

**UNDIFFERENTIATED SARCOMAS:
WHAT TO DO ? AND DOES IT MATTER ?
A SURGICAL PATHOLOGY PERSPECTIVE**

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Bullet points

- Whether or not to label a sarcoma 'undifferentiated' depends heavily on available diagnostic resources and available treatment options.
- Pathologic subclassification of all sarcomas, where possible, is the main avenue by which advances in diagnosis, prognostication and therapeutic stratification are made.
- Attempts to subclassify round cell sarcomas are likely to have the highest yield, particularly in terms of clinical benefit.
- Subclassification of seemingly undifferentiated spindle cell sarcomas may sometimes have significant clinical impact.
- Efforts to subclassify undifferentiated pleomorphic or epithelioid malignancies arising in soft tissue is very often unrewarding.

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While, in academic ivory towers, we routinely take for granted the ability to apply a wide range of diagnostic techniques and the oncologists' ability to use a variety of different treatment modalities and chemotherapeutic regimens, on a broader scale, the range of diagnostic techniques or therapeutic approaches may be significantly more limited – or there may be a mismatch between the two. Very often, any limitations or mismatches will relate principally to issues of cost and/or proven cost-effectiveness. In general, however, it is probably not the pathologists' role to determine whether or not a tumor is worth subclassifying since, in many instances, the pathologist may not be familiar with the details of a specific clinical situation or with the possible and appropriate therapeutic interventions. Thus, the approach to cases of this type needs to be multidisciplinary and, as with all good pathology practice, should include effective communication between pathologists and clinicians. The extent to which a clinician is likely to seek detailed subclassification of a seemingly undifferentiated sarcoma will depend not only on the range of therapeutic options but on issues such as tumor stage, the patient's general clinical condition and an assessment of the likelihood that subclassification may have therapeutic impact (for example, this would be more likely in a round cell sarcoma rather than a pleomorphic sarcoma). Quite often, the more significant decision point, from the clinician's perspective, will be to make sure that the tumor is a sarcoma, rather than lymphoma, carcinoma or melanoma.

In general, undifferentiated sarcomas can best be considered in broad groups – principally those with round cell morphology, spindle cell morphology, pleomorphic morphology or epithelioid morphology. The proportion of undifferentiated sarcomas which would be labelled as myxoid is relatively small and, furthermore, most such lesions are of spindle cell type and occur in older adults, in a setting where therapeutic options are frequently more limited.

ROUND CELL SARCOMAS

Arguably, the subclassification of round cell sarcomas, most but not all of which occur in pediatric and adolescent patients, has the greatest clinical impact. The principal reasons for this are that these are among the most aggressive soft tissue sarcomas, yet also the most chemosensitive and, furthermore, there are well-defined

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and quite different therapeutic regimens which are applied to rhabdomyosarcoma and Ewing's sarcoma/PNET respectively, these being the two largest groups of round cell sarcomas. As such, pathologic subclassification will have significant impact on treatment and therefore best efforts have to be made to avoid the non-discriminatory label 'undifferentiated'. That having been said, it is also true that a significant subset of the round cell sarcomas occurring principally in young infants may be extremely hard to classify reproducibly, either because they appear to be undifferentiated by whatever techniques are applied, or because they show bizarre or complex polyphenotypic differentiation or, more rarely, because there are well-documented examples in which the morphology and immunophenotype may not fit well with the genetic findings. In general, immunohistochemistry allows reliable subclassification of the large majority of round cell sarcomas occurring in young patients and, in the small subset which remain unclassified by this technique, most can be further resolved by using cytogenetics or, increasingly, molecular genetic analysis on paraffin-embedded or frozen tissue. While, in the past, electron microscopy was often quite helpful in this context, molecular testing has increasingly become the gold standard in this clinical setting, in part because of reproducibility and also in part because ultrastructural evidence of specific cytodifferentiation may be focal or limited in extent in a given tumor. In addition, tissues suitably fixed for electron microscopy are commonly not available. Furthermore, molecular genetic testing can be extremely helpful when there is immunophenotypic overlap between potential diagnoses – for example, keratin-positive Ewing's/PNET, desmin-positive desmoplastic small round cell tumor and CD99-positive mesenchymal chondrosarcoma to name just a few.

Subclassification of round cell sarcomas in adult patients (of any age) also has direct relevance, now that pediatric-type rhabdomyosarcomas and Ewing's/PNET are increasingly recognized in this age group. Subclassification is important because it is nowadays believed, principally by medical oncologists, that the use of pediatric-type chemotherapeutic regimens in adult patients with these diseases is associated with a significantly improved outcome.

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Introduction

In the context of the program laid out for this session, which includes presentations devoted to the ultrastructure, cytogenetics and molecular genetics of poorly differentiated or undifferentiated sarcomas, then it is the purpose of this presentation to address specifically what surgical pathologists might do, in the routine clinical/diagnostic setting, to facilitate the classification of an otherwise seemingly undifferentiated sarcoma. Comprehensive lists of useful immunostains and specific cytogenetic/molecular genetic aberrations are not included here as they have been featured in many textbooks and review articles. In most institutions, it is the surgical pathologist who decides whether or not immunohistochemical stains, electron microscopy, cytogenetics or molecular genetic analysis should be initiated and, in these circumstances, it is important to consider what the cost-benefit of such testing might be and what impact any work-up (by whatever technique) may have either on prognostication or therapeutic decision making.

What is an undifferentiated sarcoma?

Either obvious or ridiculous as this may seem, the decision to label any sarcoma as undifferentiated is very much in the eye of the beholder. Furthermore, that beholder's interpretation may be significantly influenced by the availability of resources to pursue the precise line of differentiation in a given neoplasm, as well as the likely influence of any more specific diagnosis on local treatment decisions. Factors which may have significant impact on whether or not to label a sarcoma as undifferentiated include:

- Clinical context/patient age
- Extent of tissue sampling
- Availability of immunohistochemistry (and range of antibodies)
- Availability of electron microscopy (including suitably fixed tissue)
- Availability of cytogenetic and molecular genetic testing
- Experience of the pathologist
- Availability of sophisticated oncologic intervention (including range of different therapies)

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SPINDLE CELL SARCOMAS

The true clinical relevance of subclassifying an otherwise seemingly undifferentiated spindle cell sarcoma is more limited than that in round cell sarcomas, mainly because of the more limited therapeutic options and smaller likelihood of chemoresponsiveness. While there is a good argument to make that pathologic subclassification of all sarcomas is worthwhile, since this is the only means by which clinical, prognostic and therapeutic differences will be identified, nevertheless, in the course of daily practice, the whole is relatively less sensitive to currently available chemotherapeutic agents and, when either very large or else disseminated, is generally treated by one of the standard 'broad spectrum' adult-type sarcoma regimens (usually including either adriamycin and/or ifosfamide).

In terms of the techniques to apply, most examples of leiomyosarcoma and synovial sarcoma will be identified successfully using immunohistochemistry but, in poorly differentiated examples of synovial sarcoma, molecular genetic testing is often helpful. With regard to MPNST, greater problems arise since less than 50% of these lesions stain with either S-100 protein or GFAP and there are no well-defined molecular genetic 'markers' for this tumor type. In this setting, electron microscopy may well be useful in proving the presence of nerve sheath differentiation although, as mentioned above, the therapeutic impact of such classification may be more limited.

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Molecular Profiling in the Diagnosis and Treatment of High Grade Sarcomas

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High density microarrays are used to measure in a comprehensive manner on a large scale ("profile") gene expression patterns (when tumor RNA is tested) or gene copy number changes (when tumor genomic DNA is tested). Microarray-based studies are increasingly useful in addressing a wide variety of questions in sarcoma biology. During this talk, a brief description of the microarray methodology and data analysis will be provided, followed by few examples of the recent applications of gene expression in the diagnosis, classification and treatment of sarcomas.

I. DNA Microarray Technology

Expression profile refers to the process of measuring the expression of thousands of individual genes simultaneously in a given tissue sample. The basic principle is hybridization-based.

Microarrays: a collection of spots on a solid surface (often a glass slide) arranged in neat rows and columns, so that the origin of each spot is known. Depending on the type, the spots can contain DNA sequences (as in gene microarrays), tissue fragments (tissue microarrays) protein (protein microarrays), etc.

Types of high-density microarrays:

a) **cDNA spotted arrays.** Libraries of cDNA clones (20,000-40,000 most commonly expressed genes) are robotically arrayed on standard 1x3 glass microscope slides and usually produced by the investigator or by a core facility. This method requires competitive

discriminate tumors from normals? Are there correlations between expression profiles and other molecular or pathological properties of the tumor? Are there correlations between expression profiles and clinical outcome and response to therapy? With what degree of certitude these results are not due to chance alone? Are there subsets within tumors of the same apparent class? Are these pathways relevant to the tumor phenotype or as potential targets for therapy? These important questions and the need to develop the mathematical tools to address them have attracted the attention of computer scientists, engineer, and biostatisticians. Numerous computational approaches have been developed.

Microarray analysis whether supervised or unsupervised ultimately generates lists of genes that discriminates among samples. Making sense of these gene lists presents a significant challenge. Gene names can be misleading and the majority of genes are linked to little or no functional information. Most of the interpretations that arise from exploring microarray data should be considered hypotheses, rather than conclusions. Additional forms of experimentation is most likely necessary to establish a conclusive connection between a gene and the tumor in which is expressed.

Validation the microarray data: How reliable is the data? Tissue microarrays for in situ mRNA hybridization or immunohistochemistry provide the possibility of confirmatory studies on large number of samples.

III. Applications of the DNA microarray technology to cancer diagnosis and prognosis

Cancer is a disease of disturbed genome function. Irrespective of whether this is the result of a point mutation, deletion, translocation, gene amplification, or methylation, the malignant phenotype is mediated by a characteristic pattern of gene expression. Identifying these genes whose expression differs between normal tissues and tumors and among tumor types is the focus of today's research. Investigators in the field initially performed studies in which tumors of different morphology or different primary sites were shown to have clearly distinguishable patterns of gene expression. This type of diagnostic classification by analysis of expression profile is called "class prediction". These proof-of-principle studies served to validate cDNA microarray technology and the researchers then shifted their focus to

hybridization to a mixture of reference and test mRNA, each labeled with a distinct fluorochrome (red-tumor and green-reference).

b) **Affymetrix** (Santa Clara, CA) **oligonucleotide microarrays.** Affymetrix uses similar equipment to that used for making silicon chips for computers, allowing mass production of chips at reasonable costs. Affymetrix uses masks to control synthesis of oligonucleotides on the surface of the chip. Light is directed through a mask to deprotect and activate selected sites, and protected nucleotides couple to the activated sites. The process is repeated, activating different sets of sites and coupling different several hundred thousand squares (cells) each containing many copies of one oligonucleotide. So the result is several hundred thousand different oligos, each of them in millions of copies. It is not used for gene discovery because this approach requires knowledge of the sequence in order to choose probes for a given gene or EST. 25 base oligos are synthesized *in situ*, by **light directed oligonucleotide synthesis**. Each gene represented by at least 20 oligos distributed along the 5' to 3' length of the gene. Each is paired with a single base mismatch oligo. The arrays are constructed on rigid material (glass) they can be inverted and mounted in a temperature-controlled hybridization chamber. It is based on an important observation that single stranded DNA binds strongly to nitrocellulose membranes in a way that does not re-associate with each other but permits hybridization with cRNA. A fluorescently tagged nucleic acid sample injected into the chamber hybridizes to complementary oligonucleotides on the array. Laser excitation enters through the back of the glass support, fluorescence emission is collected by a lens and passes through a series of optical fibers to a sensitive detector. By scanning the laser beam (translating the array) a quantitative two-dimensional fluorescence image of hybridization intensity is quickly obtained.

Probe redundancy: the use of multiple oligonucleotides of different sequence designed to hybridize to different regions of the same RNA. The use of multiple independent detectors for the same molecule greatly improves signal-to-noise ratios, improves the accuracy of RNA quantitation, drastically reduces the rate of false positives and miscalls. An additional level of redundancy comes from the use of mismatch (MM) control probes that are identical with perfect match (PM) probes except for a single base difference in a central position. The MM probes act as specificity controls that allow the direct subtraction of both background and

identification of molecularly defined tumor entities that were inapparent by conventional pathologic analysis ("class discovery"). New unsuspected biological subsets were detected among cutaneous melanomas, breast carcinomas, and pediatric acute lymphoblastic leukemias. In other cases, there has been "class rediscovery" or "class confirmation".

Variability in the results, more notable in prognostic studies than in class prediction, have already appeared in the literature and is most likely related to inter-laboratory and inter-platform reproducibility. For example, two large breast cancer expression profiling studies differed considerably in the results of unsupervised clustering: one identified 3 major subsets (ER-positive luminal cell type, basal cell type, and ERBB2-amplified type), while the other detected only 2 major subgroups according to ER status and lymphocytic infiltration. Moreover, in the latter study, well-established clinical markers, such as ERBB2 and ER were not found within a list of 70 genes linked to outcome.

IV. Molecular Profiling in the Diagnosis and Treatment of High Grade Sarcomas

Studies involving gene expression technology have been mainly applied as a source of diagnostic markers for sarcoma diagnosis and to their role in clarifying sarcoma classification. A general observation of microarray-based expression profiling studies of sarcomas is that translocation-associated sarcomas are robustly clustered by expression profiling using cDNA microarrays, whereas so-called complex karyotype sarcomas tend to be less tightly clustered. Thus, complex karyotype sarcomas that often show different gains and losses from case to case are also likely to show more variability in gene expression patterns, leading to less robust unsupervised clustering of the expression profiles. Indeed, this tumor group may be better studied by profiling of gene copy number changes using array-based CGH.

Our study on 51 soft tissue sarcoma specimens using hierarchical cluster analysis demonstrated distinct clusters for GIST, synovial sarcoma, clear cell sarcoma and round cell liposarcoma. Several fibrosarcoma tumors fall in close proximity to synovial sarcoma. Pleomorphic sarcomas exhibited overall poor correlation and consistency by boot strap

cross-hybridization signals, and allow discrimination between "real" signals and those due to non-specific and semi-specific hybridization (hybridization of the intended RNA molecules produces more signal on PM probes than for MM probes, resulting in consistent patterns that are highly unlikely to occur by chance: the pattern recognition rules are codified in the analysis software).

c) **array-based comparative genomic hybridization (array-CGH):** uses microarrays with genomic DNA sequences and used to interrogate tumor DNA samples in order to provide information regarding gene copy number (amplification and deletions).

II. Data Analysis

Data from a single 22,000-gene Affymetrix GeneChip occupies 100 MB of storage and require significant manipulation before interpretation. Normalization of all values to a mean of 500 is commonly used. Data cleanup is an important first step to reduce the noise, increase sensitivity, and manage artifacts of data set transformation. Filtering and statistical analyses constraints are applied to exclude those genes that did not vary significantly between comparison groups or that are not expressed at high enough levels. Data from a series of tumor samples with expression levels for thousands of genes can present a challenge for analysis. A method for data storage and retrieval in a database is essential.

Hierarchical Clustering or Cluster Analysis: uses standard statistical algorithms to arrange genes according to similarity in pattern of gene expression. The output is displayed graphically, conveying the clustering and the underlying expression data simultaneously. Relationship among genes is represented by a tree whose branch lengths reflects the degree of similarity between genes (dendrogram). This method recognizes groups of genes that are co-expressed, providing a new insight of their possible function. It also identifies samples whose gene expression patterns correlate. The basic idea is joining together points into clusters.

Appropriate selection of analysis tools will depend on the questions to be addressed. Certain key questions predominate cancer-related microarray research: "Can two types of cancer be discriminated? What genes discriminate them more clearly? Are there genes that

analysis. However, within this group certain prominent clusters were observed for both malignant fibrous histiocytoma (MFH) and leiomyosarcoma.

One example of "class confirmation" study in sarcomas includes the comparison of **GIST versus leiomyosarcoma**. Previous RNA expression profiling studies of different soft tissue sarcomas indicated that GIST expression profiles were distinct and quite homogeneous in part due to the unique derivation of GIST from ICC. GISTs are characterized by a distinctive transcriptional signature, as a result of overexpression of *KIT*, *PRKC#*, *DOG1*, which can be applied in tumor diagnosis, even when compared with their closest pathologic mimic, leiomyosarcomas. Likewise, several studies have used microarrays to identify genes differentially expressed between *PAX-FKHR* fusion-positive alveolar and *PAX-FKHR* fusion-negative embryonal rhabdomyosarcoma.

Molecular profiling was also applied to clarify the conflicting data on the relationship of **clear cell sarcoma (CCS) to cutaneous melanoma**. While the two are clearly genetically distinct, as CCS lack the *BRAF* mutations commonly seen in melanomas, whereas melanomas do not contain the *EWS-ATF1* fusion, by expression analysis CCS share a melanocytic gene signature with melanomas.

In the study by Baird et al, the unsupervised hierarchical analysis on MFH cases alone identified 2 distinct groups: one carrying a muscle profile (myosin X, sarcoglycan β , tenascin C) and the second group revealed an immune regulatory gene profile (HEM1, MX1, DAP10). Interestingly, the storiform-pleomorphic and the myxoid subtypes equally populate both groups of tumors. However the distinction between MFH with myogenic differentiation versus inflammatory characteristics might have clinical relevance. Myogenic differentiation as reflected by IHC in MFH or pleomorphic sarcoma, NOS, has previously shown to have an adverse clinical outcome.

New IHC markers emerging from microarray studies include DOG1 in GIST, TLE1 in synovial sarcoma, AP2- β in alveolar rhabdomyosarcoma, and Apo D in dermatofibrosarcoma protuberans (DFSP).

Still in incipient phases, microarrays studies have been used to predict outcome or therapy response. Poor outcome Ewing sarcoma has been linked with alterations in cell cycle regulatory genes. In a recent study, West et al suggested that expression signatures of either fibromatosis or solitary fibrous tumor can be identified in the stromal component of most breast carcinoma and can be correlated with clinical outcome. The fibromatosis signature is associated with good outcome breast tumors, while solitary fibrous tumor profile is found in poor clinical outcome.

Three different studies have been assessed the correlation of transcriptional profile of pre-treatment osteosarcoma biopsy and response to chemotherapy. Disappointingly, limited overlap has been noted among the results of these studies, which can be explained as a consequence of different platforms or bioinformatics approaches used, differences in patient population or chemotherapy regimens applied, etc.

The ability of mining candidate genes for targeted therapies through microarray analysis has been validated with the GIST paradigm, where the overexpressed KIT or PDGFRA provided the biologic basis for imatinib mesylate therapy. Other examples include dermatofibrosarcoma protuberans, where through an autocrine/paracrine mechanism, the overexpressed PDGFB up-regulates its receptor, PDGFRB, therefore providing another susceptible target to selective tyrosine kinase inhibition. However, targeted therapy appears to be most successful in sarcomas with a distinct underlying molecular biology, and has been failed in sarcomas with non-recurrent complex karyotype. Since drugs target proteins rather than genes, protein level validation is critical and can be performed on the tissue microarray by using IHC and ISH techniques.

In summary, microarray studies applied so far in sarcomas support their classification into genetically simple and genetically complex categories and have provided useful diagnostic markers, as well as insight for novel and targeted therapeutic approaches. However, there is a considerable vacuum between the practical world of hospital-based

molecular diagnostic laboratories and some of the predictions prompted by high-throughput genomics work. Hopeful statements, such as one made in 1999 that "doctors will be offering gene expression profiles to some patients in the next 3 years", failed to consider the many issues in moving complex assays from research laboratories to clinical laboratories. Because of the regulatory, billing, quality control, and test validation concerns, combined with limited resources, academic molecular diagnostic laboratories are extremely selective in their test menus. Furthermore, it is not clear yet that the present cost of microarray, large-scale expression profiling for "class prediction" is more cost-effective than established diagnostic approaches, i.e., histopathology supplemented in selected cases by IHC, cytogenetics, or molecular assays.

In short term however the impact of tumor expression profiling is in the identification of new diagnostic and prognostic markers, which can be studied individually by more conventional techniques. The availability of TMA accelerates the validation of these new IHC assays. The next step will require efforts in academic and commercial laboratories to generate new antibodies for the products of differentially expressed genes without currently available antibodies.

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Contribution of Cytogenetics to the Management of Poorly Differentiated Sarcomas

Table 1: Characteristic and Variant Chromosomal Aberrations and Associated Molecular Events in Bone and Soft Tissue Sarcomas

Neoplasm	Chromosomal aberration	Molecular event
Alveolar soft part sarcoma	der(17)t(X;17)(p11.2;q25.3)	<i>ASPL/TFE3</i>
Alveolar rhabdomyosarcoma	t(2;13)(q35;q14) t(1;13)(p36;q14)	<i>PAX3/FOXO1</i> <i>PAX7/FOXO1</i>
Clear cell sarcoma	t(X;2)(q13;q35) t(2;2)(q23;q35) t(12;22)(q13;q12) t(2;22)(q32;q12)	<i>PAX3/NCG1</i> <i>EWS/ATF1</i> <i>EWS/CREB1</i>
Congenital fibrosarcoma*	t(12;15)(p13;q25)	<i>ETV6/NTRK3</i>
Dermatofibrosarcoma protuberans ^b	t(17;22)(q22;q13) ^c	<i>COL1A1/PDGFB</i>
Epithelioid hemangioendothelioma	t(1;3)(p36;q25)	?
Ewing sarcoma/pPNET	t(11;22)(q24;q12) t(21;22)(q22;q12) t(7;22)(q25;q12) t(17;22)(q21;q12) t(2;22)(q33;q12) inv(22)(q12q12) t(16;21)(p11;q22)	<i>EWS/FLI1</i> <i>EWS/ERG</i> <i>EWS/ETV1</i> <i>EWS/ELF</i> <i>EWS/FEY</i> <i>EWS/ZSG</i> <i>FUS/ERG</i> <i>EWS/NRA43</i>
Extraskeletal myxoid chondrosarcoma	t(9;17)(q22;q11) t(9;15)(q22;q21)	<i>TAF2/NRA43</i>
Gastrointestinal stromal tumor	Monosomy 14, 22 1p deletion	<i>KIT</i> or <i>PDGFRA</i> mutation
Inflammatory myofibroblastic tumor	2p23 rearrangements	<i>ALK</i> rearrangement
Liposarcoma, well-differentiated	Ring/giant marker chromosome; 12q13-15 amplification	<i>MDM2</i> , <i>CDK4</i> , <i>HMG42</i> amplification
Liposarcoma, poorly-differentiated	Ring/giant marker chromosome + more complex; 12q13-15 amplification	<i>MDM2</i> , <i>CDK4</i> , <i>HMG42</i> amplification
Low grade fibromyxoid sarcoma	t(7;16)(q33;q37)	<i>FUS/CREB3L2</i>
Malignant hemangiopericytoma	t(12;19)(q13;q13)	?
Myxoid round cell liposarcoma	t(12;16)(q13;p11) t(12;22)(q13;q12)	<i>TLS⁸/CHOP</i> <i>EWS/CHOP</i>
Parosteal osteosarcoma	Ring chromosome; 12q13-15 amplification	<i>CDK4</i> , <i>MDM2</i> , <i>SAS</i> amplification
Pericytoma	t(7;12)(p22;q13)	<i>AGTB/GLI</i>
Synovial sarcoma	t(X;18)(p11.2;q11.2) t(X;20)(p11.2;q13.3)	<i>SYT/SSX1</i> <i>SYT/SSX2</i> <i>SYT/SSX4</i> <i>SS18L/SSX1</i>

*This translocation is seen also in congenital mesoblastic nephromas, confirming a relationship with congenital fibrosarcoma.

^bThis translocation is seen also in giant cell fibroblastoma, confirming a relationship with dermatofibrosarcoma protuberans.

^cRearrangement also frequently seen as a ring chromosome.

SARCOMA LOOK-ALIKES: AN ULTRASTRUCTURAL APPROACH

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Due to the variable light microscopic appearance of sarcomas, there are a large number of neoplasms that may need to be considered in the differential diagnosis in some situations. In a number of difficult cases, the surgical pathologist must approach the differential diagnosis using ancillary diagnostic techniques and should do so in an orderly fashion, recognizing that arriving to the correct diagnosis is without doubts the most important goal. It is also imperative to take into account the expense and time that may be involved in arriving to the definitive diagnosis. Therefore, the most reasonable route to address the differential diagnosis and to make a final unequivocal should be taken. Whether immunohistochemistry, electron microscopy, cytogenetics and/or molecular diagnostics should be employed in the case in question becomes the dilemma. In some cases a combination of the above mentioned techniques is important not only to solidify the diagnosis but also to provide important additional information regarding prognosis and other important factors.

Differentiation of sarcomas from sarcomatoid carcinomas, spindle and pseudo sarcomatous reactions can create a challenging diagnostic dilemma. Differentiation of these entities by light microscopy can be almost impossible in some cases. Keratin expression by immunohistochemistry is quite variable in poorly differentiated carcinomas in general, where the entity of sarcomatoid carcinoma usually falls. While keratin staining using a variety of cocktails may be of value, it is not uncommon for immunoreactivity to be weak and focal or entirely absent. A similar difficult differential arises between sarcomatoid mesothelioma and sarcoma. Most immunohistochemical markers that are expressed by epithelial mesotheliomas are not present in the sarcomatoid variety. The "positive" mesothelioma markers: podoplanin, calretinin, keratins 5 and 6, WT1 protein, thrombospondin and mesothelin are usually not present in the sarcomatoid variety of mesotheliomas. Of these, calretinin is the one most commonly expressed in sarcomatoid mesotheliomas. Another differential diagnosis that electron microscopy is very helpful in addressing is that of pseudomesotheliomatous adenocarcinoma of the lung associated with florid stromal (spindle-cell) reaction vs. mesothelioma. The differential with mesothelioma arises from the finding of an apparent biphasic neoplasm microscopically and the gross appearance encasing the lung, as is typically seen in mesotheliomas from where the descriptive name "pseudomesotheliomatous" originates. A significant number of these pseudomesotheliomatous adenocarcinomas reveal immunohistochemical profiles that are not characteristic, creating diagnostic controversies. Some of the characteristic lung adenocarcinoma markers such as TTF-1, B72.3, MOC-31 and carcino-embryonic (CEA) antigen may be expressed weakly and/or focally. Furthermore, some of these adenocarcinomas (as well as other adenocarcinomas originating in other sites) express mesothelioma-markers such as calretinin, cytokeratins 5/6 and WT-1 making the

interpretation of immunohistochemical profiles confusing and at times impossible to decipher. Mucin is most commonly seen in adenocarcinomas but can be found in mesotheliomas. These situations have medico legal ramifications, as cases in which a diagnosis of mesothelioma is considered may end up in litigation. Electron microscopy plays an important role in addressing the above mentioned differential diagnoses and, in the hands of experienced electron microscopists, is considered the preferred ancillary diagnostic tool to establish an unequivocal diagnosis. The long (tall)-ratio of height to width greater than 10-15-, sinuous, bushy and complex microvilli in mesotheliomas surrounding glandular spaces differ dramatically from the short, non-branching and blunt microvilli typical of adenocarcinomas in general. Epithelial mesotheliomas often have tonofilaments in the cytoplasm-often perinuclear- of the neoplastic cells and long, well developed desmosomes, further supporting the diagnosis. The differentiating features may be focal and a careful and sometimes extensive evaluation is needed to establish a definitive diagnosis in some cases. Morphological parameters provide clear and irrefutable evidence that can be of great value in defending (confirming) the diagnosis. Pleomorphic sarcomas may also be a source of diagnostic confusion with poorly differentiated carcinomas (see below) and occasionally, melanomas. In this situation a panel of immunohistochemical stains may be quite helpful in narrowing the differential diagnoses or solidifying a final diagnosis. Electron microscopy can then be reserved for those cases in which confusion still exists after the panel of initial immunohistochemical stains is reviewed. Distinguishing some sarcomas from melanoma can be quite difficult by light microscopy. Both are composed of poorly cohesive cells sometimes with abundant eosinophilic cytoplasm. This differential diagnosis arises predominantly in those melanomas lacking identifiable melanin by light microscopy. In this particular situation, immunohistochemical markers for melanoma can be used to make a definitive diagnosis. Melan A and HMB-45 expression can be definitive in making an unequivocal diagnosis. There are cases where ultrastructural evaluation may be needed to solidify the diagnosis or to resolve discrepant or unclear immunohistochemical findings. It should be remembered that benign and malignant peripheral nerve sheath tumors can have melanosomes and express melanocytic markers, and in this situation the presence of a well defined basal lamina surrounding neoplastic cells can be definitive in supporting schwannian differentiation. Of course, clear cell sarcomas, (deep seated melanomas) arising in soft tissues can not be differentiated from metastatic melanoma, regardless of what ancillary diagnostic techniques are employed, as they essentially represent the same entity (melanoma) and their immunomorphological expressions are the same, as expected.

Another potential unique use of electron microscopy is in the evaluation of fine needle aspirates from soft tissue lesions. In this situation, neoplastic cells are usually scanty and pattern recognition is absent. Immunohistochemistry in fine needle aspirates may be difficult to interpret, especially if specimens are bloody and/or the number of neoplastic cells available for evaluation is small. Electron microscopy can provide accurate determination of cell type by identifying specific morphological markers, even when only a handful of cells are available in the sample. Ultrastructural evaluation can also be very important in the evaluation of small blue cell neoplasms presenting in soft tissues, abdominal, mediastinal or retroperitoneal locations, as the differential diagnoses of these soft tissue lesions include carcinomas, sarcomas or lymphomas. Routine assessment of

fine needle aspirates from soft tissue neoplasms may be extremely important and decisive in establishing a definitive diagnosis which will have an immediate impact on patients' management and treatment.

However, in many, if not all situations, it is highly recommended that a combined diagnostic approach using immunohistochemistry and electron microscopy be used, taking advantage of the additive power of both ancillary diagnostic techniques. A factor to be considered is the availability of some of the ancillary diagnostic techniques. In some practices immunohistochemistry is the only readily available diagnostic tool and it is reasonable that in such situations an initial immunohistochemistry work-up be requested to approach the differential diagnosis. However, most sarcomas do not have immunohistochemically distinctive or specific phenotypes. The risk is that since the immunohistochemical findings are generally not diagnostic of specific entities but rather consistent with a diagnosis considering the particular differential diagnosis that is being addressed and the clinical setting. This may result in the wrong diagnosis when a given immunohistochemical profile is considered to fit the favored light microscopic diagnosis. One situation that is particularly vulnerable to mistakes, is when the correct diagnosis is not even suspected on the basis of the light microscopic evaluation, as the requested immunohistochemical panel may not include markers that if expressed may suggest the unexpected diagnosis. Electron microscopy, not only serves to rule out specific entities but the findings may be suggestive or diagnostic of an entity not previously considered in the differential diagnosis. Cytogenetics may play an important role in that certain sarcomas display specific chromosomal aberrations. Good examples are synovial sarcoma which characteristically shows a typical reciprocal translocation involving chromosome X and 18. (X;18) (p11.2;q11.2), Ewings sarcoma (EWS-Fl1), clear cell sarcoma (EWS-ATF1) and myxoid liposarcoma (FUS-CHOP), among other sarcomas. But there are many others, as reviewed by Miettinen in the reference given below. This topic will be addressed in details by another speaker.

Sending a difficult case to a regional electron microscopy laboratory should be always a serious consideration when difficulties in making a definitive diagnosis arise in poorly differentiated tumors. For ultrastructural evaluation to be of most value, proper tissue fixation is very important. Even though tissue obtained from paraffin blocks may retain enough differentiating ultrastructural features that is often not the case. It is quite inappropriate and risky to address difficult differential diagnoses with electron microscopy without adequate tissue preservation that allows proper evaluation of fine ultrastructural details. Tissue fixation in formalin, provided that the tissue is cut in small pieces which assures penetration of the fixative is quite adequate for electron microscopy. It is imperative that areas with necrosis be avoided. Sampling of several areas of a sarcoma is recommended, as the appearance and differentiation of the neoplasm may vary from one area to another. If the specimen available for ultrastructural evaluation is from only a particular area of the tumor, precise classification may be compromised. Data obtained from molecular diagnostics is often devoid of morphological parameters (with the exception of FISH) and although quite useful at times, caution should be employed in the interpretation of this information. Careful correlation with morphological parameters is a must and if a discrepancy arises, there should be no hesitation to proceed with additional work up to clarify the situation.

There are certain ultrastructural features that are particularly useful in the differential diagnosis of sarcomas vs. look-alikes. In the differential diagnosis of sarcomas from poorly differentiated carcinomas, careful evaluation of cell junctions may be of importance. In sarcomas, tight junctions and rudimentary cell junctions can be seen; however, true desmosomes are only found in synovial sarcomas and epithelioid sarcoma. True lumens are also absent in the great majority of sarcomas with the exception of biphasic synovial sarcomas where tonofilaments can also be seen. Tonofilaments are not present in any other sarcomas. On the other hand, these epithelial characteristics (i.e. true desmosomes and rudimentary but well defined lumens lined by microvilli and with apical tight junctions) in a poorly differentiated spindle cell neoplasm may be all that is needed to make a definitive diagnosis of synovial sarcoma. Specific electron microscopic features of certain types of sarcomas can also be helpful in arriving to a definitive diagnosis. Among these are myofibrils with spindle densities or dense bodies as evidence of smooth muscle differentiation, thin and thick filaments, Z lines (discs or band material), myosin-ribosome complexes and/or rudimentary sarcomere structures to establish a diagnosis of rhabdomyosarcoma, abundant rough endoplasmic reticulum and fibronexus for fibroblastic differentiation, interdigitating cell processes, pseudo-mesaxons, external basal lamina and pinocytotic activity as features of schwannian lineage, glycogen "lakes" in Ewings sarcoma and Weibel-Palade bodies, pinocytotic vesicles and basal lamina for endothelial differentiation, just to mention a few of the important ultrastructural criteria that are routinely employed for diagnostic purposes. It is important to emphasize that the overall evaluation of cellular details in the neoplastic cellular elements, rather than individual findings, is the best way to approach the diagnosis from an ultrastructural point of view. Not only what is seen in the neoplastic cells, but also what is absent can be of importance when making a diagnosis. The comprehensive diagnostic approach with use of several diagnostic techniques as soon as the workup of the case begins, often provides a solid unequivocal diagnosis in the shortest period of time. Even though this is perceived by some as a more expensive approach and, therefore, less desirable, the savings to the health care industry in general that result from a prompt and correct diagnosis usually more than justify the additional expense incurred in the pathologic work up of a given case. This approach is also by far the safest in terms of obtaining the correct diagnosis.

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BULLETS:

Immunohistochemical profiles of most soft tissue sarcomas are non-specific

Electron microscopy not only addresses the differential diagnosis in question but may provide crucial information that suggests or is diagnostic of a totally unexpected entity

Electron microscopy has a significant advantage over immunohistochemistry in fine needle aspirate specimens from soft tissue masses

Proper fixation is imperative for ultrastructural evaluation to be able to be most helpful

A combined diagnostic approach using more than one ancillary diagnostic technique (i.e. electron microscopy and immunohistochemistry) is highly recommended, especially in difficult cases

RECOGNIZING HIDDEN PHENOTYPES IN SARCOMAS THROUGH THE ELECTRON MICROSCOPE

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The transmission electron microscope was developed in Germany during the 30s. In diagnostic pathology, the era of electron microscopy occurred between the 60s and the 80s in Western Europe and the United States. Its major role was, and still is, the identification of intracellular details, not identifiable by light microscopy, that may indicate, especially in tumors, a line of differentiation, and a cytological classification, that could not otherwise be appreciated. In this light, electron microscopy was hoped to be histogenetically enlightening and diagnostically useful.

Nowadays, many limiting factors affecting electron microscopy have been resolved. Preparation time has been minimized and embedding, sectioning and scrutiny of grids can be performed in less than 24 hours, resulting in little or no delay in furnishing additional, sometimes essential information that can clarify therapeutic or prognostic implications for the patient. The absolute need of using special fixatives is no longer mandatory and tissue fragments properly fixed in buffered formaldehyde are perfectly suitable for diagnostic studies. Finally, the problem of sample error, because of cellular heterogeneity of tumors, has been documented to be not relevant. In an established electron microscopy unit, the expense of examining a case is comparable to that for other procedures and, therefore, for soft tissue sarcomas, a sample should always be collected in glutaraldehyde, and all cases in which electron microscopy is indicated should be evaluated by laboratories with appropriately experienced personnel.

A major and ongoing role of electron microscopy is as an educational tool in correlating the submicroscopic profile of the cells with their conventional morphology or immunohistochemical properties. In the teaching of pathology, the electron microscope is

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part sarcoma, some examples of extraskeletal myxoid chondrosarcomas, and sclerosing epithelioid fibrosarcoma. Occasionally, synovial sarcoma and leiomyosarcoma can have an epithelioid morphology. For the majority of these, immunohistochemistry is the most useful additional technique. The differential diagnosis include lymphomas and leukaemic deposits, cases of carcinoma, mesothelioma, and melanoma involving soft tissue. Electron microscopy is often helpful in this distinction and in identifying the source of a metastatic carcinoma.

Small Round Cell Soft Tissue Neoplasms

Small round cell soft tissue tumors occur more frequently but not exclusively in childhood and include several entities (Ewing's sarcoma/PNET, desmoplastic small round cell tumor, neuroblastoma, rhabdomyosarcoma, poorly differentiated synovial sarcoma, mesenchymal chondrosarcoma, small cell osteosarcoma, small cell MPNST). Lymphomas and leukaemic deposits, and small cell/neuroendocrine carcinoma have to be included in the differential diagnosis because these tumors can have similar histomorphology; they are easily distinguishable by immunohistochemistry, cytogenetics and molecular techniques. Electron microscopy is not always required for diagnosis of small round cell tumors, but can be extremely contributory when markers are inconclusive, or when there is immunophenotypic overlap and/or a genetic service is unavailable [11].

Anaplastic/Pleomorphic Sarcomas

Many types of soft tissue sarcomas (including examples of myogenic sarcomas, fibrosarcoma, myofibrosarcoma, and MPNST) are characterized by a highly heterogeneous cytology and prominent cellular pleomorphism with almost overlapping architectural and cytological features. The distinction between pleomorphic sarcomas and pleomorphic non-mesenchymal tumors, i.e. lymphoma, melanoma, carcinoma, may also be quite difficult. Relying solely on immunohistochemistry, cytogenetics and molecular techniques may be misleading or play a limited role in the diagnosis of pleomorphic sarcomas. In this group of lesions, electron microscopy can be either enlightening or frustrating, the latter when neoplastic cells are indeed cytologically undifferentiated, showing no key to the identification of their origin or line of differentiation.

In particular, in the group of pleomorphic sarcomas, ultrastructural analysis allows the identification of tumors with myofibroblastic phenotype, a category which is not

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still an invaluable and irreplaceable tool, that can enhance the pathologist's diagnostic skills in the traditional light microscopic routine.

The goal of this lecture is to set electron microscopy in a sensible diagnostic context concerning the undifferentiated and poorly differentiated sarcomas. The determination of the line of differentiation in soft tissue sarcomas is not only crucial in order to ensure proper classification, but also to provide prognostic information [1] and, in relation with the continuous development of new drugs, to assign patients to specific treatment groups. In addition, the correct classification of poorly differentiated soft tissue neoplasm is an essential prerequisite for the analysis of the results of new treatment study protocols.

Ultrastructural studies of undifferentiated/poorly differentiated sarcomas have proved of considerable value [2-4]. Neoplasms that are equivocal at the light microscopic level may exhibit sufficient submicroscopic morphologic features to allow an accurate identification and classification. Despite the use of electron microscopy has declined—due to the relative easiness of use and interpretation of immunohistochemistry—it still retains a significant role because few antibodies are completely specific and sensitive, some sarcomas show divergent differentiation or are polyphenotypic, and others have no specific immunohistochemical markers.

Several studies have attempted to assess the relative contributions of electron microscopy and immunohistochemistry in the diagnosis of soft tissue tumors [2,4-6]. In general, immunohistochemistry can be more useful for smooth muscle neoplasms, epithelioid tumors, and the majority of small round cell neoplasms, while electron microscopy is most useful in cases of malignant peripheral nerve sheath tumor (MPNST), marker-negative synovial sarcoma, fibroblastic/myofibroblastic neoplasms, and pleomorphic soft tissue malignancies. Not unexpectedly, a combination of the two techniques results in a higher rate of specific diagnosis than either alone.

Diagnostically difficult sarcomas tend to fall into several groups on the basis of their light microscopic features. These groups are: (i) spindle cell sarcomas, made up of fusiform, mesenchymal-looking cells; (ii) sarcomas with epithelioid morphology, made up of large round/polygonal cells with uniform, poorly differentiated cytological features; (iii) small round cell soft tissue tumors, composed of undifferentiated-appearing small cells

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identifiable based on histological and immunohistochemical profile, due to the overlap of their immunophenotype with that of leiomyosarcoma and of undifferentiated pleomorphic sarcoma/storiform pleomorphic malignant fibrous histiocytoma [4, 6]. In addition, electron microscopy may allow the recognition of fibroblastic differentiation in so-called undifferentiated pleomorphic sarcoma [12-14], a category encompassing lesions expressing vimentin as the only immunohistochemical marker, and may help in the diagnosis of pleomorphic liposarcoma, when convincing evidence of lipoblastic differentiation cannot be demonstrated at the histological level [3, 15].

In conclusion, electron microscopy still maintains a significant role in surgical pathology of soft tissue neoplasms, even though possibly more focused than in the past. Immunohistochemistry does not allow the identification of every tumor and some diagnostic problems can go unsolved. In many such cases, electron microscopy can furnish the answer, especially in the areas discussed above.

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with scanty amounts of cytoplasm; and (iv) anaplastic/pleomorphic sarcomas, composed of highly heterogeneous neoplastic cells.

Spindle Cell Sarcomas

While typical cases of sarcoma with spindle cell morphology can easily be diagnosed by conventional light microscopy and immunohistochemistry, many tumors lack the typical markers or show profiles overlapping with those of other sarcomas. The electron microscopic study of spindle cell sarcomas is quite often of great diagnostic value and can permit distinction among the members of this group of neoplasms which include both mesenchymal neoplasms, such as fibroblastic, smooth muscle and nerve sheath tumors, and tumors of epithelial origin, such as myoepithelioma and spindle cell (sarcomatoid) carcinoma. Relevant submicroscopic features in the differential diagnosis are surface specializations, cytoplasmic filaments, cell processes, and the presence of typical intracellular organelles.

In particular, the ultrastructural analysis allows the recognition of myofibroblastic differentiation in spindle cell sarcomas (myofibrosarcoma), when neoplastic cells show the presence of abundant, often dilated RER cisternae and bundles of actin filaments with focal densities in the peripheral cytoplasm [4,7-8]. Fibronexus junctions may complete the picture, but they are not considered a required features to define the myofibroblastic phenotype in neoplastic cells.

Another diagnosis in which electron microscopy may furnish a relevant support is that of MPNST. MPNST has no reliable immunohistochemical marker and no specific genetic alteration, and therefore electron microscopy is often useful in the distinction of poorly differentiated lesions, arising in patients not affected by Von Recklinghausen syndrome, from other spindle cell sarcomas, like synovial sarcoma and adult type fibrosarcoma. Ultrastructural features to be searched for are the presence of elongated interdigitating cytoplasmic processes, fragments of external lamina and pynocytotic vesicles [9-10].

"Epithelioid" Sarcomas

Sarcomas which predominantly display an epithelioid appearance include epithelioid sarcoma, malignant rhabdoid tumor, epithelioid endothelial tumors, alveolar soft

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PLEOMORPHIC SARCOMAS

The subclassification of pleomorphic sarcomas which, following at least initial morphologic and immunohistochemical evaluation, appear to be undifferentiated is often frustrating and unrewarding. Among this group of sarcomas, evidence of a specific line of differentiation may be quite focal either by light microscopy (e.g. lipoblasts or osteoid) or, especially so, by electron microscopy. Thus, in this context, if immunostains fail to provide good evidence of a specific line of differentiation, electron microscopy is only rarely of help. Furthermore, this group of soft tissue sarcomas, with the sole exception of dedifferentiated liposarcoma (which retains the ring and giant marker chromosomes derived from the long arm of chromosome 12, as seen in well-differentiated liposarcoma), have non-specific complex karyotypes with no reproducible or diagnostically helpful aberrations. Similarly, these tumors generally lack any evidence of specific translocations that might be identified using FISH or RT-PCR. Importantly, however, as many as 95% of pleomorphic sarcomas can be subclassified using conventional immunostains, supplemented by EM where appropriate and it is nowadays recognized that such subclassification has clinical and prognostic relevance, since tumors showing any type of myogenic differentiation are associated with a much more aggressive clinical course. Thus, it is not good practice (and not good for patient care) to simply rename lesions formerly known as so-called 'MFH' as undifferentiated pleomorphic sarcoma, without first making reasonable efforts to subclassify the lesion by conventional means. The subset of pleomorphic sarcomas which do truly remain unclassifiable or undifferentiated generally seem to have an intermediate prognosis (with a 5-year metastatic rate in the range of 50%) and, when clinically indicated, these lesions, as with spindle cell sarcomas, are most often treated with one of the 'broad spectrum' adult-type sarcoma chemotherapeutic regimens.

SARCOMAS WITH EPITHELIOID MORPHOLOGY

Most well-defined forms of sarcoma with epithelioid morphology (principally epithelioid sarcoma, epithelioid angiosarcoma, epithelioid GIST, epithelioid MPNST, etc.) can readily be recognized and reproducibly diagnosed based on clinical context, morphology and immunostains. However, at least in my experience, there exists a significant subset of poorly differentiated epithelioid malignant neoplasms, presenting in

soft tissue with no apparent evidence of a primary neoplasm elsewhere, in which the differential diagnosis lies between an undifferentiated sarcoma with epithelioid morphology, metastatic carcinoma or metastatic melanoma. Very commonly, extensive immunostains fail to reveal any evidence of epithelial or melanocytic differentiation and, in the setting of clinical evidence that the soft tissue mass is the primary lesion, then there is little option but to regard these as some type of undifferentiated sarcoma.

Tumors in this general category have not been well documented or extensively described in either the pathologic or clinical literature and there are virtually no data as to the behavior and treatment response of such neoplasms. In general, it seems that these lesions can only be labelled as sarcoma as a diagnosis of exclusion, since there are no markers which specifically recognize mesenchymal differentiation. Having said that, if all epithelial markers are negative and there is readily identified CD34 positivity, then this generally would argue quite strongly against a diagnosis of carcinoma. When dealing with tumors in this category, at the present time, it seems that pathologists can do little more than to exclude the possibility of a metastatic epithelial/melanocytic neoplasm (or perhaps anaplastic large cell lymphoma), but further attempts to identify a specific line of differentiation thereafter are almost always fruitless. Lesions of this type warrant further more detailed pathologic study, particularly since this may assist in better definition of the optimal therapeutic approach to such enigmatic neoplasms.

CONCLUSION

In general terms, careful sampling combined with immunohistochemistry and molecular genetic testing, where appropriate, allows meaningful classification of many of the lesions initially thought to be undifferentiated. In my personal experience, it seems that the role of electron microscopy is becoming increasingly limited, although in part this also reflects diminished availability of this element of technical infrastructure. In general, subclassification of undifferentiated round cell sarcomas, where possible, has the greatest clinical impact and the subclassification of seemingly undifferentiated spindle cell sarcomas, if feasible, is also sometimes useful. At this point in time, so long as reasonable efforts are made to subclassify any pleomorphic or epithelioid malignancy arising in soft tissue by conventional light microscopic means, then further attempts to subclassify the subset of these lesions which appear to be truly

undifferentiated are, unfortunately, often unhelpful and the clinically beneficial yield is low.

Molecular Profiling in the Diagnosis and Treatment of High Grade Sarcomas

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High density microarrays are used to measure in a comprehensive manner on a large scale ("profile") gene expression patterns (when tumor RNA is tested) or gene copy number changes (when tumor genomic DNA is tested). Microarray-based studies are increasingly useful in addressing a wide variety of questions in sarcoma biology. During this talk, a brief description of the microarray methodology and data analysis will be provided, followed by few examples of the recent applications of gene expression in the diagnosis, classification and treatment of sarcomas.

I. DNA Microarray Technology

Expression profile refers to the process of measuring the expression of thousands of individual genes simultaneously in a given tissue sample. The basic principle is hybridization-based.

Microarrays: a collection of spots on a solid surface (often a glass slide) arranged in neat rows and columns, so that the origin of each spot is known. Depending on the type, the spots can contain DNA sequences (as in gene microarrays), tissue fragments (tissue microarrays) protein (protein microarrays), etc.

Types of high-density microarrays:

a) **cDNA spotted arrays.** Libraries of cDNA clones (20,000-40,000 most commonly expressed genes) are robotically arrayed on standard 1x3 glass microscope slides and usually produced by the investigator or by a core facility. This method requires competitive

discriminate tumors from normals? Are there correlations between expression profiles and other molecular or pathological properties of the tumor? Are there correlations between expression profiles and clinical outcome and response to therapy? With what degree of certitude these results are not due to chance alone? Are there subsets within tumors of the same apparent class? Are these pathways relevant to the tumor phenotype or as potential targets for therapy? These important questions and the need to develop the mathematical tools to address them have attracted the attention of computer scientists, engineer, and biostatisticians. Numerous computational approaches have been developed.

Microarray analysis whether supervised or unsupervised ultimately generates lists of genes that discriminates among samples. Making sense of these gene lists presents a significant challenge. Gene names can be misleading and the majority of genes are linked to little or no functional information. Most of the interpretations that arise from exploring microarray data should be considered hypotheses, rather than conclusions. Additional forms of experimentation is most likely necessary to establish a conclusive connection between a gene and the tumor in which is expressed.

Validation the microarray data: How reliable is the data? Tissue microarrays for in situ mRNA hybridization or immunohistochemistry provide the possibility of confirmatory studies on large number of samples.

III. Applications of the DNA microarray technology to cancer diagnosis and prognosis

Cancer is a disease of disturbed genome function. Irrespective of whether this is the result of a point mutation, deletion, translocation, gene amplification, or methylation, the malignant phenotype is mediated by a characteristic pattern of gene expression. Identifying these genes whose expression differs between normal tissues and tumors and among tumor types is the focus of today's research. Investigators in the field initially performed studies in which tumors of different morphology or different primary sites were shown to have clearly distinguishable patterns of gene expression. This type of diagnostic classification by analysis of expression profile is called "class prediction". These proof-of-principle studies served to validate cDNA microarray technology and the researchers then shifted their focus to

hybridization to a mixture of reference and test mRNA, each labeled with a distinct fluorochrome (red-tumor and green-reference).

b) **Affymetrix** (Santa Clara, CA) **oligonucleotide microarrays.** Affymetrix uses similar equipment to that used for making silicon chips for computers, allowing mass production of chips at reasonable costs. Affymetrix uses masks to control synthesis of oligonucleotides on the surface of the chip. Light is directed through a mask to deprotect and activate selected sites, and protected nucleotides couple to the activated sites. The process is repeated, activating different sets of sites and coupling different several hundred thousand squares (cells) each containing many copies of one oligonucleotide. So the result is several hundred thousand different oligos, each of them in millions of copies. It is not used for gene discovery because this approach requires knowledge of the sequence in order to choose probes for a given gene or EST. 25 base oligos are synthesized *in situ*, by **light directed oligonucleotide synthesis**. Each gene represented by at least 20 oligos distributed along the 5' to 3' length of the gene. Each is paired with a single base mismatch oligo. The arrays are constructed on rigid material (glass) they can be inverted and mounted in a temperature-controlled hybridization chamber. It is based on an important observation that single stranded DNA binds strongly to nitrocellulose membranes in a way that does not re-associate with each other but permits hybridization with cRNA. A fluorescently tagged nucleic acid sample injected into the chamber hybridizes to complementary oligonucleotides on the array. Laser excitation enters through the back of the glass support, fluorescence emission is collected by a lens and passes through a series of optical fibers to a sensitive detector. By scanning the laser beam (translating the array) a quantitative two-dimensional fluorescence image of hybridization intensity is quickly obtained.

Probe redundancy: the use of multiple oligonucleotides of different sequence designed to hybridize to different regions of the same RNA. The use of multiple independent detectors for the same molecule greatly improves signal-to-noise ratios, improves the accuracy of RNA quantitation, drastically reduces the rate of false positives and miscalls. An additional level of redundancy comes from the use of mismatch (MM) control probes that are identical with perfect match (PM) probes except for a single base difference in a central position. The MM probes act as specificity controls that allow the direct subtraction of both background and

identification of molecularly defined tumor entities that were inapparent by conventional pathologic analysis ("class discovery"). New unsuspected biological subsets were detected among cutaneous melanomas, breast carcinomas, and pediatric acute lymphoblastic leukemias. In other cases, there has been "class rediscovery" or "class confirmation".

Variability in the results, more notable in prognostic studies than in class prediction, have already appeared in the literature and is most likely related to inter-laboratory and inter-platform reproducibility. For example, two large breast cancer expression profiling studies differed considerably in the results of unsupervised clustering: one identified 3 major subsets (ER-positive luminal cell type, basal cell type, and ERBB2-amplified type), while the other detected only 2 major subgroups according to ER status and lymphocytic infiltration. Moreover, in the latter study, well-established clinical markers, such as ERBB2 and ER were not found within a list of 70 genes linked to outcome.

IV. Molecular Profiling in the Diagnosis and Treatment of High Grade Sarcomas

Studies involving gene expression technology have been mainly applied as a source of diagnostic markers for sarcoma diagnosis and to their role in clarifying sarcoma classification. A general observation of microarray-based expression profiling studies of sarcomas is that translocation-associated sarcomas are robustly clustered by expression profiling using cDNA microarrays, whereas so-called complex karyotype sarcomas tend to be less tightly clustered. Thus, complex karyotype sarcomas that often show different gains and losses from case to case are also likely to show more variability in gene expression patterns, leading to less robust unsupervised clustering of the expression profiles. Indeed, this tumor group may be better studied by profiling of gene copy number changes using array-based CGH.

Our study on 51 soft tissue sarcoma specimens using hierarchical cluster analysis demonstrated distinct clusters for GIST, synovial sarcoma, clear cell sarcoma and round cell liposarcoma. Several fibrosarcoma tumors fall in close proximity to synovial sarcoma. Pleomorphic sarcomas exhibited overall poor correlation and consistency by boot strap

cross-hybridization signals, and allow discrimination between "real" signals and those due to non-specific and semi-specific hybridization (hybridization of the intended RNA molecules produces more signal on PM probes than for MM probes, resulting in consistent patterns that are highly unlikely to occur by chance: the pattern recognition rules are codified in the analysis software).

c) **array-based comparative genomic hybridization (array-CGH):** uses microarrays with genomic DNA sequences and used to interrogate tumor DNA samples in order to provide information regarding gene copy number (amplification and deletions).

II. Data Analysis

Data from a single 22,000-gene Affymetrix GeneChip occupies 100 MB of storage and require significant manipulation before interpretation. Normalization of all values to a mean of 500 is commonly used. Data cleanup is an important first step to reduce the noise, increase sensitivity, and manage artifacts of data set transformation. Filtering and statistical analyses constraints are applied to exclude those genes that did not vary significantly between comparison groups or that are not expressed at high enough levels. Data from a series of tumor samples with expression levels for thousands of genes can present a challenge for analysis. A method for data storage and retrieval in a database is essential.

Hierarchical Clustering or Cluster Analysis: uses standard statistical algorithms to arrange genes according to similarity in pattern of gene expression. The output is displayed graphically, conveying the clustering and the underlying expression data simultaneously. Relationship among genes is represented by a tree whose branch lengths reflects the degree of similarity between genes (dendrogram). This method recognizes groups of genes that are co-expressed, providing a new insight of their possible function. It also identifies samples whose gene expression patterns correlate. The basic idea is joining together points into clusters.

Appropriate selection of analysis tools will depend on the questions to be addressed. Certain key questions predominate cancer-related microarray research: "Can two types of cancer be discriminated? What genes discriminate them more clearly? Are there genes that

analysis. However, within this group certain prominent clusters were observed for both malignant fibrous histiocytoma (MFH) and leiomyosarcoma.

One example of "class confirmation" study in sarcomas includes the comparison of **GIST versus leiomyosarcoma**. Previous RNA expression profiling studies of different soft tissue sarcomas indicated that GIST expression profiles were distinct and quite homogeneous in part due to the unique derivation of GIST from ICC. GISTs are characterized by a distinctive transcriptional signature, as a result of overexpression of *KIT*, *PRKC#*, *DOG1*, which can be applied in tumor diagnosis, even when compared with their closest pathologic mimic, leiomyosarcomas. Likewise, several studies have used microarrays to identify genes differentially expressed between *PAX-FKHR* fusion-positive alveolar and *PAX-FKHR* fusion-negative embryonal rhabdomyosarcoma.

Molecular profiling was also applied to clarify the conflicting data on the relationship of **clear cell sarcoma (CCS) to cutaneous melanoma**. While the two are clearly genetically distinct, as CCS lack the *BRAF* mutations commonly seen in melanomas, whereas melanomas do not contain the *EWS-ATF1* fusion, by expression analysis CCS share a melanocytic gene signature with melanomas.

In the study by Baird et al, the unsupervised hierarchical analysis on MFH cases alone identified 2 distinct groups: one carrying a muscle profile (myosin X, sarcoglycan β , tenascin C) and the second group revealed an immune regulatory gene profile (HEM1, MX1, DAP10). Interestingly, the storiform-pleomorphic and the myxoid subtypes equally populate both groups of tumors. However the distinction between MFH with myogenic differentiation versus inflammatory characteristics might have clinical relevance. Myogenic differentiation as reflected by IHC in MFH or pleomorphic sarcoma, NOS, has previously shown to have an adverse clinical outcome.

New IHC markers emerging from microarray studies include DOG1 in GIST, TLE1 in synovial sarcoma, AP2- β in alveolar rhabdomyosarcoma, and Apo D in dermatofibrosarcoma protuberans (DFSP).

Still in incipient phases, microarrays studies have been used to predict outcome or therapy response. Poor outcome Ewing sarcoma has been linked with alterations in cell cycle regulatory genes. In a recent study, West et al suggested that expression signatures of either fibromatosis or solitary fibrous tumor can be identified in the stromal component of most breast carcinoma and can be correlated with clinical outcome. The fibromatosis signature is associated with good outcome breast tumors, while solitary fibrous tumor profile is found in poor clinical outcome.

Three different studies have been assessed the correlation of transcriptional profile of pre-treatment osteosarcoma biopsy and response to chemotherapy. Disappointingly, limited overlap has been noted among the results of these studies, which can be explained as a consequence of different platforms or bioinformatics approaches used, differences in patient population or chemotherapy regimens applied, etc.

The ability of mining candidate genes for targeted therapies through microarray analysis has been validated with the GIST paradigm, where the overexpressed KIT or PDGFRA provided the biologic basis for imatinib mesylate therapy. Other examples include dermatofibrosarcoma protuberans, where through an autocrine/paracrine mechanism, the overexpressed PDGFB up-regulates its receptor, PDGFRB, therefore providing another susceptible target to selective tyrosine kinase inhibition. However, targeted therapy appears to be most successful in sarcomas with a distinct underlying molecular biology, and has been failed in sarcomas with non-recurrent complex karyotype. Since drugs target proteins rather than genes, protein level validation is critical and can be performed on the tissue microarray by using IHC and ISH techniques.

In summary, microarray studies applied so far in sarcomas support their classification into genetically simple and genetically complex categories and have provided useful diagnostic markers, as well as insight for novel and targeted therapeutic approaches. However, there is a considerable vacuum between the practical world of hospital-based

molecular diagnostic laboratories and some of the predictions prompted by high-throughput genomics work. Hopeful statements, such as one made in 1999 that "doctors will be offering gene expression profiles to some patients in the next 3 years", failed to consider the many issues in moving complex assays from research laboratories to clinical laboratories. Because of the regulatory, billing, quality control, and test validation concerns, combined with limited resources, academic molecular diagnostic laboratories are extremely selective in their test menus. Furthermore, it is not clear yet that the present cost of microarray, large-scale expression profiling for "class prediction" is more cost-effective than established diagnostic approaches, i.e., histopathology supplemented in selected cases by IHC, cytogenetics, or molecular assays.

In short term however the impact of tumor expression profiling is in the identification of new diagnostic and prognostic markers, which can be studied individually by more conventional techniques. The availability of TMA accelerates the validation of these new IHC assays. The next step will require efforts in academic and commercial laboratories to generate new antibodies for the products of differentially expressed genes without currently available antibodies.

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