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Intratumor versus Intertumor Heterogeneity in Gene Expression Profiles of Soft-Tissue Sarcomas

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Soft-tissue sarcomas (STSs) constitute more than 30 histologic entities. In addition, within each entity, tumors are often heterogeneous in macroscopic features, genetic alterations, microscopic appearance, and clinical course. Therefore, there has been concern about whether a single tumor sample can provide a gene expression profile representative of the entire tumor. We used 27-k cDNA microarray slides to assess the importance of intratumor versus intertumor heterogeneity of the gene expression profiles of 2 morphologically heterogeneous STSs. Multiple pieces of tumor (8 and 10 pieces) were obtained from a myxoid variant of malignant fibrous histiocytoma (MFH) and a leiomyosarcoma (LMS), respectively, and the expression patterns were compared with single tumor samples from 20 MFHs and 16 LMSs. Hierarchical clustering analysis of the expression profiles showed that samples from the same tumor clustered together. The average intratumor distance was considerably shorter than the average intertumor distance in both LMS and MFH. In addition, tumor subclusters that distinguished different macroscopic parts of the tumor could be discerned. We concluded that intratumor variability exists but that accurate gene expression profiling also could be obtained using single samples from a large STS.

INTRODUCTION

Soft-tissue sarcomas (STSs) of the extremities and the trunk wall account for about 1% of adult malignancies and comprise more than 30 histologic types, with malignant fibrous histiocytoma (MFH), leiomyosarcoma (LMS), and liposarcoma being the most common (Fletcher et al., 2002). Tumor subclassification is based on distinguishing a line of differentiation within the tumor, and such careful identification provides the basis for reclassification of pleomorphic STS or MFH to a type-specific subset, which may also be aided by the identification of specific cytogenetic alterations or gene fusions. Although histopathologic appearance is still the basis for STS classification, the reproducibility of this method is not optimal, and novel diagnostic methods are needed. Major aims in the ongoing studies applying gene expression profiling to STS include distinguishing new subtypes within the currently identified histotypes and identifying novel predictive and prognostic markers. Many STS, such as synovial sarcomas, myxoid liposarcomas, and clear-cell sarcomas, are classified by recurrent cytogenetic alterations and type-specific fusion proteins (Fletcher et al., 2002; Mandahl et al., 2004). In other STS types, such as MFH, LMS, and pleomorphic liposarcoma, no specific diagnostic genetic alterations have been identified, and most of these tumors are characterized by multiple complex chromosomal aberrations. The data available from studies that have applied gene expression profiling to STS have shown that several of the tumor types characterized by specific translocations display quite homogenous and distinct gene expression profiles (Nielsen et al., 2002; Lee et al., 2003; Segal et al., 2003; Skubitz and Skubitz, 2004). Among the pleomorphic tumors, some form distinct subclusters, whereas others fall close to other tumor categories (Ren et al., 2003; Segal et al., 2003; Skubitz and Skubitz, 2004). The latter probably indicates that some types of pleomorphic tumors contain tumors that represent distinct entities, and others that have undergone extensive dedifferentiation that prevents distinguishing the origin of the primary tumor.

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TABLE 1. Histopathologic Data on Intratumor Pieces

Tumor piece	Necrosis[a]	Vascular invasion	Myxoid differentiation[a]	Mitoses per 10 HPF	Cellular pleomorphism[a]
M1A	3	−	1	8	3
M1B	2	−	2	15	2
M1C	1	−	1	19	2
M1D	2	+	2	11	3
M1E	2	−	1	23	2
M1F	3	−	3	28	2
M1G	2	−	1	34	3
M1H	2	−	2	31	2
L1A	0	−	0	4	2
L1B	0	−	0	1	2
L1C	1	−	0	3	2
L1D	1	+	0	8	2
L1E	0	−	1	0	2
L1F	1	−	0	1	2
L1G	1	−	1	6	2
L1H	1	−	0	2	2
L1I	2	−	0	2	3
L1J	1	−	0	1	3

[a]Parameters graded from 1 to 3: 1 < 10%, 2 10%–50%, 3 > 50%.
+ present; − absent; HPF: high-power fields.

STS are often large; the median size of tumors of the extremities is 7 cm. Morphologically, STS often display intratumor macroscopic and microscopic heterogeneity. Can a gene expression profile determined from a single tumor sample obtained from a large, morphologically heterogeneous STS be used for diagnostic, predictive, and prognostic purposes? To what extent do differences in gene expression profiles within a tumor or between tumors of the same histopathologic type influence the results and the reproducibility? To determine the importance of intratumor versus intertumor variability, we performed gene expression profiling on 8 pieces from a myxoid MFH and on 10 pieces from a LMS and compared the expression profiles to those obtained for single tumor samples from 20 MFHs and 16 LMSs.

MATERIALS AND METHODS

Tumor Samples

Multiple tumor pieces were obtained from 2 deep-seated, high-grade pleomorphic STS: a MFH and a LMS. The MFH measured 17 × 15 × 10 cm and developed in the thigh of an 88-year-old man. The 8 tumor pieces, labeled M1A through M1H, were histopathologically characterized separately and showed variable degrees of vascular invasion, myxoid degeneration, and necrosis (Table 1). The tumor was classified as a myxoid variant of a MFH. The LMS measured 11 × 8 × 7 cm and developed in the groin of an 82-year-old man. The 10 tumor pieces, labeled L1A–L1J, showed variable degrees of necrosis, vascular invasion, and cellular pleomorphism (Table 1). The tumors used for comparison were single samples from 20 MFHs, 6 of which were myxoid MFHs, and from 16 LMSs (Table 2). Tumor morphology was reviewed by a sarcoma pathologist (H.A.D.) in all cases. Malignancy grading was based on a four-tiered grading system, and immunostaining included muscle-specific actin, smooth-muscle actin, S-100, EMA, cytokeratins, and markers to exclude melanoma and lymphoma. Ethical permission for the study was obtained from the ethics committee at Lund University.

Microarray Analyses

We used spotted cDNA microarrays with 27,648 spots containing sequence-verified IMAGE clones from the Research Genetics IMAGE clone library. The slides were produced at the Swegene DNA Microarray Resource Center, Department of Oncology, Lund University. Clone information was linked to gene names using build 164 of the Unigene database (http://www.ncbi.nlm.nih.gov/UniGene), and 17,517 unique Unigene clusters were represented on the array.

RNA Extraction, cDNA Synthesis, Labeling, and Hybridization

Frozen tumor tissue (80–120 mg) was pulverized under liquid nitrogen. Total RNA was extracted

TABLE 2. Clinicopathologic Data on the Whole Tumor Series

Case	Patient no.	Age/sex	Tumor site	Tumor size (cm)	Necrosis (+/−)	Vascular invasion (+/−)	Malignancy grade (I–IV)
M1A-H	MFH-myx	88/M	LE/D	17	+	+	IV
M2	MFH-myx	75/F	LE/D	18	+	−	IV
M3	MFH-myx	71/F	UE/S	4	+	−	IV
M4	MFH-myx	65/M	LE/D	11	+	−	IV
M5	MFH-myx	49/M	UE/D	2	−	−	I
M6	MFH-myx	74/M	UE/S	3	+	−	III
M7	MFH-myx	76/M	LE/S	5	−	−	III
M8	MFH-sp	69/M	LE/D	19	+	+	IV
M9	MFH-sp	74/M	LE/D	4	−	−	III
M10	MFH-sp	70/M	LE/S	3	+	−	IV
M11	MFH-sp	54/M	LE/S	5	+	−	IV
M12	MFH-sp	68/M	LE/D	6	+	−	IV
M13	MFH-sp	54/F	LE/S	2	−	−	IV
M14	MFH-sp	77/M	UE/D	8	+	−	IV
M15	MFH-sp	58/M	LE/D	9	+	−	IV
M16	MFH-sp	76/F	LE/S	4	+	−	IV
M17	MFH-sp	77/F	TW/D	11	+	+	IV
M18	MFH-sp	59/M	LE/D	17	+	−	IV
M19	MFH-sp	37/M	LE/D	10	+	−	IV
M20	MFH-sp	72/M	LE/D	20	+	+	IV
M21	MFH-sp	80/F	UE/D	6	−	−	III
L1 A-J	LMS	82/M	LE/D	11	+	+	IV
L2	LMS	51/M	TW/D	8	+	−	IV
L3	LMS	52/F	LE/D	11	+	−	III
L4	LMS	73/F	LE/D	5	+	−	IV
L5	LMS	68/F	LE/D	20	+	−	IV
L6	LMS	82/M	LE/D	7	−	−	IV
L7	LMS	50/M	LE/D	16	+	−	IV
L8	LMS	63/F	LE/S	4	−	−	IV
L9	LMS	74/F	LE/D	10	+	+	IV
L10	LMS	48/M	LE/S	4	−	−	IV
L11	LMS	82/F	LE/D	8	+	−	IV
L12	LMS	88/F	LE/D	11	+	−	IV
L13	LMS (LR)	77/M	LE/S	7	−	−	III
L14	LMS	77/M	LE/S	12	+	−	IV
L15	LMS	40/F	LE/D	27	−	−	IV
L16	LMS	61/M	LE/D	12	−	+	IV
L17	LMS	82/M	LE/D	7	+	−	IV

+ present; − absent.

Abbreviations: MFH: malignant fibrous histiocytoma, LMS: leiomyosarcoma, myx: myxoid, sp: storiform-pleomorphic, LR: local recurrence, S: superficial, D: deep, LE: lower extremity, UE: upper extremity, TW: trunk wall.

using TRIzol® reagent (Invitrogen™ Life Technologies, Carlsbad, CA) and the RNeasy® Midi Kit (Qiagen Inc., Valencia, CA) according to the manufacturers' instructions. Universal Human Reference RNA (Stratagene, La Jolla, CA) was used as the reference. cDNA was synthesized from 25–35 μg of tumor RNA and 20 μg of reference RNA and indirectly labeled with Cy3 and Cy5 for the tumor and reference RNA, respectively, using the CyScribe cDNA Post labeling kit (Amersham Biosciences, Amersham, UK). Reverse transcription was primed with anchored oligo(dT) and catalyzed by CyScript™ reverse transcriptase in the presence of amino allyl-dUTP. After reverse transcription, the remaining RNA templates were degraded by alkaline hydrolysis treatment using NaOH, and the cDNA was ethanol-precipitated. The amino allyl–modified cDNA from the tumor and the reference were labeled separately, purified using the Cyscribe GFX purification kit (Amersham Biosciences), and pooled before being vacuum-dried to a pellet. The blocking reagents poly d(A) (Amersham Biosciences), yeast tRNA (Sigma, St. Louis, MO), and human Cot-1 DNA® (Invitrogen™ Life Technologies) were added to the pooled sample in order to reduce nonspecific hybridization. The hybridization steps were carried out manually with the help of the Pronto!™

Universal Hybridization Kit (Corning Life Sciences, Corning Inc., NY) according to the manufacturer's instructions. The labeled pellet was dissolved in the Pronto!™ hybridization solution and applied to the slide, which was then sealed in a Corning® hybridization chamber at 42°C for 18–20 hr.

Image and Data Analyses

The slides were scanned using the Agilent DNA microarray scanner (Agilent Technologies, Palo Alto, CA) to obtain images with a resolution of 5 μm. Image analysis and data extraction were carried out using GenePix™ Pro 4.1.1.4 version (Axon Instruments Inc., Foster City, CA). Automatic spot detection was manually verified for inaccuracies in order to eliminate artifacts and bad spots. All data management and analyses were carried out using the Web-based BioArray Software Environment (BASE; http://base.onk.lu.se/int/; Saal et al., 2002). Background corrections, filtering, transformations, and analyses were performed uniformly on the data. Expression ratios were calculated from background-corrected spot intensities, and spots were used that had diameters greater than or equal to 55 μm and had been flagged as "found" in GenePix Pro. Expression values, defined as the 2 logarithm of the expression ratios, were normalized using the local LOWESS (Cleveland et al., 1979) method in 8 print-tip groups to correct for spatial bias. For each spot, the uncertainty of the expression value was estimated as $u = (SNR_1)^{-2} + (SNR_2)^{-2}$, where SNR_i is the signal-to-noise ratio for channel i. Nineteen samples were hybridized in duplicate (18) or triplicate (1), and replicate assays were merged in a weighted fashion. The weighted mean of a set of values, x_i, was defined as $m = \Sigma_i w_i x_i / \Sigma_i w_i$, where the weight, w_i, is $\exp(-3u_i^{1/2}/|x_i - m|)$. This set of equations was solved numerically by simple iteration. The error of the merged value was defined as $U = 1/\Sigma_i(1/u_i) + \Sigma_i w_i^2 (x_i - m)^2 / (\Sigma_i w_i)^2$. The merged data were transformed again using an error model that moves intensity values of poor-quality spots (spots with low SNR) toward the mean of that particular gene across all hybridizations, reducing the importance of the poor-quality spot in later analysis. The modified expression value was given by $x_i' = w_i(x_i - m)$. Filters for variation and presence of expression across hybridizations were set to reject all spots with a standard deviation of the modified expression value that was smaller than 0.3 and expression in less than 90% of the samples, which resulted in 5,724 spots (representing 5,345 reporters) in the expression analysis. Unsupervised agglomerative hierarchical clustering was carried out in order to group together tumor samples on the basis of the expression profiles across all spots. The distance between genes was measured using the Euclidean distance metric, whereas the Pearson correlation distance metric (distance, $d = 1 -$ Pearson correlation coefficient) was used to measure the distance between samples in the hierarchical cluster analysis. Measurement of pair-wise Pearson correlation distances between samples and two-dimensional-multidimensional scaling (2D-MDS) analysis also were performed.

RESULTS

The final expression analysis was based on 5,345 reporters, and the unsupervised cluster analysis showed that the samples obtained from the same tumor (the myxoid MFH M1 and the LMS L1) clustered together and separately from the individual pieces (Fig. 1).

The intratumor pieces were hybridized on the same prints of slides. The single tumor pieces from the different MFHs and LMSs were all hybridized on 4 print batches of slides (one of which also was used for the intratumor LMS hybridizations). Three intratumor pieces from the LMS and 15 of the single LMSs were hybridized in duplicate, and one single-piece tumor was hybridized in triplicate on different prints. All repeats clustered next to the first sample were run regardless of the difference in prints (data not shown), which motivated the merging of the data described. The distance between the 3 duplicate experiments from the LMS L1 were comparable to the distance between the closest intratumor pieces, L1A-L1B and L1E-L1F (data not shown). Hence, the difference between the closest pieces within a tumor was similar to the experimental variability. The 20 MFHs and the 16 LMSs analyzed were used as controls for intertumor variability of the gene expression profiles and were therefore not analyzed further for subclustering or possible discriminating genes because this was not within the scope of the study.

The intratumor distances between the 8 pieces of the myxoid MFH M1 and the 10 pieces of the LMS L1 were compared to the intertumor distances between the 20 MFHs and the 16 LMS from which single pieces were obtained. The Pearson correlation (centered, unsquared) coefficient r was measured for all pairs of samples within the M1 (M1A–H), MFH (M2–21), L1 (L1A–J), and LMS (L2–17) groups, and the corresponding pair-wise distance was calculated as $d = 1 - r$ (Table 3). The average distance calculated within the groups

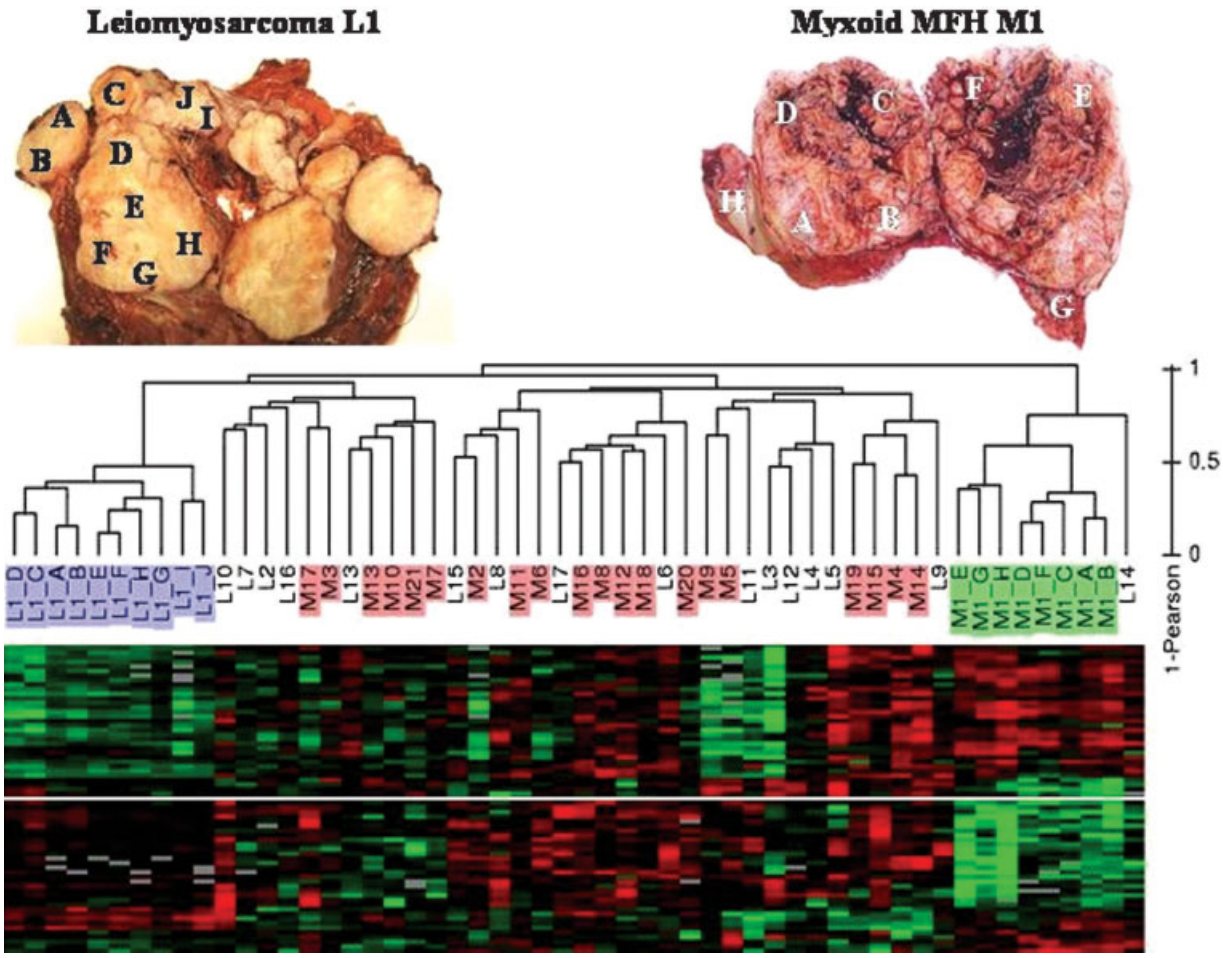

Figure 1. Macroscopic pictures of the 2 soft-tissue sarcomas from which multiple pieces were obtained: the 11 × 8 × 7 cm macroscopically multilobulated and microscopically heterogeneous LMS, on the left, and the 17 × 15 × 10 cm high-grade and pleomorphic myxoid MFH, on the right. Cluster analysis showed separate clusters for the 2 multiple-piece tumors compared to the 16 LMSs and the 20 MFHs from which single pieces were obtained.

TABLE 3. Intratumor and Intertumor Distances

Group	No. of samples	No. of pairs	Minimum distance	Maximum distance	Average distance
M1/intratumor	8	28	0.2036	0.8339	0.5043
MFH/intertumor	20	190	0.474	1.247	0.9617
L1/intratumor	10	45	0.1424	0.7745	0.429
LMS/intertumor	16	120	0.528	1.2835	0.9809

showed that this value was close to 1 for the LMS and MFH groups and close to 0.5 for the L1 and M1 groups. Thus, the average intratumor distance was considerably lower than the average intertumor distance for both LMS and MFH. The average intratumor distances in both tumor types also were smaller than the minimal intertumor variability (except for 1 intertumor distance in the MFH group). In the LMS, maximal intratumor variability was at the same level as minimal intertumor variability. The MFH showed somewhat higher intratumor heterogeneity, with a maximal intratumor heterogeneity that was at the same level as the average intertumor variability.

The relationships between the intratumor and intertumor pieces in their expression profiles also were estimated in a 2D-MDS plot, which was based on the minimized squared error of Euclidean distances (Fig. 2). The MFH M1 showed somewhat higher intratumor heterogeneity because the L1 pieces clustered closer together than did the pieces from M1.

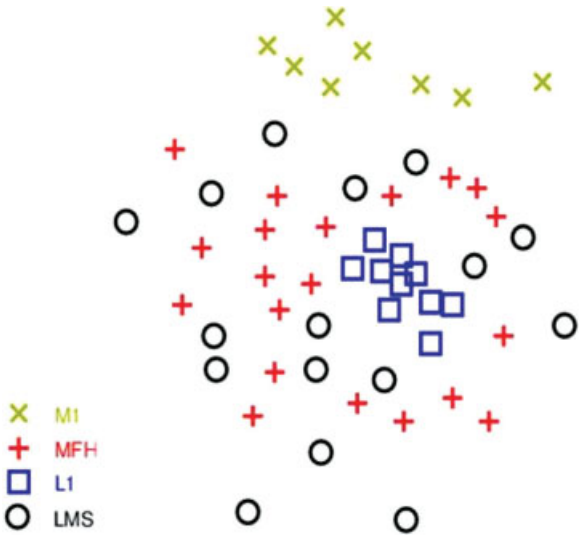

Figure 2. Multidimensional scaling (MDS) plot demonstrating intratumor (L1A-J and M1A-H) versus intertumor (L2-17 and M2-21) heterogeneity.

DISCUSSION

Morphologic heterogeneity is evident in STS. Örndal et al. (1994) demonstrated variability of karyotypic abnormalities in MFH, LMS, liposarcoma, synovial sarcoma, and chondrosarcoma. Different, albeit related, cytogenetic clones were found in 21 of 73 single pieces of tumor, and different clones in different parts of the tumor were detected in 12 of 16 tumors. Hence, cytogenetic heterogeneity and clonal evolution seem to be common in STS and have been detected most often in MFH and LMS (Örndal et al., 1994). Because gene expression profiles in STS may identify hitherto unrecognized but clinically relevant tumor subsets, we aimed to assess to what extent the intratumor and intertumor variability of expression profiles affect the reproducibility of the findings.

The 2 tumors investigated, a myxoid MFH and an LMS, are heterogeneous tumor types. Both tumors were large and displayed pronounced macroscopic heterogeneity, which was particularly evident in the multilobulated LMS (Fig. 1). Both tumors also showed histopathologic variability in differentiation, myxoid appearance, necrosis, and vascular invasion within the tumors (Table 1). We analyzed gene expression profiles from multiple pieces of these tumors and compared them to single pieces from 36 MFHs and LMSs. In the cluster analysis, the 9 pieces from the myxoid MFH and the 10 pieces from the LMS formed separate subclusters from the tumors from which single pieces were obtained (Fig. 1). When the distances within these 2 tumors were compared to the distances between the remaining tumors, the maximal intratumor distance was lower than the average intertumor distance. However, the maximal intratumor distance was greater than the intertumor distance between the 2 single-piece tumors that were most closely related. These findings not only suggest that single tumor samples from macroscopically as well as microscopically heterogeneous STSs provide expression profiles that cluster closely together, but also demonstrate that the variability within tumors may be as high as that between different tumors of the same histopathologic subtype.

Shmulevich et al. (2002) evaluated intratumor heterogeneity in 3 LMSs from which 3 peripheral tumor samples and 1 core sample were obtained. Euclidian distances, used to assess the proximities between the samples, demonstrated a high degree of concordance in the global gene expression profiles between the samples from the tumor periphery and core. Shmulevich et al., (2002) concluded that variability between the different tumor sections was within the experimental variability between replicate experiments. In the cluster analysis in our study, the different pieces from the 2 tumors also showed a tendency to cluster according to their original position within the tumors. In the myxoid MFH, the peripheral tumor pieces G, E, and H formed one subcluster, whereas the more central pieces, A–D and F, formed another subcluster. Also, in the LMS, which was exceptionally macroscopically heterogeneous with multiple connected tumor nodules (Fig. 1), subclustering of pieces next to each other was evident; pieces A and B (which formed one nodule) clustered together and were most closely related to nearby pieces C and D. Pieces E–H, which formed the largest nodule, clustered together, and finally, I and J, which formed the last nodule, clustered together. This tumor also demonstrated that variability between 2 pieces was approximately as pronounced as intraexperimental variability for pieces that were repeated (H, I, and J).

Although some of the same genes that have been reported by different studies to show altered expression in STS overlap, many genes do not (Allander et al., 2002; Nielsen et al., 2002; Segal et al., 2002; Lee et al., 2003). These discrepancies may be explained by several factors, including differences in sample size, in technical platforms used, in the number of genes investigated, in the references used, and in the approaches and methods used for the statistical evaluation of the results. On the basis of the results of our study, intratumor

heterogeneity seems to have only a small impact on the variability of expression profiles.

The level of intratumor heterogeneity in the gene expression profiles in the present study also suggests that in STS, reliable gene expression profiles can be obtained regardless of tumor size. Expression profiling in STS for diagnostic and prognostic purposes can be performed reliably from single tumor samples, but intratumor heterogeneity may have a larger impact on the results in small tumor series and may thereby limit the validity of the findings. This is one reason why large sample sets should preferably be run: the effects of tumor-related heterogeneity and experimental variability are minimized, providing reproducible expression patterns.

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