>t(12;16)(q13;p11)</td>
<td>FUS-DD1T3 (TLS-CHOP)</td>
</tr>
<tr>
<td></td>
<td>t(12;22)(q13;q12)</td>
<td>EWSR1-DD1T3 (EWSR1-CHOP)</td>
</tr>
<tr>
<td>pulmonary myxoid sarcoma</td>
<td>t(2;22)(q34;q12)</td>
<td>EWSR1-CREB1</td>
</tr>
<tr>
<td>Sclerosing epithelioid fibrosarcoma</td>
<td>t(7;16)(p22;q24)</td>
<td>FUS-CREB3L2</td>
</tr>
<tr>
<td>Alveolar rhabdomyosarcoma</td>
<td>t(2;13)(q35;q14)</td>
<td>PAX3-FKHR</td>
</tr>
<tr>
<td></td>
<td>t(1;13)(p36;q14)</td>
<td>PAX7-FKHR</td>
</tr>
<tr>
<td></td>
<td>t(X;2)(q13;q35)</td>
<td>PAX3-AFX</td>
</tr>
<tr>
<td>Alveolar soft-part sarcoma</td>
<td>der(17)t(X;17)(p11;q25)</td>
<td>ASPSCR1-TFE3</td>
</tr>
<tr>
<td>Endometrial stromal sarcoma</td>
<td>t(7;17)(p15;q11)</td>
<td>JAZF1-JJAZ1(SUZ12)</td>
</tr>
<tr>
<td></td>
<td>t(6;7)(p21;p15)</td>
<td>JAZF1-PHF1</td>
</tr>
<tr>
<td></td>
<td>t(6;10)(p21;p11)</td>
<td>EPC-PHF1</td>
</tr>
<tr>
<td>Epithelioid hemangioendothelioma</td>
<td>t(1;3)(p36;q25)</td>
<td>WWTR1-CAMTA1</td>
</tr>
<tr>
<td>Mesenchymal Chondrosarcoma</td>
<td>t(8;8)(q13;q21)</td>
<td>HEY1-NCOA2</td>
</tr>
<tr>
<td>Synovial sarcoma</td>
<td>t(X;18)(p11;q11)</td>
<td>SS18-SSX1, SSX2, SSX4</td>
</tr>
<tr>
<td>Undifferentiated small round blue cell tumor</td>
<td>t(4;19)(q35;q13)</td>
<td>CIC-DUX4</td>
</tr>
<tr>
<td></td>
<td>t(10;19)(q26;q13)</td>
<td></td>
</tr>
</tbody>
</table>
### Translocations/Amplifications Resulting in Gene Overexpression

<table>
<thead>
<tr>
<th>Tumor Type</th>
<th>Translocation</th>
<th>Gene(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Well differentiated liposarcoma/atypical lipomatous tumor†/dedifferentiated liposarcoma</td>
<td>12q14-15 (ring chromosomes, giant marker chromosomes)</td>
<td>Amplification of MDM2, CDK4, HMGA2, GLI, SAS</td>
</tr>
<tr>
<td>Osteosarcoma, low grade (parosteal and intramedullary)</td>
<td>12q14-15 (ring chromosomes, giant marker chromosomes)</td>
<td>amplification of CDK4, MDM2, HMGA2, GLI, SAS</td>
</tr>
<tr>
<td>Aneurysmal bone cyst*</td>
<td>t(16;17)(q22;p13)</td>
<td>CDH11-USP6</td>
</tr>
<tr>
<td></td>
<td>t(1;17)(p34.3;p13)</td>
<td>THRAP3-USP6</td>
</tr>
<tr>
<td></td>
<td>t(3;17)(q21;p13)</td>
<td>CNBP-USP6</td>
</tr>
<tr>
<td></td>
<td>t(9;17)(q22;p13)</td>
<td>OMD-USP6</td>
</tr>
<tr>
<td></td>
<td>t(17;17)(q21;p13)</td>
<td>COL1A1-USP6</td>
</tr>
<tr>
<td>Congenital/infantile fibrosarcoma</td>
<td>t(12;15)(p13;q25)</td>
<td>ETV6-NTRK3</td>
</tr>
<tr>
<td>Dermatofibrosarcoma protuberans</td>
<td>t(17;22)(q21;q13)</td>
<td>COLIA1-PDGFB</td>
</tr>
<tr>
<td>Giant cell fibroblastoma</td>
<td>t(17;22)(q21;q13)</td>
<td>COLIA1-PDGFB</td>
</tr>
<tr>
<td>Myxoinflammatory fibroblastic sarcoma/hemosiderotic fibrolipomatous tumor</td>
<td>t(1;10)(p22;q24)</td>
<td>TGFBR3-MGEA5</td>
</tr>
<tr>
<td></td>
<td>3p11-12 (ring chromosome)</td>
<td>Amplification of VGLL3, CHMP2B</td>
</tr>
<tr>
<td>Nodular fasciitis*</td>
<td>t(17;22)(p13;q13)</td>
<td>MYH9-USP6</td>
</tr>
<tr>
<td>Radiation-induced angiosarcoma</td>
<td>8q24</td>
<td>Amplification of MYC</td>
</tr>
<tr>
<td>Tenosynovial giant cell tumor /pigmented villonodular synovitis†</td>
<td>t(1;2)(p13;q35-37)</td>
<td>COL6A3-CSF1</td>
</tr>
</tbody>
</table>

### Translocations Resulting in Altered Subcellular Localization/Activation

<table>
<thead>
<tr>
<th>Tumor Type</th>
<th>Translocation</th>
<th>Gene(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inflammatory myofibroblastic tumor</td>
<td>t(1;2)(q22;p23)</td>
<td>TPM3-ALK</td>
</tr>
<tr>
<td></td>
<td>t(2;19)(p23;p13)</td>
<td>TPM4-ALK</td>
</tr>
<tr>
<td></td>
<td>t(2;17)(p23;q23)</td>
<td>CLTC-ALK</td>
</tr>
<tr>
<td></td>
<td>t(2;2)(p23;q13)</td>
<td>RANBP2-ALK</td>
</tr>
<tr>
<td></td>
<td>t(2;11)(p23;p15)</td>
<td>CARS-ALK</td>
</tr>
<tr>
<td></td>
<td>inv(2)(p23;q35)</td>
<td>ATIC-ALK</td>
</tr>
</tbody>
</table>
### Table 2: Selected Diagnostically Helpful Mutations in Mesenchymal Neoplasms

<table>
<thead>
<tr>
<th>Neoplasm</th>
<th>Locus</th>
<th>Alteration</th>
<th>Detection</th>
<th>Immunohistochemical surrogate marker</th>
</tr>
</thead>
<tbody>
<tr>
<td>GIST</td>
<td>KIT, PDGFRA, BRAF</td>
<td>Activating point mutation</td>
<td>PCR</td>
<td>Kit (CD117), PDGFR-α</td>
</tr>
<tr>
<td>Desmoid-type fibromatosis†</td>
<td>CTNNB1</td>
<td>Activating point mutation</td>
<td>PCR</td>
<td>β-catenin</td>
</tr>
<tr>
<td>Malignant rhabdoid tumor</td>
<td>INI1</td>
<td>Deletion, inactivating mutation</td>
<td></td>
<td>Loss of Ini-1 expression</td>
</tr>
<tr>
<td>Epithelioid sarcoma</td>
<td>INI1</td>
<td>Heterogenous (inactivating mutation, translocation, non-genomic silencing)</td>
<td></td>
<td>Loss of Ini-1 expression</td>
</tr>
<tr>
<td>Fibrous dysplasia*</td>
<td>GNAS</td>
<td>Activating point mutation</td>
<td>PCR</td>
<td></td>
</tr>
<tr>
<td>Myxoma*</td>
<td>GNAS</td>
<td>Activating point mutation</td>
<td>PCR</td>
<td></td>
</tr>
</tbody>
</table>

*Benign neoplasm; † Intermediate (locally aggressive) neoplasm
REFERENCES


The Electronic Sarcoma Update Newsletter

ESUN is an online, peer-reviewed newsletter that contains articles of interest to patients, caregivers, physicians and nurses. ESUN’s feature articles discuss specific sarcomas and the issues involved in dealing with these rare cancers. Regular columns cover clinical trials, recent research findings, community news, patient stories, and a variety of topics of interest to the sarcoma community.

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