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Tissue Microarray Validation of Epidermal Growth Factor Receptor and SALL2 in Synovial Sarcoma with Comparison to Tumors of Similar Histology

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Histological diagnosis of synovial sarcoma can be difficult. Genome-wide expression profiling has identified a number of genes expressed at higher levels in synovial sarcoma than in other soft tissue tumors, representing excellent candidates for diagnostic immunohistochemical markers. A tissue microarray comprising 77 sarcomas, including 46 synovial sarcomas, was constructed to validate identified markers and investigate their expression in tumors in the differential diagnosis of synovial sarcoma. Immunostaining was performed for two such markers, epidermal growth factor receptor and SAL (drosophila)-like 2 (SALL2), and for fifteen established markers used in the differential diagnosis of sarcomas. As predicted by expression profiling, epidermal growth factor receptor (a potential therapeutic target) and SALL2 stained most cases of synovial sarcoma; staining was significantly less common among other tested sarcomas. Hierarchical clustering analysis applied to immunostaining results for all 18 antibodies showed that synovial sarcomas, leiomyosarcomas, hemangiopericytomas, and solitary fibrous tumors cluster distinctly, and assigned one case with indeterminate histology as a Ewing sarcoma. Digital images from over 2500 immunostained cores analyzed in this study were captured and are made accessible through the accompanying website: http://microarray-pubs.stanford.edu/tma_portal/synsarc. *(Am J Pathol 2003, 163:1449–1456)*

The subclassification of sarcomas has become increasingly sophisticated with the introduction of routine immunohistochemistry into diagnostic pathology. The combination of morphological examination and immunohistochemistry has resulted in the evolution of current diagnostic terminology and practice. Synovial sarcoma, for example, was initially recognized as a biphasic tumor with epithelial and uniform spindle-cell components, but now encompasses a wider morphological spectrum of tumors, including monophasic spindle cell and poorly differentiated subtypes,[1,2] such that classic biphasic tumors now account for a minority of cases of synovial sarcoma encountered in practice.[3] However, despite progress in subclassification of sarcomas, there remain a significant number of cases for which the exact diagnosis is uncertain.

Expression profiling of tumors with cDNA microarrays can identify genes expressed in association with distinct tumor types, and has the potential to identify subgroups of tumors that cannot be recognized by morphological examination.[4] These classes can be identified by hierarchical cluster analysis of the gene expression data, a statistical method of grouping tumors based on degree of relatedness of their gene expression profiles,[5] or by other classification methods.[6] Through cDNA microarray profiling of 46 soft-tissue tumor specimens, we identified a group of more than 100 genes and expressed sequence tags (ESTs) that are characteristically expressed at higher levels in synovial sarcomas than in leiomyosarcomas, schwannomas, liposarcomas, malignant fibrous histiocytomas, or gastrointestinal stromal tumors.[7] Several of these results have since been corroborated in independent studies.[8,9] These genes fall into several classes, including homeotic transcription factors, genes involved in chondrogenesis and skeletal development, regulators of retinoic acid response, neuronal proteins, and others.[7]

Tissue microarrays[10] can be used to test the diagnostic utility of antibodies against proteins encoded by differentially expressed genes, using large numbers of archival patient specimens. By including other tumors that are in the differential diagnosis in the same tissue microarray, the precise specificity of staining can be directly assessed. Immunostaining of serial sections of the tissue microarray also permits comparison with established immunohistochemical markers.

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Table 1. Cases Used for Tissue Microarray

46 synovial sarcoma
35 monophasic
11 biphasic*
30 grade 2
16 grade 3
30 arising in limb
16 arising in other site
5 Ewing sarcoma
5 solitary fibrous tumor
5 malignant peripheral nerve sheath tumor
5 leiomyosarcoma
5 low-grade fibromyxoid sarcoma
4 soft tissue Hemangiopericytoma
7 sarcomas of uncertain subtype

*Glandular epithelial elements were present in the tissue array cores for only 4 of 11, and scoring was based on the spindle cell component in these cases.

Our objectives in this study were to 1) test whether genes found to be characteristically expressed in synovial sarcomas by cDNA expression profiling were differentially expressed in these tumors at the protein level, 2) test the new candidate immunohistochemical markers against a series of well-characterized sarcomas in the differential diagnosis, as well as several cases with diagnostic uncertainty, 3) apply hierarchical cluster analysis to immunostaining data to determine the ability of a panel of diagnostic antibodies to allow meaningful grouping of sarcoma immunoprofiles, and 4) use the World Wide Web to make the immunohistochemistry results accessible.

Materials and Methods

Tissue Array Construction

Representative archival paraffin blocks were retrieved from a total of 82 cases, as detailed in Table 1. This included all available cases of synovial sarcoma at Vancouver General Hospital accessioned during the years 1982 to 2000, as well as 29 recent cases representing six tumor types that can histologically mimic variants of synovial sarcoma, and seven further cases of sarcoma in which there was not a consensus as to the correct subclassification (but where synovial sarcoma had been considered a diagnostic possibility). All cases were reviewed by pathologists with expertise in soft-tissue tumors (JXO and MvdR). For 44 of the 46 synovial sarcoma cases, the diagnosis was confirmed by cytogenetics, reverse transcription-polymerase chain reaction, fluorescent *in situ* hybridization, expression profiling and/or by the presence of biphasic histology (see Supplemental Table 1 at http://www.amjpathol.org). Duplicate 0.6-mm cores were taken from representative areas of tumor and inserted into a recipient paraffin block to create a tissue microarray[10] containing a total of 157 cores, including three murine kidney orientation/control cores. Clinical follow-up was available for all of the synovial sarcoma cases with a mean follow-up period of 81 months.

Immunohistochemistry

Commercially available polyclonal antibodies against SAL (Drosophila)-like 2 (SALL2) and two monoclonal antibodies recognizing epidermal growth factor receptor (EGFR) were purchased. A panel of fifteen other standard immunohistochemistry markers recognizing synovial sarcomas or the other arrayed tumors was also used. Antibody sources and staining conditions, including antigen retrieval methods are summarized in Table 2. Microwave antigen-retrieval was performed in a pressure cooker by boiling 4 minutes then incubating 20 minutes in 10 mmol/L citrate buffer (pH 6.0). Proteinase antigen-retrieval consisted of a 4-minute incubation in protease-1

Table 2. Antibodies for Immunohistochemistry

Antigen	Clone	Supplier	Dilution	Pretreatment
bcl-2	124	DAKO	1:20	Microwave
CD34*	QBEnd10	DAKO	1:200	Microwave
CD99	O13	ID Labs Biotechnology	1:20	Microwave
CD117 (c-kit)	Polyclonal	Santa Cruz Biotechnology	1:100	None
Cytokeratin 7*	OV-TL 12/30	DAKO	1:200	Proteinase
Desmin*	D33	DAKO	1:200	Microwave
EGFR*	31G7	Zymed	1:10; 1:20	Proteinase
EGFR*	2-18C9	DAKO	Prediluted	Proteinase
EMA*	E29	DAKO	1:200	None
H-caldesmon*	h-CD	DAKO	1:400	Microwave
HER2/neu	Polyclonal	DAKO	1:10000	Microwave
Low mw keratins*	CAM5.2	BD Biosciences	1:50	Proteinase
Muscle actin*	HHF35	DAKO	1:100	None
Neurofilaments	2F11	DAKO	1:2000	Proteinase
Pankeratins*	Polyclonal	DAKO	1:4000	Proteinase
S-100*	Polyclonal	Dr. A. Marks†	1:200	None
SALL2*	Polyclonal	Chemicon	1:100	Microwave
Smooth muscle actin*	1A4	DAKO	1:200	None

*Immunohistochemistry results scanned and posted at http://microarray-pubs.stanford.edu/tma_portal/synsarc for these antibodies.
†From Banting and Best Department of Medical Research, University of Toronto, Ontario, Canada.
mw, molecular weight.
Locations of suppliers: DAKO, Carpinteria, CA; ID Labs Biotechnology, London, Ontario, Canada; Santa Cruz Biotechnology, Santa Cruz, CA; Zymed, San Francisco, CA; BD Biosciences, San Jose, CA; Chemicon, Temecula, CA.

Table 3. Immunostaining Results with Potential Novel Markers for Synovial Sarcoma

Antibody		Synovial sarcoma			Other sarcomas in differential diagnosis*			Other sarcomas with strong staining
		Strong	Weak	Neg.	Strong	Weak	Neg.	
EGFR	*n*	16	12	14	1	9	19	MPNST (1/5)
31G7	%	38%	29%	33%	3%	31%	66%	
2-18C9	*n*	22	16	4	8	6	15	EWS (1/5), SFT (1/5), MPNST (3/5), LGFMS (3/5)
	%	52%	38%	10%	28%	21%	52%	
SALL2	*n*	18	19	5	2	10	17	SFT (2/5)
	%	43%	45%	12%	7%	34%	59%	

*5 Ewing sarcomas (EWS), 5 solitary fibrous tumors (SFT), 5 MPNST, 5 leiomyosarcomas (LMS), 5 LGFMS, and 4 hemangiopericytomas (HPCT). Neg., negative.

solution (Ventana, Tucson, AZ) according the supplier's recommended protocol. Antigen retrieval was achieved by microwaving the slides in citrate buffer (pH 6.0. Visualization was by biotin-avidin immunoperoxidase methodology, with staining done on a Ventana automated immunostainer. For SALL2, slides were stained manually using the EnVision+ System, HRP (DAB) kit (DAKO, Carpinteria, CA), following manufacturer's instructions except that PBS (not Tris) was used as wash buffer.

For all synovial sarcoma cases, immunostain scoring was based on the spindle cell component only, regardless of whether a biphasic epithelial component was present or not. For all antibodies, staining of tissue array cores was scored as strong (diffuse and/or intense positive-staining in at least 20% of the cells), weak (any lesser degree of staining), negative, or uninterpretable (insufficient tumor cells present). Where duplicate cores gave discordant results, the higher score was used. Studies have shown that duplicate cores in tissue microarrays correct for focal expression of antigen in the majority of cases.[11]

Hierarchical Clustering Analysis

Hierarchal clustering analysis of our tissue microarray data were performed using software tools (the Cluster and TreeView programs) that were originally developed for analyzing cDNA microarray data. Cluster and TreeView software are freely available programs that can be accessed at http://rana.lbl.gov/EisenSoftware.htm. An Excel macro, TMA-Deconvoluter,[12] was designed and written specifically for converting raw tissue microarray staining data from a workbook with multiple worksheets in Excel, into a tabular format compatible for use with Cluster. This software also allows for a direct link between clustered immunostain data and digital images of the stained tissue cores that are stored on a server. The software is freely available at: http://genome-www.stanford.edu/TMA/. Average-linkage hierarchical clustering[5] was then performed on the reformatted data using the Cluster software, with filters set to require at least 50% interpretable immunostaining data for each tumor (which excluded two cases of synovial sarcoma from the analysis). Hierarchical clustering works in two dimensions: tumors are grouped together based on the relatedness of their immunostaining profile, and in a second dimension antibodies are grouped based on which tumors they stain. To give equal weight to the results for each specific antigen target in the cluster analysis, results from the two anti-EGFR antibody stains were given one-half weighting relative to the other antibody immunostains. The output was visualized using TreeView, which graphically displays the results of the cluster analysis as dendrograms and arrays, wherein the rows and columns correspond to the raw staining data, presented in the order determined by hierarchical clustering.

A χ^2 test with Bonferroni correction for multiple comparisons was used to analyze the significance of immunohistochemical scoring differences between synovial sarcoma and the other types of sarcoma in the differential diagnosis.

Results

Using a tissue microarray (Table 1), we tested the performance of 15 antibodies recognizing established sarcoma markers (Table 2), as well as three antibodies against novel markers that appeared promising from cDNA microarray analysis[7] for their ability to distinguish synovial sarcoma from other histologically similar tumors.

Novel Markers of Synovial Sarcoma

Results of immunostaining with potential novel markers of synovial sarcoma are shown in Table 3. Tissue microarrays with 0.6-mm cores failed to represent glandular areas of biphasic synovial sarcomas in 7 of 11 cases. For this reason, scoring was based on the spindle cell component.

The EGFR is an oncogene with tyrosine kinase activity[13] for which commercial antibodies are available. We tested two different monoclonal antibodies against EGFR, Zymed 31G7, and the DAKO 2–18C9. The 31G7 antibody (at 1:20 dilution) showed strong membranous immunostaining of spindle cells in 16 of 42 synovial sarcomas, and weaker staining in a further 12 of 42 (Figure 1A). With this antibody, EGFR staining was observed significantly more frequently in synovial sarcoma than in the other arrayed tumors which are important in the differential diagnosis of this cancer ($P = 0.006$). Strong immunohistochemical staining for EGFR with 31G7 had a sensitivity

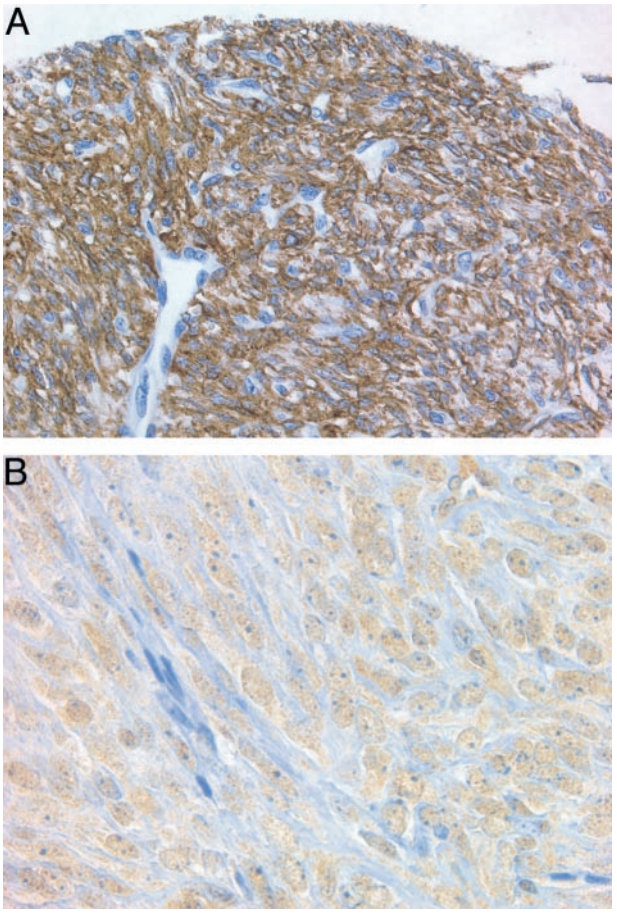

Figure 1. A: Strong, predominantly membranous EGFR immunostaining of a monophasic synovial sarcoma (original magnification, ×400). **B:** Nuclear immunostaining of synovial sarcoma by SALL2 (original magnification, ×600).

of 38% and a specificity of 97% for synovial sarcoma in this series of tumors. No correlation was observed between EGFR staining and patient outcome (data not shown). The 2–18C9 anti-EGFR monoclonal antibody showed strong staining in 22 of 42, and weaker staining in 16 of 42 of the synovial sarcomas. The difference between synovial sarcoma and the other sarcomas (Table 3) remained statistically significant ($P = 0.001$). While this antibody was more sensitive, its staining was less specific in comparison to the other 29 arrayed tumors in the differential diagnosis, with some other sarcomas showing strong positive immunostaining, including 3 of 5 malignant peripheral nerve-sheath tumors (MPNST) and 3 of 5 low-grade fibromyxoid sarcomas (LGFMS). Strong staining with antibody 2–18C9 was 52% sensitive and 72% specific for synovial sarcoma, whereas the presence of any (weak or strong) staining was 90% sensitive and 52% specific.

SALL2, a poorly characterized zinc-finger-containing transcription factor with homology to homeotic genes of *Drosophila melanogaster*, [14] showed strong nuclear immunostaining of spindle cells in 18 of 42 synovial sarcomas, and weak staining in a further 19 of 42 (Figure 1B). SALL2 immunoreactivity was observed significantly more frequently in synovial sarcomas compared to the other sarcomas present in the microarray ($P < 0.001$), although strong nuclear SALL2 positivity was observed in 2 of 5 cases of solitary fibrous tumor. The sensitivity of strong nuclear SALL2 staining for the diagnosis of synovial sarcoma was 43% and the specificity was 93%. No correlation was observed between SALL2 staining and patient outcome (data not shown).

Four of the biphasic synovial sarcomas had interpretable epithelial areas included in the arrayed tissue cores. EGFR immunostaining of this component was weakly positive in one of these cases, and negative in three. SALL2 nuclear immunostaining was strongly positive in two cases and weakly positive in the other two.

The preferential staining of synovial sarcomas with the antibodies against EGFR and SALL2 was highly significant, by a χ^2 test with Bonferroni's adjustment for multiple comparisons. For each of the three antibodies, adjusted standardized deviates were >2 for strong staining of synovial sarcomas and for negative staining of the other tested sarcomas. Considering the synovial sarcoma cases only, the subset with strong staining by either EGFR antibody was not the same as the subset identified by strong SALL2 immunostaining (correlation not significant by χ^2).

Tissue Microarray Analysis of Previously Characterized Immunohistochemical Markers

Results of staining with the panel of 15 established immunohistochemical markers are shown in Table 4. The most sensitive marker for synovial sarcoma was bcl-2, which stained 91% of cases, followed by pancytokeratin (77%), EMA (75%), and cytokeratin 7 (67%). Among these, the most specific marker of synovial sarcoma *versus* other sarcomas was cytokeratin 7, with only 2 of 29 non-synovial sarcomas staining positively, compared to 29 of 43 synovial sarcomas. These data are consistent with previously published reports.[15]

Hierarchical Clustering Analysis of Tissue Microarray Immunostains

Hierarchical cluster analysis is able to sort antibodies according to similarities among the tumors that they stain. Different titrations of the same antibody (eg, anti-EGFR monoclonal 31G7) clustered together (data not shown). Antibodies clustered into groups that reflected the tissue specificity of expression of the cognate antigens, including epithelial, muscle, and nerve markers (Figure 2). Interestingly, the top-most branch of the antibody dendrogram clearly separates the eight markers which stain most synovial sarcomas (keratins, EMA, bcl2, and the new markers EGFR and SALL2) from the 10 that do not. The staining patterns observed for EGFR and SALL2 were otherwise not closely correlated with each other nor with those of any other antibodies in the panel, suggesting that the expression of both EGFR and SALL2 may be largely independent from that of other examined markers.

Table 4. Immunostaining Results on Tissue Array with Established Sarcoma Markers

Antigen	Synovial sarcoma	EWS	SFT	MPNST	LMS	LGFMS	HPCT
bcl-2	29/11/4	1/1/3	4/1/0	0/4/1	0/2/3	4/0/1	3/1/0
CAM 5.2	13/4/27	2/0/3	0/0/5	0/1/4	0/1/4	0/0/5	0/0/4
CD117 (c-kit)	0/1/42	0/0/5	0/0/5	0/1/4	0/0/5	0/0/5	0/0/4
CD34	1/0/43	0/0/5	1/4/0	0/1/4	0/0/5	0/0/5	3/1/0
CD99	1/11/32	5/0/0	4/1/0	3/1/1	1/2/2	1/1/3	0/1/3
Cytokeratin 7	19/10/14	0/0/5	0/0/5	0/0/5	0/0/5	0/0/5	0/2/2
Desmin	0/0/44	0/0/5	0/0/5	1/0/4	0/0/5	0/0/5	0/0/4
EMA	20/13/11	0/0/5	0/0/5	0/0/5	0/2/3	0/0/5	0/1/3
H-caldesmon	0/0/42	0/0/5	0/0/5	0/0/5	5/0/0	0/0/5	0/0/4
HER-2/NEU	0/11/32	0/0/5	0/0/5	0/0/5	0/0/4	0/0/4	0/0/4
Muscle-specific actin	0/1/43	0/0/5	0/0/5	1/0/4	5/0/0	0/0/5	0/0/4
Neurofilaments	3/3/39	0/0/5	0/0/5	0/0/5	0/0/5	0/0/5	0/0/4
Pancytokeratin	24/10/10	1/2/2	0/0/5	2/1/2	0/1/4	0/2/3	0/1/3
S-100	2/4/37	0/0/5	0/0/5	1/1/3	0/0/5	0/1/4	0/0/4
Smooth muscle actin	1/1/42	0/1/4	0/0/5	0/1/4	5/0/0	0/0/5	0/0/4

Staining results are displayed as strong/weak/negative.

The tumors were also subjected to a hierarchical cluster analysis, on the basis of similarity in their patterns of staining with this panel of antibodies (Figure 2). Most synovial sarcomas clustered onto a single major branch, but because of negative or weak detection of epithelial markers in the tissue array cores, 15 of 44 cases clustered outside this main branch. Eleven of these 15 synovial sarcoma cases nevertheless had positive SALL2 staining, and 12 were positive for EGFR using the 2–18C9 antibody.

All leiomyosarcomas and hemangiopericytomas clustered onto distinct branches. All five solitary fibrous tumors clustered onto a terminal branch at high correlation, although this branch did include several other single tumors which shared a pattern of strong staining for bcl2 and CD99, and negative staining for epithelial, neural and muscle markers. LGFMS and Ewing sarcomas displayed only a very loose grouping with this panel of antibodies, and MPNST did not present a sufficiently consistent immunoprofile to cluster in any recognizable pattern.

Among the seven tumors in which there was diagnostic uncertainty about the proper subclassification, case 49 clustered quite distinctly with two Ewing sarcoma cases. On review of clinical records from case 49, where the histological features had not permitted a definitive diagnosis, it became clear that a later cytogenetic analysis had shown t(11;22), confirming the diagnosis of Ewing sarcoma. The other tumors which had lacked a consensus diagnosis did not cluster tightly with any other sarcoma subtypes.

Discussion

cDNA expression profiling of soft tissue tumors[7] defined a group of genes characteristically expressed by synovial sarcomas. The present study was undertaken to determine whether the products of these genes were present in tissues from synovial sarcoma patients. Immunohistochemisty provides a practical confirmatory test of the specificity of the expressed proteins, can immediately be applied to clinical diagnosis, and allows the precise

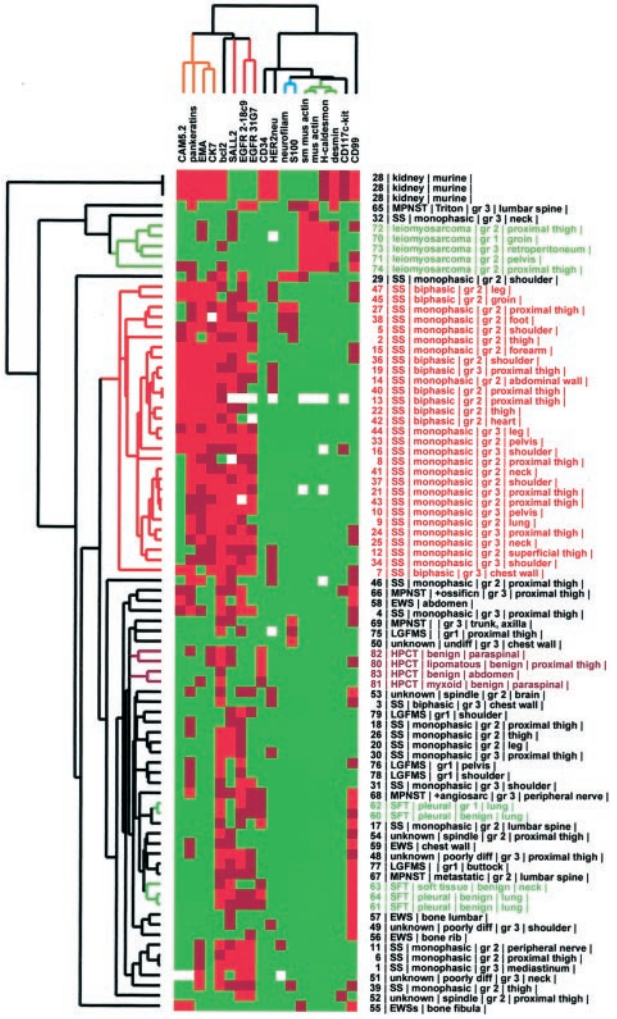

Figure 2. Hierarchical cluster analysis of synovial sarcoma tissue microarray immunostaining results. For each of the antibodies indicated at the **top** of the figure, strong positive staining is indicated by a red square, weak positive by brown, absence of staining as green, and no available data as white. The dendrogram at the **top** shows the clustering of the antibodies based on the relatedness of tumors stained by each antibody. Individual case details are listed at the **right**; the dendrogram at the **left** shows the clustering of the tumors based on the degree of similarity of their immunohistochemical staining results.

localization of expression to the tumor cells themselves.[4] The tissue microarray technique allows practical use of archival material for which clinical outcome data are available. Construction of a synovial sarcoma tissue array allows high-throughput analysis of new markers, parallel comparison of their performance *versus* known markers of this disease, and, by inclusion on the same array of other tumor types that need to be considered in the differential diagnosis, provides an immediate direct assay for the specificity of a new marker in a diagnostic setting.

EGFR as detected by antibody 31G7 showed strong membranous immunostaining in 38% of synovial sarcomas, and weaker staining in a further 29%, whereas cDNA microarray analysis[7] showed strong EGFR RNA expression in 4 of 8 synovial sarcomas, and moderate expression in a further 3 of 8. Thus, these methods are in good agreement with analysis at the nucleic acid level that appears more sensitive. On review of the literature, in one report that included two frozen synovial sarcoma specimens, EGFR antibody positively stained the spindle cell component,[16] and in a recent report on a small series of synovial sarcomas from 17 patients, 68% were reported as immunoreactive.[17] Of note, inhibitory small molecules and antibodies targeting EGFR are currently under investigation as carcinoma treatments.[13] The demonstration of EGFR expression at the protein level in many synovial sarcomas reiterates the potential for treating synovial sarcomas with specific EGFR inhibitors. The 2–18C9 EGFR antibody has been used in defining EGFR expression in clinical trials of EGFR inhibitors, and was also applied to the synovial sarcoma tissue microarray. This antibody appeared to be more sensitive; 52% of synovial sarcomas were strongly positive, 38% weakly positive, and 10% were negative, values still very much in keeping with the expression profiling data. However, the 2–18C9 antibody staining protocol was also less specific for synovial sarcoma, producing strong staining in the majority of MPNST and LGFMS specimens.

Strong SALL2 nuclear immunostaining in 43% of synovial sarcomas and weaker staining in a further 49% again agrees well with cDNA microarray analysis (strong RNA expression in 3/8 synovial sarcomas, moderate expression in a further 4/8).[7] Interestingly, SALL2 has recently been identified as strongly associated with Wilms tumor by gene expression profiling, with confirmatory immunohistochemistry demonstrating protein expression in condensing fetal kidney mesenchyme, predominantly in stromal cells but also in early developing epithelial structures.[18]

Thus, two synovial sarcoma-associated genes identified by gene expression profiling were confirmed by immunohistochemistry on tissue microarrays. Neither SALL2 nor EGFR has previously been studied in large numbers of synovial sarcomas. These markers were present in a majority of keratin-negative synovial sarcomas and thus may have utility in identifying these more diagnostically-challenging cases. Strong staining for either of these markers appears to be highly specific for synovial sarcoma when compared to six other tumors that complicate the differential diagnosis of a keratin-negative non-pleomorphic cellular soft tissue tumor, particularly where MPNST is unlikely on clinical grounds and a negative CD34 stain argues against solitary fibrous tumor. EGFR represents a possible therapeutic target, and currently a trial has been opened by the European Organization for Research and Treatment of Cancer to test the response of synovial sarcoma to EGFR inhibitors (EORTC protocol 62022).

With regard to tissue microarray analysis of known markers, we found that synovial sarcomas, as well as the other arrayed soft tissue tumors, are negative for CD117 (c-kit) immunostaining (one case of weak staining among 43 cases). This result is in agreement with the recent study of Hornick and Fletcher,[19] who found 0 of 20 cases stained positively, but contrasts with a report of positive c-kit immunostaining in 20/20 synovial sarcomas, where faint, diffuse, and cytoplasmic staining of spindle cells was considered positive and no negatively-staining tissues were included.[20] While our negative CD34 and positive keratin, EMA, and bcl-2 immunostain findings agree with published results, CD99 and S100 positivity was seen in a smaller fraction of synovial sarcomas than previously reported.[21,22] Despite the use of two cores per case, focal staining may be under-recognized in tissue microarray cores when compared with whole sections, and may account for this discrepancy, along with differences in the antibody suppliers. Both cases of synovial sarcoma that did show focally strong S100 staining (requiring both nuclear and cytoplasmic staining) also had overlapping focal neurofilament staining, suggesting that, although these were not tumors arising in identifiable peripheral nerves, axons running through the tumor might be responsible for their S100 positivity.[23]

Hierarchical cluster analysis has been applied to gene expression data, and has been remarkably successful in its ability to group tumors according to their primary site, as well as in subgrouping tumors that are not reproducibly subclassifiable based on conventional morphology.[7,24–27] This same methodology can be applied to the analysis of multiple immunohistochemical staining results on tissue arrays with recently developed software,[12] creating a convenient way to display and present large amounts of data, and convert complex results into a visual format that displays large-scale patterns within the data. Hierarchical clustering, followed by visualization of the data in TreeView, allows new antibody markers to be compared with other known markers. In a second dimension, unknown tumors can be tested for relationships to known tumor classes on the basis of their whole immunohistochemical profile. The algorithm takes into account not only strong positive stains, but also gives weight to negative and weakly positive results. In this fashion, one unknown case could be assigned as a Ewing sarcoma, a diagnosis independently confirmed by cytogenetic analysis. A similar partial clustering phenomenon of tumors, based on their reaction pattern with a large number of markers, has previously been seen by us in a study of lymphomas[12] and a study on the comparison between endocervical and endometrial adenocarcinomas.[28] In general, hierarchical clustering analysis is less powerful when applied to immunohistochemical as opposed to expression profiling data, because semiquantitative scor-

ing causes data loss and introduces an element of subjectivity. Additionally, the overall data matrix is smaller; whereas tissue microarrays do facilitate testing of an order of magnitude more specimens than are available for typical immunohistochemistry studies, cDNA microarrays permit analysis of many thousands of genes as compared to the several dozen immunohistochemical markers applicable to serial tissue microarray sections.

The TreeView and Stainfinder software allow for a rapid retrieval of archived digital images of stained tissue cores. The images from the current study are available through the accompanying website and allow for a direct inspection of all our reported immunostaining results (http://microarray-pubs.stanford.edu/tma_portal/synsarc). This tissue microarray-based study is one of the first where the pertinent digitalized immunostain images data are posted on the internet for public review. Tissue microarray analysis has led to an enormous increase in the number of cases that can be studied by immunohistochemistry. While software tools have been developed to allow for a better evaluation of the immunostain interpretations we believe that access to the digital primary immunostain images is also a very important aspect of the dissemination of findings using tissue microarray technology.

Tumors exhibit molecular heterogeneity, and it is therefore the pattern of expression of a panel of markers, rather than any single marker, that is needed to define a diagnosis. Hierarchical clustering is one way to efficiently relate a large panel of tumor data to known knowledge, and can be applied to immunostaining results as easily as it is to gene expression profiles. Within these larger patterns, the expression of specific markers such as c-kit[29] or EGFR may define tumors that can be treated with specific agents.

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