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Classification and Subtype Prediction of Adult Soft Tissue Sarcoma by Functional Genomics

Neil H. Segal,* Paul Pavlidis,† Cristina R. Antonescu,‡ Robert G. Maki,§ William S. Noble,† Diann DeSantis,¶ James M. Woodruff,‡ Jonathan J. Lewis,*¶ Murray F. Brennan,¶ Alan N. Houghton,*§ and Carlos Cordon-Cardo‡

From the Swim Across America Laboratory, Departments of Surgery,¶ Medicine,§ and Pathology,‡ Memorial-Sloan Kettering Cancer Center, New York and Columbia Genome Center,† Columbia University, New York, New York*

Adult soft tissue sarcomas are a heterogeneous group of tumors, including well-described subtypes by histological and genotypic criteria, and pleomorphic tumors typically characterized by non-recurrent genetic aberrations and karyotypic heterogeneity. The latter pose a diagnostic challenge, even to experienced pathologists. We proposed that gene expression profiling in soft tissue sarcoma would identify a genomic-based classification scheme that is useful in diagnosis. RNA samples from 51 pathologically confirmed cases, representing nine different histological subtypes of adult soft tissue sarcoma, were examined using the Affymetrix U95A GeneChip. Statistical tests were performed on experimental groups identified by cluster analysis, to find discriminating genes that could subsequently be applied in a support vector machine algorithm. Synovial sarcomas, round-cell/myxoid liposarcomas, clear-cell sarcomas and gastrointestinal stromal tumors displayed remarkably distinct and homogenous gene expression profiles. Pleomorphic tumors were heterogeneous. Notably, a subset of malignant fibrous histiocytomas, a controversial histological subtype, was identified as a distinct genomic group. The support vector machine algorithm supported a genomic basis for diagnosis, with both high sensitivity and specificity. In conclusion, we showed gene expression profiling to be useful in classification and diagnosis, providing insights into pathogenesis and pointing to potential new therapeutic targets of soft tissue sarcoma. ***(Am J Pathol 2003, 163:691–700)***

Soft tissue sarcomas (STS) define a group of histologically and genetically diverse cancers that account for approximately 1% of all adult malignancies with an annual incidence in the United States of approximately 8000 cases.[1] There are over 50 subtypes of this disease, which are currently diagnosed by genetic and morphological criteria.[2,3] Those most frequently seen include liposarcoma, leiomyosarcoma, malignant fibrous histiocytoma (MFH), fibrosarcoma, and synovial sarcoma.[4] The molecular classification of STS includes two major categories on the basis of 1) a single recurrent genetic alteration, such as chromosomal translocations (synovial sarcoma, myxoid/round-cell liposarcoma, clear-cell sarcoma) or activating mutation (*KIT*), or 2) non-recurrent genetic aberrations, which form part of a complex abnormal karyotype.[5]

It is possible to classify some STS by their recurrent chromosomal translocations or somatic mutation,[6] such as the presence of *SYT-SSX* fusion transcript in synovial sarcoma,[7,8] *EWS-ATF1* in clear-cell sarcoma,[9,10] *TLS-CHOP* in myxoid/round-cell liposarcoma[11,12] and *ASPL-TFE3* in alveolar soft-part sarcoma.[13] Most of these translocations produce chimeric transcription factors, which presumably deregulate the expression of several target genes.[14] In the case of gastrointestinal stromal tumors (GIST), a distinct somatic mutation has been described in *KIT*,[15–17] which leads to ligand-independent constitutive activation of its encoded receptor tyrosine kinase. This in turn results in altered cell proliferation and tumorigenesis.

The group of tumors characterized by numerous, non-recurrent chromosomal alterations includes MFH, conventional fibrosarcoma, leiomyosarcoma, de-differentiated liposarcoma and pleomorphic liposarcoma. In particular, the diagnosis of MFH has been long controversial. Originally described in the 1960s as a fibrous xanthoma,[18–20] MFH was considered a true histiocytic tumor displaying facultative fibroblastic properties. Subsequent ultrastructural evaluation found the predominant cell type to be in fact a fibroblast or one of its variants, leading to the conclusion that MFH should be reclassified as pleomorphic fibrosarcoma.[21,22] Others consider MFH to be a final common pathway for certain types of STS and represent tumor progression or de-differentiation.[23–25]

The molecular classification of cancer has recently been prompted by the sequencing and annotation of the

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Address reprint requests to Neil H. Segal c/o Carlos Cordon-Cardo, Division of Molecular Pathology, Memorial Sloan-Kettering Cancer Center, Rm S801, 1275 York Avenue, New York, NY, 10021. E-mail: segaln01@med.nyu.edu.

human genome and technical advancement in gene transcription profiling.[26–28] These profound scientific advancements have permitted high-throughput analysis and molecular correlation between tumors that provides insight into molecular pathways and mechanisms. The support vector machine (SVM) model has, in particular, been shown to be useful in classification tasks using gene expression data.[29–31]

In this study, we investigated the gene expression profiles of 51 high-grade STS, representing nine different histological subtypes. We focused on high-grade lesions, as these often pose a diagnostic challenge and would potentially benefit from molecular-based classification and a diagnostic algorithm. Using hierarchical cluster analysis, multidimensional scaling and SVM analysis, we determined the molecular relationship of STS and compared this to the current histological classification, for the purpose of a novel biology-based model of STS.

Materials and Methods

Tumor Specimens

Tumor specimens, obtained from 51 patients undergoing surgery at Memorial Sloan-Kettering Cancer Center, included MFH ($n = 11$), conventional fibrosarcoma ($n = 8$), leiomyosarcoma ($n = 6$), round-cell liposarcoma ($n = 4$), pleomorphic liposarcoma ($n = 3$), de-differentiated liposarcoma ($n = 5$), clear-cell sarcoma ($n = 4$), synovial sarcoma ($n = 5$), and GIST ($n = 5$). Specimens were collected under an IRB-approved tissue procurement protocol. Representative tumor tissue was embedded in OCT compound and frozen as tissue blocks using liquid nitrogen. Tumor specimens were selected for analysis according to validation of histological diagnosis. Round-cell liposarcoma, de-differentiated liposarcoma and pleomorphic liposarcoma were dissected from microscopically identified regions within the frozen tumor block, to ensure selection of high-grade areas only. Prior therapy was not considered an exclusion criterion, as we showed in a pilot study that tumors did not cluster differently by prior treatment. For additional details on genotype, subtype, prior therapy, site and stage, see Supplemental Data at http://www.amjpathol.org, or http://www.mskcc.org/genomic.sts.[32] Tumor specimens have been used in a similar study in the classification of clear-cell sarcoma.[33]

Histological and Molecular Diagnosis

In all cases histological slides were available from the primary resection specimen and were reviewed independently by two soft-tissue pathologists (C.R.A., J.M.W.). Histological diagnosis was supported in every case by an appropriate immunohistochemical panel and/or molecular genetic evaluation. RT-PCR using total RNA extracted from frozen tissue was performed for detection of specific fusion transcripts such as *SYT-SSX*, *TLS-CHOP*, and *EWS-ATF1*, used in the molecular diagnosis of synovial sarcoma,[34] myxoid/round-cell liposarcoma,[12] and clear-cell sarcoma,[10] respectively. All GIST tumors were tested for the presence of *KIT* mutations, using PCR amplification of genomic DNA, followed by direct sequencing.[35] These studies were performed in the laboratories of the Division of Molecular Pathology, Memorial Sloan-Kettering Cancer Center.

RNA Isolation and Gene Expression Profiling

Cryopreserved tumor sections were homogenized under liquid nitrogen by mortar and pestle. Total RNA was extracted in Trizol reagent and purified using the Qiagen Rneasy kit. RNA quality was assessed on ethidium bromide agarose gel electrophoresis. cDNA was then synthesized in the presence of oligo(dT)24-T7 from Genset Corp. (La Jolla, CA). cRNA was prepared using biotinylated UTP and CTP and hybridized to HG U95A oligonucleotide arrays (Affymetrix Inc., Santa Clara, CA). Fluorescence was measured by laser confocal scanner (Agilent, Palo Alto, CA) and converted to signal intensity by means of Affymetrix Microarray Suite v4.0 software. For complete expression data, see Supplemental Data at http://www.amjpathol.org, or http://www.mskcc.org/genomic.sts.[32]

Hierarchical Cluster Analysis

Hierarchical cluster analysis was performed using XCluster (http://genome-www.stanford.edu/~sherlock/cluster.html), using a centered Pearson correlation coefficient distance metric and average linkage to measure cluster distances during partitioning.[36] A nonparametric bootstrap was used to estimate confidence of the cluster structure.[37] For each bootstrap sample, the clustering obtained was compared to the clustering obtained with the original data set. Two clusters (branches of the hierarchy) were considered identical if they contained the same members.

Multidimensional Scaling Analysis

As an alternative and independent way of visualizing the cluster structure of the data a multidimensional scaling analysis was done. To deal with both the large range and the negative values of the expression data we took as the distance function $\frac{1}{2}(1 - r)$, where r is the Spearman rank-order correlation coefficient. The multidimensional scaling was done using S-PLUS[38] projecting the data into three dimensions.

Support Vector Machine Analysis

The ability of a machine-learning algorithm to correctly classify each tumor type was measured using SVM analysis with hold-one-out cross-validation.[29,30] In brief, during the training phase the SVM takes as input a microarray data matrix, and labels each sample as either belonging to a given class (positive) or not (negative). The SVM treats each sample in the matrix as a point in a high-dimensional feature space, where the number of genes on the microarray determines the dimensionality of the space. The SVM learning algorithm then identifies a hyperplane in this space that best separates the positive and negative training examples. The trained SVM can then be used to make predictions about a test sample's membership in the class. This approach allows us to collect unbiased measurements of the ability of the SVM to classify each sample. We used a standard "hold-one-out" training/testing scheme, in which the SVM is trained separately on training sets made up of all but one of the samples, and then tested on the single "held out" sample. Because a classifier's performance can be hindered by the inclusion of irrelevant data, we used feature selection to identify genes that are most important for classification. The genes in the training data set were ranked in order of their proposed importance in distinguishing the positives from the negatives, as described in more detail in the next section, and the top *N* genes were taken for each trial. The value *N* was varied in 12 powers of 2, ranging from 4 to 8192. Thus, the SVM was run 51 times on each of 12 different numbers of features (genes), for each of the tumor classes. Each held-out test sample was counted as either a false positive, false negative, true positive, or true negative.

Gene Ranking for Feature Selection

To select genes that were the most informative for the SVM, we tested a variety of methods including the Fisher score method[30] and parametric and nonparametric statistics. Data reported here were derived from Student's *t*-test, because it yielded the best SVM performance overall. Each gene in each training data set was subjected to the following procedure. A standard Student's *t*-test was used to compare the expression in one tumor type to that in the remaining samples. The resulting *P* values were then used to rank the genes, and the desired number of genes was then selected for use. The corresponding data from the training set was used to train the SVM, and the same genes were used for the test data. It is important to note that the genes were selected solely on the basis of the training data. Finally, a *t*-test statistic as determined for all samples was used to provide an overall ranking of the genes in order of relevance for each tumor classification. This ranking was used to provide an overview of the most important genes for distinguishing the class.

Results

Cluster Analysis

We determined the gene expression profile of 51 adult soft tissue sarcomas using 12,559 oligonucleotide probe sets on the U95A GeneChip from Affymetrix. Tumor specimens included nine different histological subtypes, which taken together cover more than 75% of STS cases diagnosed in the United States.

We explored three approaches to data analysis. In the first, we used unsupervised cluster analysis to identify groups of tumors related by similarity in overall gene expression profile using all genes represented on the U95A GeneChip (Figure 1). We identified two principal clusters that discriminate specimens by karyotypic and morphological features. STS characterized by non-recurrent genetic aberrations and karyotypic complexity show poor overall similarity in both gene expression profile and bootstrap analyses. In contrast, STS characterized by single recurrent genetic events clustered distinctly in strong groups. This was shown for all cases of GIST, synovial sarcoma, clear-cell sarcoma and round-cell liposarcoma. Similarly, visualized using multidimensional scaling analysis once again using all genes represented on the U95A GeneChip (MDS) (Figure 2).

Five of 8 conventional fibrosarcomas were observed to cluster in close proximity to the synovial sarcoma cluster. These 5 specimens were retrospectively tested for the presence of *SYT-SSX* fusion transcript by RT-PCR, and were found to be negative. Similarly, a single case of pleomorphic liposarcoma was observed to cluster in proximity to the round-cell liposarcoma group and was shown to be negative for the *TLS-CHOP* fusion transcript (data not shown).

Although the pleomorphic STS were not strongly related overall by gene expression profile, predominant groups were observed on hierarchical cluster analysis in concordance with histological classification. In particular, 5 of 6 leiomyosarcoma specimens (S20-S24) co-clustered with a de-differentiated liposarcoma (S29). This de-differentiated liposarcoma was noted previously to contain divergent leiomyosarcomatous differentiation on routine histological and immunohistochemical assessment. These 6 specimens were designated as "genomic leiomyosarcoma group #1" for further discussion. Similarly, 9 of 11 MFH specimens (S36-S40, S43-S46), including 5 of 6 lesions with myxoid features, clustered together with a single fibrosarcoma (S5). This was designated as "genomic MFH group" for further discussion. The remaining specimens appeared heterogeneous.

Support Vector Machine Analysis

Our second approach incorporated the use of SVM analysis to explore the outcome of genomic diagnosis in both previously-defined histological subtypes and potential novel genomic groups. Specimens were divided into two groups to establish training classes for each diagnostic category. The positive class contained all specimens that belong to a specific category. The negative class contained the remaining specimens. We performed hold-one-out cross-validation, in which one specimen was hidden from the SVM during training and was subsequently given to the "machine" as a test specimen. This was performed over a range of gene numbers to identify the range in which the "machine" operates optimally in diagnosing an unknown specimen. The outcome of the analysis was compared to the predicted subtype of the test specimen and indicated as true/false positive or true/false negative.

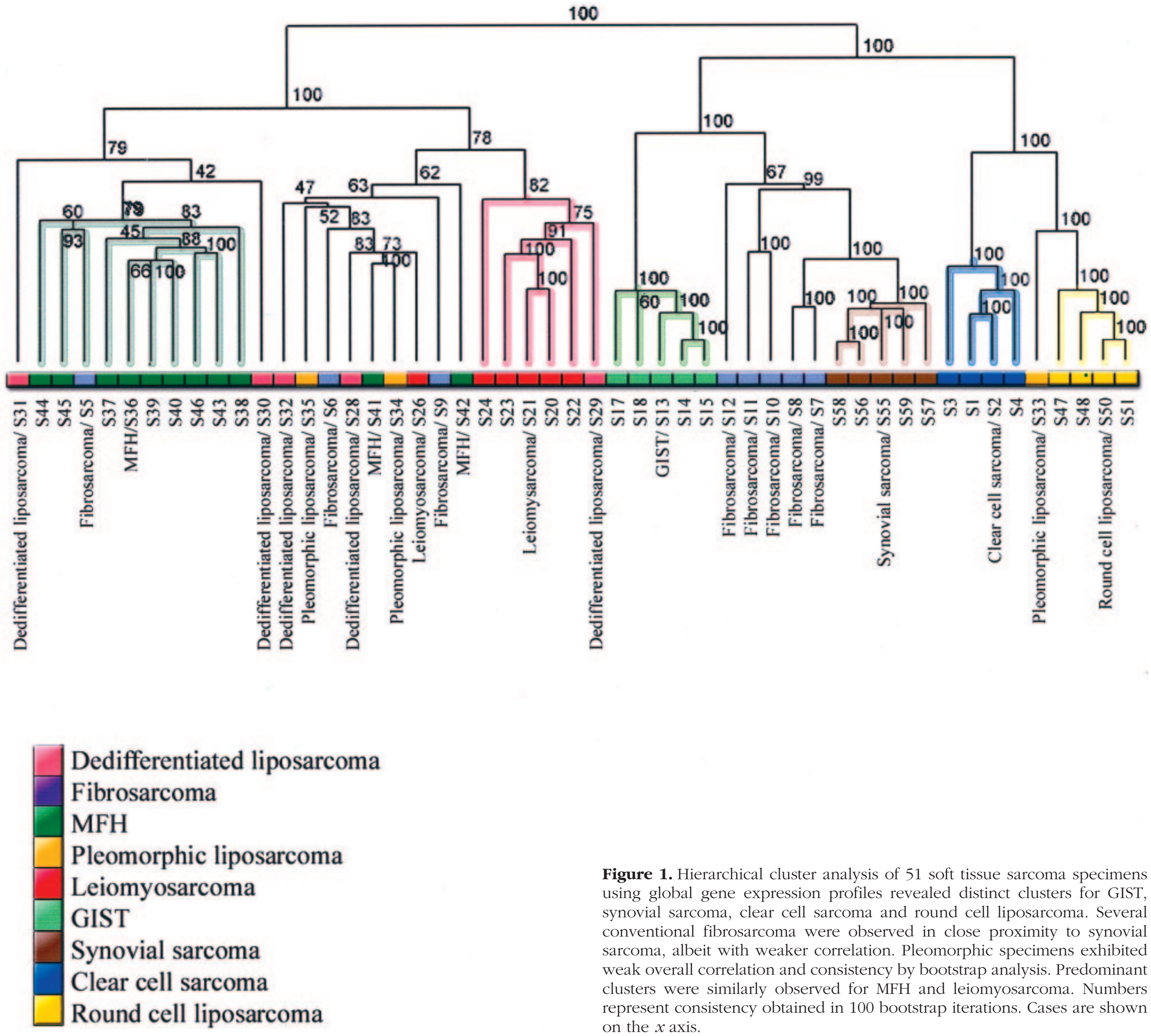


Figure 1. Hierarchical cluster analysis of 51 soft tissue sarcoma specimens using global gene expression profiles revealed distinct clusters for GIST, synovial sarcoma, clear cell sarcoma and round cell liposarcoma. Several conventional fibrosarcoma were observed in close proximity to synovial sarcoma, albeit with weaker correlation. Pleomorphic specimens exhibited weak overall correlation and consistency by bootstrap analysis. Predominant clusters were similarly observed for MFH and leiomyosarcoma. Numbers represent consistency obtained in 100 bootstrap iterations. Cases are shown on the *x* axis.

SVM analysis achieved both high sensitivity and high specificity in GIST, synovial sarcoma, round-cell liposarcoma, and clear-cell sarcoma. In the case of MFH, leiomyosarcoma, and de-differentiated liposarcoma, genomic reclassification of these tumors by cluster analysis improved SVM performance (Figure 3). Interestingly, de-differentiated liposarcomas were diagnosed accurately using as few as four genes, but only up to 64 genes. This limited range of sensitivity is consistent with a genomic-based relationship over few genes that is sufficient for SVM diagnosis yet insufficient to generate clusters using global gene expression. In the case of leiomyosarcoma, the designated "genomic leiomyosarcoma group #1" behaved poorly in SVM analysis, as observed by consistent misclassifications as false positive and false negative. We explored this further by hypothesizing an alternative "genomic leiomyosarcoma group #2" which included the outlier leiomyosarcoma specimens S26. This hypothetical cluster gained support by demonstrating consistently perfect SVM performance over a large range in the number of genes used. These results, taken together, demonstrate the efficacy of a diagnostic algorithm in validating and, in particular, exploring the outcome of cluster analysis techniques.

Gene with Potential Biological and Therapeutic Relevance

Our third approach to data analysis was the identification of genes, consistent with each tumor subtype for the purpose of useful biological discovery (Figure 4). In the case of MFH, leiomyosarcoma, and de-differentiated liposarcoma, genomic classification was used. This was performed using Student's *t*-test analysis and cross-referencing the top scoring 500 genes against both the published literature and the gene ontology consortium database (http://www.geneontology.org/) using NetAffx (http://www.affymetrix.com). We further limited this analysis to the top 50 genes for any particular STS subtype.

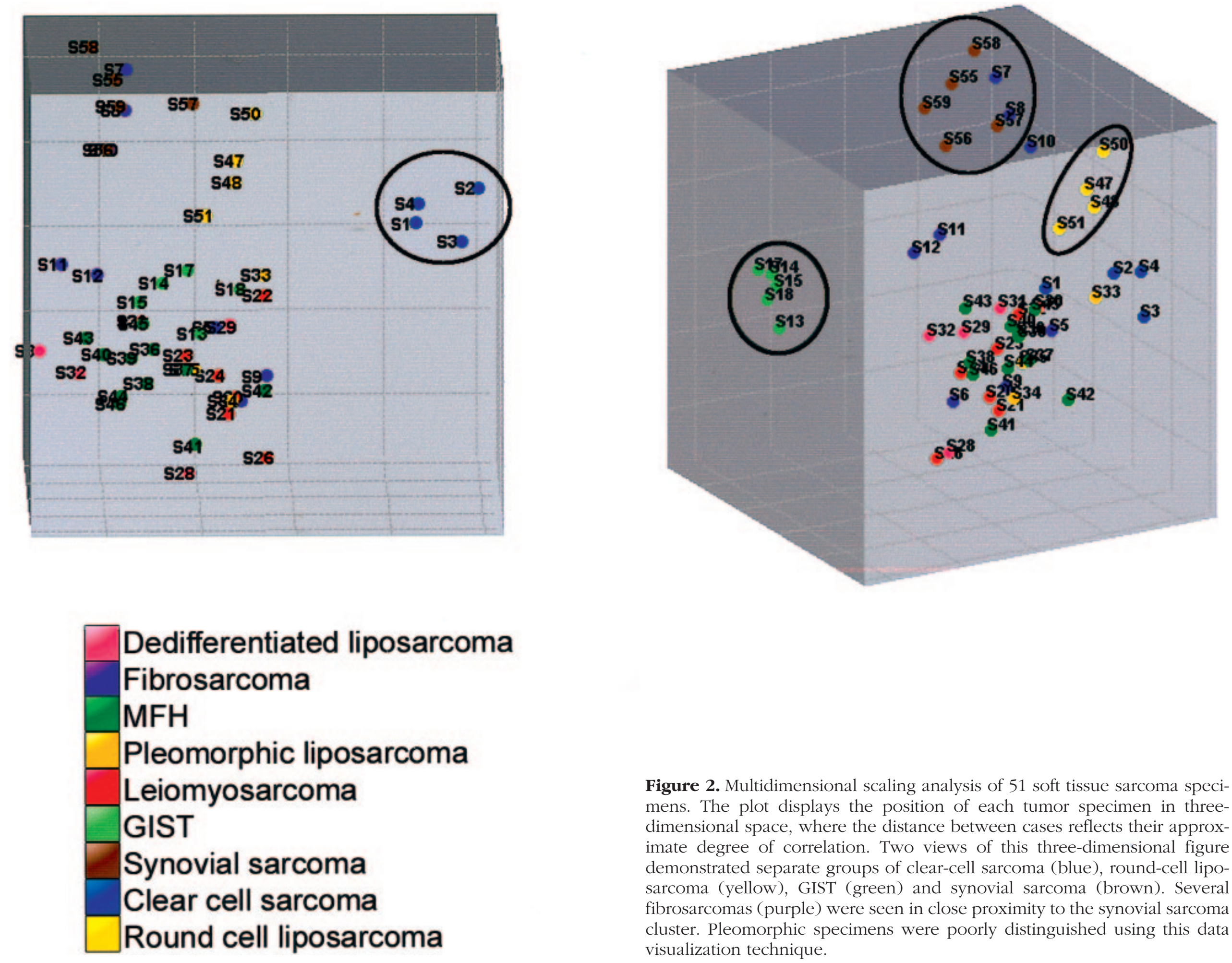


Figure 2. Multidimensional scaling analysis of 51 soft tissue sarcoma specimens. The plot displays the position of each tumor specimen in three-dimensional space, where the distance between cases reflects their approximate degree of correlation. Two views of this three-dimensional figure demonstrated separate groups of clear-cell sarcoma (blue), round-cell liposarcoma (yellow), GIST (green) and synovial sarcoma (brown). Several fibrosarcomas (purple) were seen in close proximity to the synovial sarcoma cluster. Pleomorphic specimens were poorly distinguished using this data visualization technique.

We identified the known genetic markers for distinct subtypes of STS, including KIT (GIST), SYT-SSX (synovial sarcoma), PPARγ (round-cell liposarcoma) and MITF (clear-cell sarcoma). In addition, we discovered several genes that are implicated in diverse biological processes, pathways, and states of differentiation.

GISTs were characterized by genes involved in receptor tyrosine kinase signal pathways, including *KIT, putative G protein-coupled receptor*, and *activin type II A receptor.* We similarly observed genes encoding ion channels, as well as the neuropeptide precursor *preproenkephalin*. Enkephalin has been implicated in gastrointestinal motility,[39] consistent with GISTs deriving from the interstitial cell of Cajal (ICC). We next searched for genes that were selectively expressed in the KIT pathway and identified *phosphatidylinositol 3 (PI-3) kinase* γ[40] in 5 of 5 specimens and the KIT ligand, *stem cell factor (SCF),* in 2 of 5 specimens (S15, S17). This finding was not related to any particular mutation in *KIT* (Table 1).

Synovial sarcomas were characterized by genes expressed in early developmental pathways involving WNT and notch signaling, including *TLE1, FZD1, WNT5A*, and *JAG2*. Several developmentally related homeobox genes, such as *SIX1, MEOX2*, and *SALL2* were also identified. Other genes of interest in synovial sarcoma included the *retinoic acid receptor* γ and *MYC* oncogene. Clear-cell sarcomas demonstrated several genes associated with their melanocytic lineage,[33] including *SOX10, gp100*, and *MITF*.

De-differentiated liposarcoma were characterized by genes located on 12q, including *CDK4* and *MDM2*. Round-cell liposarcomas were characterized by lipid metabolism and adipogenic profiles and included several homeobox genes. Leiomyosarcomas were characterized by genes implicated in the smooth-muscle phenotype. For complete gene list data, see Supplemental Data at http://www.amjpathol.org, or http://www.mskcc.org/genomic.sts.[32]

Discussion

We report here the genomic profiling of adult STS using oligonucleotide array analysis. This study attempts to provide an overall molecular perspective on the similarities and differences as well as unique characteristics of STS. We have sought to clarify relationships across the spectrum of histological distinctness from that of the well-defined GIST to the more controversial MFH lesions.

Data from this analysis demonstrates that STS characterized by specific translocations display remarkably ho-

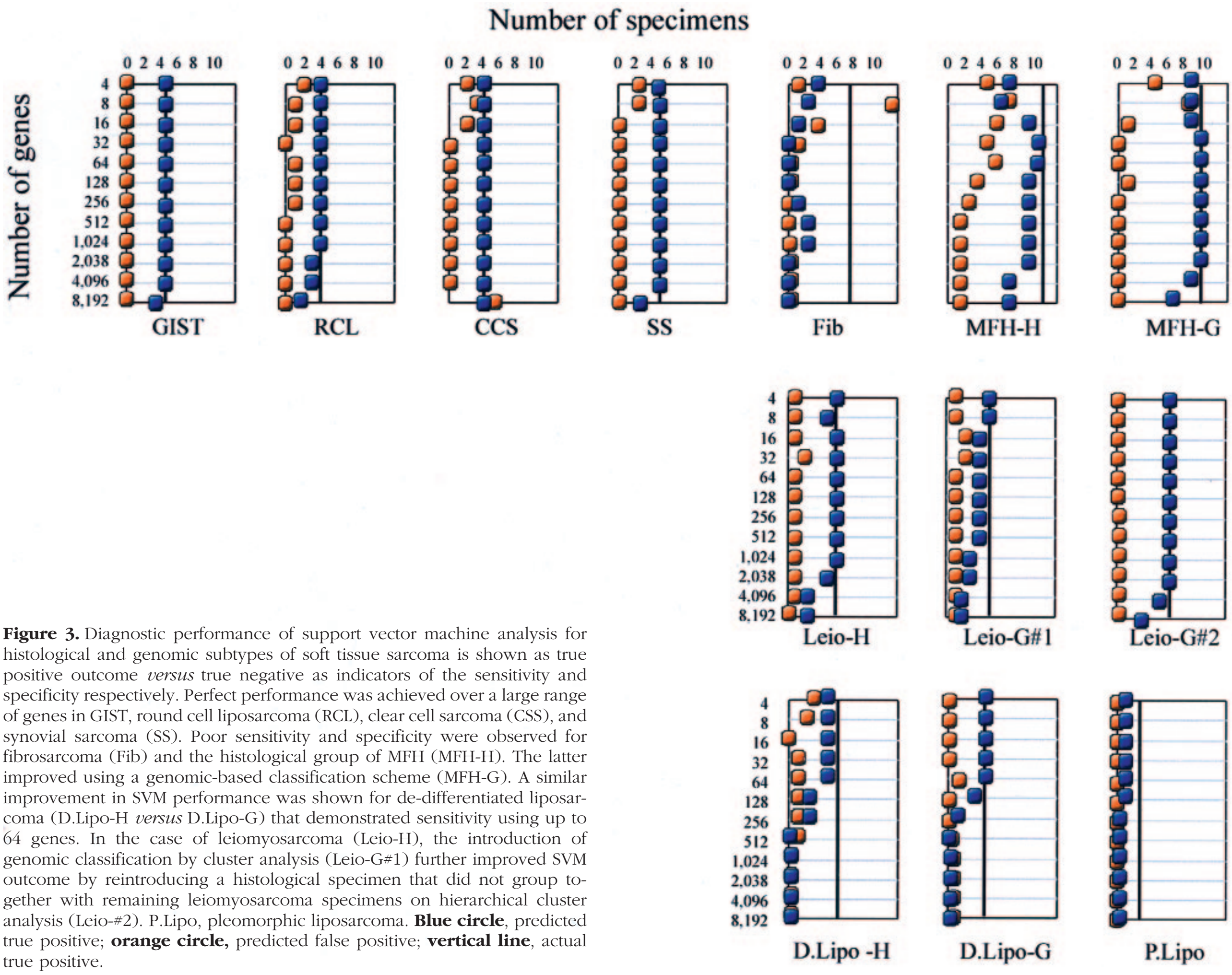


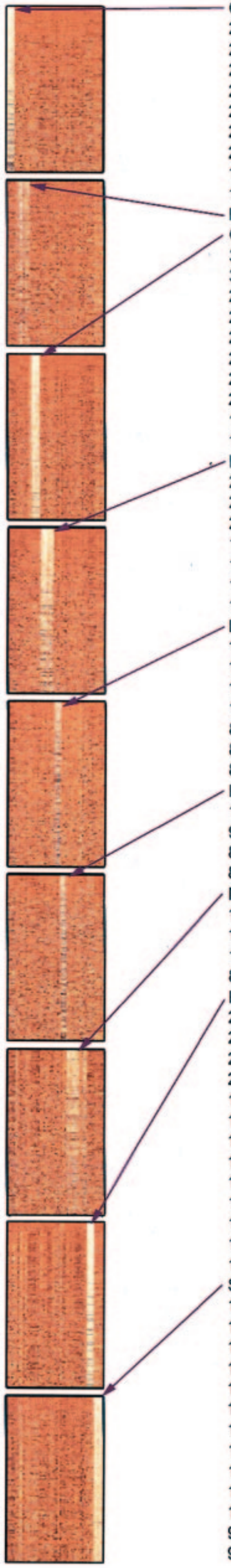
Figure 3. Diagnostic performance of support vector machine analysis for histological and genomic subtypes of soft tissue sarcoma is shown as true positive outcome *versus* true negative as indicators of the sensitivity and specificity respectively. Perfect performance was achieved over a large range of genes in GIST, round cell liposarcoma (RCL), clear cell sarcoma (CSS), and synovial sarcoma (SS). Poor sensitivity and specificity were observed for fibrosarcoma (Fib) and the histological group of MFH (MFH-H). The latter improved using a genomic-based classification scheme (MFH-G). A similar improvement in SVM performance was shown for de-differentiated liposarcoma (D.Lipo-H *versus* D.Lipo-G) that demonstrated sensitivity using up to 64 genes. In the case of leiomyosarcoma (Leio-H), the introduction of genomic classification by cluster analysis (Leio-G#1) further improved SVM outcome by reintroducing a histological specimen that did not group together with remaining leiomyosarcoma specimens on hierarchical cluster analysis (Leio-#2). P.Lipo, pleomorphic liposarcoma. **Blue circle**, predicted true positive; **orange circle,** predicted false positive; **vertical line**, actual true positive.

mogenous and distinct global gene expression profiles, as evident in the case of synovial sarcomas, round-cell liposarcomas and clear-cell sarcomas. This phenomenon was similarly observed in GISTs, characterized by recurrent genetic mutations in *KIT*. The observation of distinct gene expression profiles in these tumors is striking, in particular their consistent ability to cluster using different algorithms. This finding in GIST is consistent with a previous study that showed 13 GISTs to display a distinct gene expression profile relative to 6 spindle-cell sarcomas.[41] Furthermore, the GIST separated from leiomyosarcoma, including intraabdominal tumors, in support of their different histogenesis. Our findings are supported in a recent study by Nielsen et al.[42] Using cDNA microarray technology to profile 41 soft tissue tumors, their study identified GIST, synovial sarcoma and a subset of leiomyosarcoma as distinct groups on hierarchical cluster analysis.

Synovial sarcomas were furthermore shown to be distinct subtypes of STS in recent studies by Allander et al[43] and Nagayama et al.[44] Interestingly, the latter study suggests synovial sarcoma to be related to MPNST. We identified close proximity of several fibrosarcoma specimens to synovial sarcoma. These three tumor types are often indistinguishable on routine light microscopy and may indeed represent a common class of primitive mesenchymal tumors.

The present study also describes the use of a supervised learning algorithm, SVM analysis, in the diagnosis of STS. The diagnosis of tumors characterized by specific genetic events was highly accurate using as few as between 4 and 32 genes. Errors were predominantly confined to reduced specificity at low gene numbers and an eventual drop-off in sensitivity between 1000 and 8000 genes. These findings suggest that, aside from pathognomonic genetic changes that have been reported for these tumors, collective information from an extremely diverse number of genes may be considered in their diagnosis and underlying biology.

Data from this report also reveal that STS characterized by pleomorphic phenotypes and complex karyotypes display relatively inconsistent gene expression profiles, in keeping with their cytogenetic heterogeneity. However, within this group of pleomorphic STS, leiomyosarcoma and a subset of MFH were distinguished by their ability to cluster. This particular finding prompted us to explore the possibility of diagnosing these tumors using a genomic platform. SVM analysis attained perfect performance over a limited range in gene number when diag-



p-Value	Rank	Probe Set	Description	Symbol	Locus	Genbank
Clear cell sarcoma						
23.9	2/ 12	36018_at	SOX10	SOX10	22q13.1	AJ001183
22.3	3/ 18	38327_at	gp100	SILV	12q13-q14	M77348
22.1	5/ 20	40648_at	c-mer	MERTK	2q14.1	U08023
21	7/ 28	34674_at	S100	S100A1	1q21	X58079
20.7	8/ 29	1201_at	RAB-33A	RAB33A	Xq26.1	D14889
20.7	9/ 30	2069_s_at	α(E)-catenin	CTNNA1	5q31	L23805
20.7	10/ 31	38228_g_at	microphthalmia-associated transcription factor	MITF	3p14.1-p12.3	AB006909
19.5	14/ 44	1585_at	ERBB3/HER3	ERBB3	12q13	M34309
13.6	44/ 146	36778_at	ocular albinism 1	OA1	Xp22.3	Z48804
Fibrosarcoma>500						
GIST						
36.4	1/ 1	31792_at	annexin A3	ANXA3	4q13-q22	M20560
30	4/ 5	1888_s_at	KIT	KIT	4q11-q12	X06182
22.4	6/ 17	37221_at	cAMP-dependent protein kinase RII-β	PRKAR2B	7q22-q31.1	M31158
21.9	7/ 21	35871_s_at	pancreas sodium bicarbonate cotransporter	SLC4A4	4q21	AF011390
21.3	8/ 24	2081_s_at	protein kinase C θ	PRKCQ	10p15	L07032
21.2	9/ 26	37669_s_at	Na,K-ATPase β-1	ATP1B1	1q22-q25	U16799
20.2	10/ 36	35162_s_at	activin A receptor, type II	ACVR2	2q22.2-q23.3	D31770
20.1	-12/ 39	32351_at	G protein-coupled receptor 20	GPR20	8q24.2-q24.3	U66579
18.7	17/ 52	38291_at	preproenkephalin	PENK	8q23-q24	J00123
16.7	29/ 87	36287_at	phosphatidylinositol 3 kinase γ	PIK3CG	7q22.2	X83368
Leiomyosarcoma						
23.8	3/ 13	32314_g_at	tropomyosin 2 (β)	TPM2	9p13.2-p13.1	M12125
23.7	4/ 14	34203_at	calponin 1, basic, smooth muscle	CNN1	19p13.2-p13.1	D17408
22.8	5/ 15	37765_at	leiomodin 1 (smooth muscle)	LMOD1	1q32	X54162
18.1	12/ 65	32847_at	myosin, light polypeptide kinase	MYLK	3q21	U48959
17.3	15/ 77	37909_at	laminin, alpha 3	LAMA3	18q11.2	L34155
17	18/ 81	37407_s_at	myosin, heavy polypeptide 11, smooth muscle	MYH11	16p13.13-p13.12	AF013570
15.2	23/ 110	36790_at	tropomyosin 1 (α)	TPM1	15q22.1	M19267
Dedifferentiated liposarcoma						
17.5	1/ 71	41202_s_at	conserved gene amplified in osteosarcoma	OS4	12q13-q15	AF000152
15.3	2/ 108	1880_at	mouse double minute 2	MDM2	12q14.3-q15	M92424
14.3	4/ 127	41582_at	advillin	AVIL	12q13.11-12q14.3	AF041449
12	6/ 203	36001_at	methyltransferase-like 1	METTL1	12q13	Y18643
8.6	8/ 473	35759_at	TCP-1 beta subunit homolog	CCT2	12q15	AF026166
8.59	9/ 475	1942_s_at	cyclin-dependent kinase 4	CDK4	12q13-14	U37022
8.56	10/ 479	36083_at	sarcoma amplified sequence	SAS	12q13-q14	U01160
Pleomorphic liposarcoma						
11	11/ 250	32538_at	transferrin	TF	3q21	S95936
9.94	12/ 330	34378_at	adipophilin	ADFP	9p21.3	X97324
8.73	13/ 456	39014_at	Fc fragment of IgG binding protein	FCGBP	19q13.1	D84239
8.72	14/ 457	38320_s_at	lipase, hormone-sensitive	LIPE	19q13.1-q13.2	L11706
MFH						
12.9	1/ 173	39640_at	glutamine-fructose-6-phosphate transaminase 2	GFPT2	5q34-q35	AB016789
11.6	2/ 219	39182_at	epithelial membrane protein 3	EMP3	19q13.3	U87947
10.3	3/ 301	37403_at	annexin A1	ANXA1	9q12-q21.2	X05908
8.95	4/ 423	33437_at	homolog of yeast SPB1	FTSJ1	Xp11.23	AJ005892
Round cell liposarcoma						
26.7	1/ 8	37104_at	peroxisome proliferative activated receptor γ	PPARG	3p25	L40904
22.5	4/ 16	31531_g_at	acetyl-Coenzyme A carboxylase β	ACACB	12q24.1	U89344
21.1	5/ 27	38430_at	fatty acid binding protein 4	FABP4	8q21	AA128249
20	7/ 41	40658_r_at	adipose most abundant gene transcript 1	APM1	3q27	D45371
17.4	11/ 74	41209_at	lipoprotein lipase	LPL	8p22	M15856
16.4	12/ 91	40077_at	aconitase 1	ACO1	9p22-p13	Z11559
14.6	14/ 120	205_g_at	homeobox protein Hox-A4	HOXA4	7p15-p14	M74297
14	16/ 136	39023_at	isocitrate dehydrogenase 1	IDH1	2q33.3	AF020038
11.8	25/ 210	34009_at	LAGE-1b	CTAG2	Xq28	AJ223040
11.2	32/ 236	37809_at	homeobox protein Hox-A9	HOXA9	7p15-p14	U41813
11.1	34/ 243	33637_g_at	NY-ESO-1	CTAG1	Xq28	U87459
11.1	36/ 245	34574_at	MAGE-11	MAGEA11	Xq28	U10686
10.8	41/ 261	31983_at	homeobox protein EMX2	EMX2	10q26.1	X68880
Synovial sarcoma						
19.6	1/ 43	469_at	ephrin-B3	EFNB3	17p13.1-p11.2	U66406
18.2	2/ 63	41489_at	transducin-like enhancer of split 1	TLE1	19p13.3	M99435
18.1	3/ 66	40004_at	sine oculis homeobox homolog 1	SIX1	14q23.1	X91868
14.9	4/ 114	34069_s_at	SYT...SSX1	SYT/SSX	t(X;18)(p11.2;q11.2)	S79325
14.9	5/ 115	35178_at	Wnt inhibitory factor-1	WIF-1	12q14.2	W27944
13.8	10/ 142	40399_r_at	mesenchyme homeo box 2	MEOX2	7p22.1-p21.3	AI743406
12.8	12/ 179	38300_at	frizzled-1	FZD1	7q21	AB017363
10.7	24/ 272	1603_g_at	protein kinase C –	PRKCI	Xq21.3	L33881
10.4	25/ 290	32137_at	jagged 2	JAG2	14q32	AF029778
10.2	27/ 313	31862_at	Wnt-5a	WNT5A	3p21-p14	L20861
10.2	28/ 314	37567_at	sal (Drosophila)-like 2	SALL2	14q11.1-q12	X98834
9.65	35/ 355	1132_s_at	retinoic acid receptor γ	RARG	12q13	L12060
9.55	37/ 361	35158_at	myc	MYCN	2p24.1	Y00664

Figure 4. Identification of genes for biological discovery. The thumbnail panels represent the top 500 genes for each tumor type scored by Student's *t*-test analysis and sorted by increasing *P* value (shown as negative log *P* value). The second column shows rank according to *P* value where a higher value corresponds to a lower *P* value; the first value indicates rank within genes that discriminate the particular tumor subtype; the second value indicates rank within all genes that discriminate any tumor subtype. Light to dark color variation in the **left panel** represents high to low levels of expression. Annotated genes were selected according to biological interest from the top 500 genes that discriminate any STS subtype. All genes for fibrosarcoma failed to satisfy this criterion. See Supplemental Data at http://www.amjpathol.org for detailed gene lists.

Table 1. Gene Mutation in *KIT* and *KIT, SCF* Expression

Specimen	*KIT* mutation	*KIT* expression	*SCF* expression
S13	Exon 11 del: 557,558 WK	+	−
S14	Exon 9 ins: 6 bp	+	−
S15	Exon 11 del: 557,558 WK	+	+
S17	Exon 11 ins: 45 bp duplication	+	+
S18	Exon 11 point mutation: 559, V to G	+	−

nosing genomic MFH compared to histological MFH. This observation supports our claim that the genomic group MFH is distinct and amenable to objective diagnosis. Since MFH is diagnosed at different rates by different pathologists we do not have a good sense nationwide or worldwide if specific drugs are better for this subtype or not, beyond just using doxorubicin, ifosfamide, DTIC, or combinations thereof. The identification of a subset of MFH with a particular characteristic expression profile could potentially facilitate an objective diagnosis of this tumor type and assist in subsequent therapeutic studies.

Unlike genomic MFH, improved SVM performance with specimens selected by genomic classification was not initially shown for leiomyosarcoma. The above findings were intriguing for two reasons. First, it provided further support that the ability to diagnose the genomic MFH group by SVM analysis was not only a consequence of their ability to cluster, but in fact demonstrated that the other tumors in this study were sufficiently different so as not to be misdiagnosed as MFH by SVM analysis. Second, the observation of a consistent misclassification of genomically defined leiomyosarcoma prompted us to repeat this SVM analysis including the specimen that was excluded on cluster analysis. This removed the false positive occurrence in SVM analysis and also improved overall performance.

These observations that SVM performance improved when diagnosing genomic groups *versus* histological groups was not surprising as these tumors were selected largely on the basis of genomic correlation. However, this finding was significant and demonstrated an important and logical extension of genomic profiling. It illustrated that genomic correlation between tumors may be exploited to recognize novel classifications, against which meaningful biological/clinical correlates may be considered. We concluded that the genomic classification by cluster analysis of adult STS and SVM support is feasible and presents a user-independent reproducible mechanism by which to establish biology-based classification of soft tissue sarcoma.

Further inspection of the gene lists that discriminate subtypes of STS was particularly informative for biological discovery. In particular we identified features consistent with autocrine growth loops in a subset of GIST, involving SCF and KIT, and in synovial sarcoma, involving WNT5a and components of the downstream signaling pathway, including FRIZZLED-1.

Mutations in the KIT occur somatically in many sporadic GISTs. These mutations activate the tyrosine kinase activity of KIT and induce constitutive signaling. Inhibition of the tyrosine kinase activity of KIT by imatinib mesylate induces tumor regression in GISTs.[45] The finding of SCF, also known as KIT ligand, in subset of GISTs is a novel and noteworthy finding that may have implications in understanding potential autocrine growth effects in GIST involving the KIT pathway.

The recent study by Nagayama et al[44] similarly identified several genes related to the WNT signaling pathway in synovial sarcoma, including *WNT inhibitory factor 1* and *Frizzled homolog 10*. The finding of PRAME as a discriminating gene in several independent studies[42–44] in synovial sarcoma suggests a particularly robust association of the tumor antigen and this STS subtype.

Results of this analysis point to current treatment strategies for patients with STS, including imatinib (STI-571) for GIST and PPARγ agonists for myxoid/round-cell liposarcomas and suggest additional therapeutic considerations. These include blockade of PI-3 kinase with wortmannin or similar compounds in GIST, and the use of retinoid agonists/antagonists or blockade of WNT signaling in synovial sarcoma.

Whereas Allander et al[43] identified a strong association between *ERBB2* expression and synovial sarcoma, we did not identify a similar association. This discrepant finding is likely based on tumor subtype selection as we included only monophasic synovial sarcoma in our study and their group identified *ERBB2* to be predominantly expressed in biphasic synovial sarcoma.

We have approached the challenge of sarcoma classification using a combination of clustering techniques to propose novel groups and supervised diagnostic techniques to test the proposed grouping. This combined approach allows us to consider the distinction between groups of tumors in terms of diagnostic sensitivity and specificity rather than by similarity in gene expression profile alone. The classification of STS will continue to evolve as additional subtypes of this disease are introduced into the molecular classification scheme. More detailed analysis of the gene expression profiles of each of the more than 50 subtypes of STS will clarify the biological differences within STS and will hopefully propose therapies specific for each subclass of STS, if not therapy specific for an individual patient's tumor. The present study proposes multiple molecular pathways that may become potential targets for therapeutic intervention, and represents one step toward a comprehensive molecular understanding of this rare and heterogeneous group of diseases.

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