Array Comparative Genomic Hybridization Reveals Distinct DNA Copy Number Differences between Gastrointestinal Stromal Tumors and Leiomyosarcomas

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Abstract

Leiomyosarcomas are spindle cell tumors showing smooth muscle differentiation. Until recently, most gastrointestinal stromal tumors (GIST) were also classified as smooth muscle tumors, but now GISTs are recognized as a separate entity, defined as spindle cell and/or epithelioid tumors localized in the gastrointestinal tract. Using microarray-based comparative genomic hybridization (array CGH), we have created a detailed map of DNA copy number changes for 7 GISTs and 12 leiomyosarcomas. Considerable gains and losses of chromosomal segments were observed in both tumor types. The most frequent aberration observed in GISTs was loss of chromosomes 14 and 22, with minimal recurrent regions in 14q11.2-q32.33 (71% of the tumors) and 22q12.2-q13.31 (100%). In leiomyosarcomas, frequent loss of chromosome 10 and 13q was observed, with minimal recurrent regions in 10q21.3 (75%) and 13q14.2-q14.3 (75%). Recurrent high-level amplification of 17p13.1-p11.2 was detected in leiomyosarcomas. Expression profiling using cDNA microarrays revealed four candidate genes in this region with high expression (*AURKB, SREBF1, MFAP4*, and *FLJ10847*). Altered expression of *AURKB* and *SREBF1* has been observed previously in other malignancies. Hierarchical clustering of all samples separated GISTs and leiomyosarcomas into two distinct clusters. Statistical analysis identified six chromosomal regions, 1p36.11-p13.1, 9q21.11-9q34.3, 14q11.2-q23.2, 14q31.3-q32.33, 15q24.3-q26.3, and 22q11.21-q13.31, which were significantly different in copy number between GISTs and leiomyosarcomas. Our results show the potential of using array comparative genomic hybridization to classify histologically similar tumors such as GISTs and leiomyosarcomas. (Cancer Res 2006; 66(18): 8984-93)

Introduction

Sarcomas, malignant tumors of mesenchymal origin, are histologically classified according to the normal tissue they resemble. Leiomyosarcomas, malignant tumors displaying features of smooth muscle differentiation, are characterized by spindle cells with elongated blunt-ended nuclei and eosinophilic cytoplasm (1). Leiomyosarcomas can occur at many anatomic locations and account for 5 to 10% of all soft tissue sarcomas (2). Leiomyosarcomas develop principally in adults (50-60 years of age) and are most common in women. According to their localization, they can be subdivided into different subgroups: deep soft tissue tumors (retroperitoneum, abdominal cavity, and intramuscular), uterine tumors, (sub)cutaneous tumors, and vascular leiomyosarcomas (i.e., vena cava). The subgroups also differ in their clinical characteristics, with cutaneous tumors showing a better prognosis (2).

Several studies have identified recurrent copy number changes at the cytogenetic level in leiomyosarcomas. Using chromosome-based comparative genomic hybridization (CGH), recurrent losses of 2p, 2q, 10q, 11q, and 13q have been observed, as well as frequent gains of 1q, 5p, 8q, and 17p (3–6). Some of these regions, e.g., loss of 2p, 10q, and 12p, as well as gain of 1q and 17p, have been associated with more aggressive tumor behavior (7).

Until recently, most gastrointestinal spindle cell sarcomas were classified as smooth muscle tumors. However, based on histological data and immunoreactivity, these tumors, which show almost no smooth muscle differentiation, have been reclassified as gastrointestinal stromal tumors (GIST), a separate entity from leiomyosarcomas. GISTs have relatively simple karyotypes compared with leiomyosarcomas, including recurrent losses of 1p, 9p, 11p, 14q, and 22q, as well as gains of 8q and 17q, among other regions (8–10). Some of these aberrations, e.g., alterations of the p16/INK4a locus in 9p21.3 and gain of 5p, 8q, 17q, and 20q, have been associated with more aggressive tumor behavior (8, 11).

In 95% of GISTs, a gene encoding a type II receptor kinase (*KIT*) is expressed and is a target of activating mutations (12). Approximately 70 to 80% of these mutations occur in exon 11, leading to ligand-independent phosphorylation of the KIT tyrosine kinase, which can induce malignant transformation (12, 13). A minority of GISTs show activating mutations of the platelet-derived growth factor receptor α (*PDGFRA*), another receptor tyrosine kinase (14). Recently, small-molecule inhibitors such as imatinib that specifically target tyrosine kinases have been developed (15). These inhibitors have proven highly effective in treating GISTs (16), a fact that highlights the importance of distinguishing GISTs from leiomyosarcomas.

Microarray-based CGH (array CGH) enables genome-wide, high-resolution analysis of DNA copy number alterations. In this study, we sought to investigate the use of DNA copy number changes as a classification tool and to identify novel candidate areas important to the biology of GISTs and leiomyosarcomas. We used array CGH to create a detailed map of DNA copy number changes in 7 GISTs

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Possible conflict of interest: B. Bjerkehagen is a member of the Advisory Board in Novartis, Norway, for gastrointestinal stromal tumors.

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and 12 leiomyosarcomas. Statistical analysis identified six chromosomal regions that can distinguish the two tumor types based on changes in DNA copy number. Further analysis of recurrent high-level amplification of 17p13.1-p11.2 in leiomyosarcomas revealed four frequently overexpressed candidate genes.

Materials and Methods

Tumor samples. Eighteen human sarcomas initially classified as leiomyosarcomas were selected from a tumor collection at the Department of Tumor Biology, Norwegian Radium Hospital (Oslo, Norway). The collection and use of the tumor panel were approved by the ethical committee of Southern Norway. Sarcomas were collected immediately after surgery, cut into small pieces, frozen in liquid nitrogen, and stored at −70°C until use. In addition, one tumor sample tested was grown s.c. in immunodeficient mice as xenografts. All tumors were diagnosed at the time of collection according to the WHO International Histological Classification. At study completion, all samples were reviewed by the pathologist and classified according to the current standard. Seven leiomyosarcomas were then reclassified as GISTs. In addition, one sample initially diagnosed as malignant fibrous histiocytoma was reclassified as leiomyosarcoma and included in the study. Clinical data for all samples, as well as immunohistochemical data, are given in Table 1.

Genomic microarray construction. Genomic microarrays spanning the entire human genome at ~1 Mb resolution were made using bacterial artificial chromosomes (BAC) and P1 artificial chromosomes (PAC), based on the 1 Mb clone set kindly provided by Dr. Nigel Carter at the Wellcome Trust Sanger Institute, United Kingdom (17).

The 1 Mb clone collection was supplemented with tiling path probes between 1q12 and the beginning of 1q25 (~35 Mb) using overlapping BACs and PACs. The BAC clones were from the RPCI-11 library, and the PAC clones were from the RPCI-1, -3, -4, and -5 libraries.[5] All clones were kindly provided by Dr. Simon G. Gregory at the Wellcome Trust Sanger Institute. According to the September 2005 assembly of the human genome (Ensembl v33),[6] the tiling path is discontinuous with two gaps >260 kb. The first segment is delimited by RP3-365I19 and RP11-640M9, and the second by RP11-301M17 and RP11-14N7, estimated to be 497 and 678 kb in size, respectively. Four additional smaller gaps between 200 and 260 kb are also present.

Additional RPCI-11 clones belonging to chromosomes 1, 12, 17, X, and Y from the Cancer Chromosome Aberration Project[7] and the VGC-mapped BAC library[8] were incorporated, as well as 575 Caltech clones from the OncoBAC clone collection.[9] The genomic microarray contained a total of 4,549 unique genomic clones. A complete list can be found in the corresponding array design file in the ArrayExpress[10] microarray database (accession no. A-MEXP-253).

Isolation, amplification, and printing of BAC and PAC DNA were done as previously described (17). For detailed description, see Supplementary Materials and Methods.

Microarray-based CGH (array CGH). Genomic DNA was isolated from tumor tissue by standard phenol chloroform extraction. A pool of normal female or male genomic DNA (Promega, Madison, WI) was used as a reference, depending on patient gender. One microgram of total genomic DNA was digested overnight at 37°C using 60 units of *Dpn*II endonuclease (New England Biolabs, Beverly, MA). Digested DNA was purified using the QIAquick PCR Purification Kit, as instructed by the supplier (Qiagen, Valencia, CA). Approximately 500 ng of the digested DNA was labeled by random priming (BioPrime DNA Labeling System; Invitrogen, Carlsbad, CA) using 1.5 μL of 1 mmol/L Cy3-dCTP or Cy5-dCTP (Perkin-Elmer Life Sciences, Foster City, CA) in a 100 μL reaction volume. After overnight incubation at 37°C, labeled probes were purified using Microspin G-50 columns (GE Healthcare, Piscataway, NJ), according to the supplier's instructions.

Labeled tumor and reference DNA were combined and ethanol-precipitated together with 135 μg human Cot-1 DNA (Invitrogen). The DNA pellet was dissolved in 108 μL of hybridization buffer (50% formamide, 10% dextran sulfate, 4% SDS, 2× SSC), and 4 μL 100 mg/mL yeast tRNA (Invitrogen). The DNA was denatured for 10 minutes at 70°C and prehybridized for at least 30 minutes at 37°C.

Hybridization was done using an automated GeneTAC hybridization station (Genomic Solutions/Perkin-Elmer), agitating the hybridization solution for 42 to 46 hours at 37°C. After hybridization, slides were washed with 50% formamide/2× SSC at 48°C, 2× SSC/0.1% SDS at 48°C, and PN buffer [0.1 mol/L NaH_2PO_4/Na_2HPO_4 (pH 8.0), 0.1% NP40] at 25°C. For all three solutions, the slide was washed for five cycles; each cycle had a flow time of 20 seconds and a hold time of 40 seconds. After being removed from the hybridization station, slides were rinsed briefly in 0.05× SSC and dried by spinning in a centrifuge.

Scanning and analysis. The arrays were scanned using an Agilent G2565BA scanner (Agilent Technologies, Palo Alto, CA). The acquired grayscale images were analyzed using GenePix Pro 6.0 (Axon Laboratories, Union City, CA). The spots were automatically segmented and manually adjusted where necessary. Local background was subtracted, and the fluorescence intensities and the intensity ratio of the two dyes were calculated for each spot.

Further data processing, including filtering and normalization, was done in M-CGH, a MATLAB toolbox specifically designed for this purpose (18). In brief, empty and manually flagged spots, as well as spots with intensities lower than the background in both channels and net signal intensities below local background plus twice the SD of the background were excluded. Log_2-transformed ratios were normalized using a global intensity–dependent algorithm. The spot reproducibility was assessed by calculating the mean and SD of the signal intensity of the repeated spots; genomic clones with a SD >0.2, as well as ratios based on a single measurement, were excluded. The mean ratios of the replicate spots were exported to a text file. A detailed description of the data processing, algorithms, and image generation have been previously published (18).

Array CGH data analysis. The complete array CGH data for the 7 GISTs and 12 leiomyosarcomas can be viewed in the ArrayExpress microarray database (accession no. E-MEXP-707). Clones belonging to chromosomes 1 to 22 with a known unique chromosomal location in Ensembl (v33, September 2005) were analyzed, a total of 3,351 unique BACs and PACs, the vast majority from the 1 Mb clone set and the 1q12-q25 tiling path.

Experimental variation was assessed using 19 normal female to normal male hybridizations (data not shown). The SD for each autosomal clone across the 19 experiments was calculated, ranging from 0.00 to 0.65 with a mean of 0.05. Clones showing a SD >0.2 were eliminated. Thus, 22 of the 3,351 clones (0.007%) were discarded, and a total of 3,329 unique BAC and PAC clones were used for the analyses (see clone list in Supplementary Tables S1*A* and *B*).

Missing values were imputed via a K-Nearest Neighbour algorithm normalization using "Significance Analysis of Microarrays" (SAM; ref. 19). Clustering of all samples was done using J-Express (20) with average linkage (WPGMA) as the cluster method and Pearson correlation as the metric distance.

In order to facilitate the identification of significant copy number changes, CGH-Explorer v. 2.52 was used (21). "Analysis of Copy number Errors" (ACE) was done separately for GISTs and leiomyosarcomas using a false discovery rate of 0.0000. Frequency plots and tables of the identified regions of copy number errors were generated from the ACE analysis. Chromosomal segments showing gains or losses in at least 30% of the samples [3 of 7 GISTS (42%) and 4 of 12 leiomyosarcomas (33%)] were used in the identification of minimal recurrent regions of alteration.

To identify chromosomal regions whose DNA copy number significantly differed in leiomyosarcomas and GISTs, a two-class unpaired *t* test was done using SAM. Using 100 permutations and a false discovery rate of 1%, a

[5] Available from http://bacpac.chori.org/humanmalepac.htm.
[6] Available from http://www.ensembl.org/index.html.
[7] Available from http://cgap.nci.nih.gov/Chromosomes/CCAP.
[8] Available from http://genomics.med.upenn.edu/genmapdb/.
[9] Available from http://informa.bio.caltech.edu/Bac_onc.html.
[10] Available from http://www.ebi.ac.uk/arrayexpress.

Table 1. Clinical and immunohistochemical data for GISTs and leiomyosarcomas

Sample	Sample origin	Patient age (y)/ sex	Initial Diagnosis	Revised Diagnosis	Grade/ risk*	Location	Size (cm)†	Metastasis (mo)‡	Status	Follow-up (mo)§	Desmin	SM Actin	CD34	KIT
GIST1	Prim	73/M	LMS	GIST	High risk	Abdomen	15	11	DD	16	−	−	+	+
GIST2	Rec	52/M	LMS	GIST	High risk	Small bowel	8	11	DD	23	−	+	−	+
GIST3	Rec	61/F	LMS	GIST	High risk	Small bowel	11	18	AD	135	−	−	+	+
GIST4	Rec	47/M	LMS	GIST	Low risk	Rectum	1.5	93	DD	121	−	−	+	+
GIST5	Met	53/M	LMS	GIST	High risk	Liver	6	73	DD	101	−	f.p.	+	+
GIST7	Prim	74/M	LMS	GIST	High risk	Abdomen	20	m.d.	DD	13	−	+	+	+
GIST8	Prim	70/M	LMS	GIST	Low risk	Stomach	5.5	n.m.	NED	95	−	−	+	+
LMS1	Prim	59/F	LMS	LMS	4	Retroperitoneum	9	15	DD	176	f.p.	+	f.p.	−
LMS3	Prim	72/F	LMS	LMS	4	Retroperitoneum	20	54	DD	91	+	+	−	−
LMS5x	Prim	46/F	LMS	LMS		Uterus	13	m.d.	DD	11	f.p.	+	−	−
LMS7	Prim	71/F	LMS	LMS	4	Thigh (i.m.)	8	n.m.	DOC	67	+	+	−	−
LMS10	Prim	67/F	LMS	LMS	4	Retroperitoneum	8	n.m.	NED	123	−	+	−	−
LMS12‖	Prim	67/M	LMS	LMS	3	Retroperitoneum	20	28	DD	63	−	+	f.p.	−
LMS17	Prim	59/F	LMS	LMS		Uterus	8.5	12	DD	31	−	f.p.	−	−
LMS18	Prim	46/F	LMS	LMS		Uterus	n.a.	20	DD	62	+	+	−	−
LMS21	Prim	31/F	LMS	LMS	4	Retroperitoneum	14	1	DD	18	+	−	w.f.p.	−
LMS23	Prim	72/F	LMS	LMS	4	Thigh (vena femoralis)	10	m.d.	DD	41	−	+	−	−
LMS24	Prim	66/F	LMS	LMS	4	Perineum	3	n.m.	DOC	51	+	+	−	−
LMS28	Prim	82/M	MFH	LMS	4	Knee (s.c.)	7	n.m.	DOC	79	−	f.p.	−	−

Abbreviations: SM, smooth muscle; x, xenograft; Prim, primary tumor; Rec, recurrence; Met, metastasis; F, female; M, male; MFH, malignant fibrous histiocytoma; LMS, leiomyosarcoma; n.a., not available; m.d., metastasis at diagnosis; n.m., no metastasis; DD, dead of disease; AD, alive with disease; DOC, dead of other cause; NED, no evidence of disease; f.p., focal positive; w.f.p., weak focal positive.

*Grading is based on a four-tiered system used in the Scandinavian Sarcoma Group. Uterine leiomyosarcomas are not graded. GISTs are graded according to size and mitotic count.

† Largest diameter of the tumor.

‡ Time to first metastasis from diagnosis.

§ Time to last follow-up from diagnosis.

‖ Additional stainings have been done. Sample is estrogen- and progesterone receptor–negative.

list of genomic clones showing significant copy number differences between leiomyosarcomas and GISTs was generated. Chromosomal segments represented by multiple significant clones were considered to discriminate the two tumor histotypes.

Results

Hierarchical clustering of tumors. DNA copy number changes of 18 tumors historically diagnosed as leiomyosarcomas were analyzed blindly using a 1 Mb resolution BAC/PAC genomic microarray supplemented with a tiling path between 1q12 and the beginning of 1q25 (~35 Mb). Hierarchical clustering of the 18 tumor samples using normalized ratios identified two well-defined clusters. Pathologic reviewing (without knowledge of the results) revealed that all samples within one of these primary clusters were classified as GIST by current standards (Fig. 1*A*). After being reviewed, all of the samples in the second primary cluster, including one additional sample (LMS28) previously classified as malignant fibrous histiocytoma, were confirmed as leiomyosarcoma (Fig. 1*A*).

The leiomyosarcoma cluster was further divided into two subclusters. The only noteworthy difference between the subclusters was the anatomic location of the tumor. All leiomyosarcomas of uterine origin clustered together, along with one leiomyosarcoma of retroperitoneal origin (Fig. 1*B*).

Recurrently altered regions in leiomyosarcomas and GISTs. Significant regions of DNA copy number changes in each sample were identified using the ACE algorithm in CGH-Explorer. Minimal recurrent regions were separately identified in GISTs and leiomyosarcomas (Table 2*A* and *B*). GISTs showed considerable losses and gains of large chromosomal regions. Of the identified recurrent regions, seven represented losses of chromosomal segments and seven gains. The most frequently observed aberration was loss of the whole or parts of chromosome 22; this aberration was observed in all tumors with a minimal recurrent region in 22q12.2-q13.31 (17.8 Mb). In six of the seven samples, a loss of one copy of chromosome 22 was observed. The second most frequent alteration was loss of chromosome 14; in five of seven samples, one copy of the entire chromosome was absent. In addition, in four of seven samples, three chromosomal regions were lost; 1p36.32-p13.1 (114.1 Mb), 13q12.11-q33.2 (86.5 Mb), and 15q13.2-qtel (71.8 Mb); and in three of seven samples, 9q13-q34.2 (65.3 Mb) was lost.

Seven recurrent gained regions were observed in GISTs. Gain of 8p23.3-pcen (43 Mb) was observed in four of seven samples. All remaining six regions, 4ptel-q13.2 (67.2 Mb), 5p15.33-q35.3 (177.5 Mb), 17q21.2-q21.31 (1.1 Mb), 17q22-qtel (30.0 Mb), 18q (58.8 Mb), and 20q12-q13.12 (4.1 Mb), were observed in three of seven samples (the low-frequency limit selected for this analysis). Scattered

high-level amplifications and homozygous deletions were observed in some of the samples. No recurrent region of aberration showed frequent high-level amplification ($\log_2$ ratio >1) or homozygous deletion ($\log_2$ ratio <−1). Figure 2*A* shows a genome-wide frequency plot of copy number aberrations for the GIST group, whereas Fig. 2*B* shows a representative ratio plot for one GIST sample (GIST1). Genome-wide ratio plots for all GISTs are shown in Supplementary Fig. S1*A*.

Leiomyosarcomas showed more recurrent losses than gains. Eighteen of 32 recurrent regions were losses, compared with 14 regions of increased DNA copy number. The most frequent minimal regions of loss were in 10q21.3 and 13q14.2-q14.3, both detected in 9 of 12 samples. Chromosome 2 was a frequent target for deletion; multiple recurrent regions were identified at 2p25.1-p21 (35.9 Mb), 2p14-p13.1 (8.8 Mb), 2q24.1-q31.2 (41.8 Mb), and 2q37.1-q37.2 (9.4 Mb), with frequencies ranging from 6 of 12 samples to 7 of 12

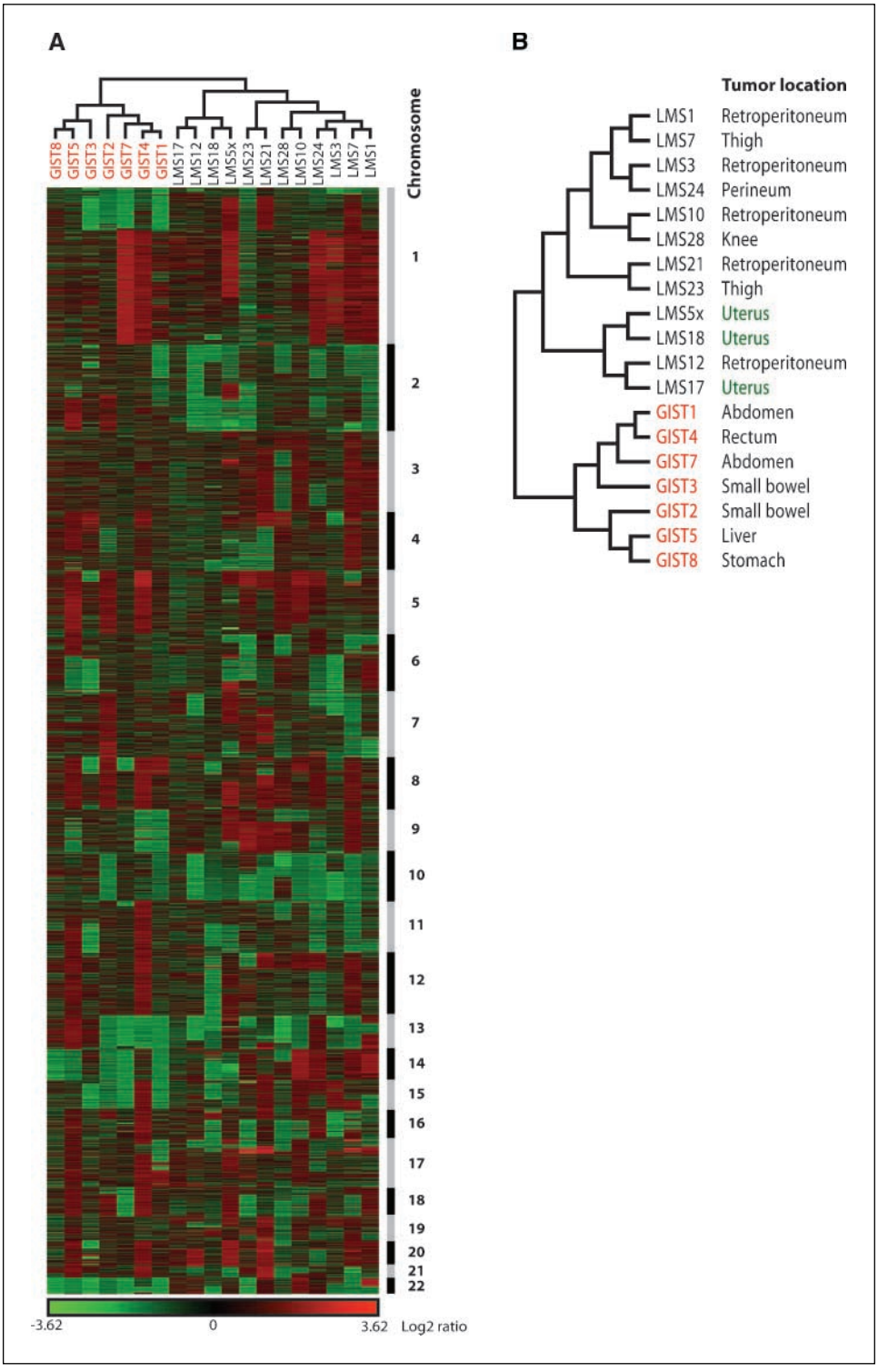

Figure 1. *A,* hierarchical clustering dendogram and heat map showing DNA copy number ratios in tumor samples compared with a pool of normal diploid DNA. A total of 3,329 unique genomic clones are shown in chromosomal order from 1ptel to 22qtel. *Red,* increases in DNA copy number; *green,* decreases in DNA copy number. Chromosomes are indicated with black and gray bars. *B,* hierarchical clustering dendogram indicating anatomic location of the tumor samples.

samples (Fig. 2*C*). Additional regions with decreased copy number were observed at 1p36.32-p36.21 (7.9 Mb), 4q31.3-qtel (36.2 Mb), 6p25.2-p22.3 (21.2 Mb), 6q14.1-q23.3 (58.6 Mb), 7p22.3-p13 (43.4 Mb), 11p15.5-p15.4 (2.4 Mb), 11q22.1-q24.1 (21.9 Mb), 16q21.2-q22.1 (20.2 Mb), 18q11.2-qtel (57.9 Mb), 21q21.1-q22.11 (18.4 Mb), and 22q13.1-q13.33 (11.4 Mb); all occurring in at least 4 of 12 samples (see Fig. 2*C*).

A considerable number of gains were also observed in leiomyosarcomas. The most frequently affected region was 17p13.1-p11.2, in which high-level amplification was found in four of six samples with increased DNA copy number (see Fig. 3*A*). The minimal recurrent region was limited to 1.9 Mb in 17p11.2, in which three samples showed high-level amplification. The long arm

Table 2. Minimal recurrent regions altered in GISTs and leiomyosarcomas

Cytoband	Aberration	Start clone	End clone	Size (Mb)	Frequency	Observation
(A) Minimal recurrent regions altered in GISTs (n = 7)						
1p36.32-p13.1	Loss	RP4-785P20	RP11-27K13	114.1	4/7	Four samples with −1p
4ptel-q13.2	Gain	CTC-36P21	RP11-211G17	67.2	3/7	
5p15.33-q35.3	Gain	CTD-2265D9	RP11-451H23	177.5	3/7	Three samples with +5
8p23.3-pcen	Gain	RP11-338B22	CTD-2115H11	43.0	4/7	Three samples with +8
9p21.3	Loss*	RP11-149I2	RP11-468C2	3.2	3/7	Two samples with −9
9q13-q34.2	Loss	RP11-274B18	RP11-153P4	65.3	3/7	Two samples with −9
13q12.11-q33.2	Loss	RP11-76K19	RP11-406G20	86.5	4/7	Three samples with −13
14q11.2-q32.33	Loss	RP11-84C10	RP11-417P24	85.1	5/7	All with −14
15q13.2-qtel	Loss	RP11-38E12	CTB-154P1	71.8	4/7	Three samples with −15
17q21.2-q21.31	Gain	RP11-506G7	RP5-905N1	1.1	3/7	Two samples with +17q
17q22-qtel	Gain	RP11-429O1	GS1-50C4	30.0	3/7	Two samples with +17q
18qcen-qtel	Gain	RP11-296E23	CTC-964M9	58.8	3/7	Two samples with +18
20q12-q13.12	Gain	RP4-600E6	RP1-138B7	4.1	3/7	Two samples with +20
22q12.2-q13.31	Loss	RP1-76B20	LL22NC03-75H12	17.8	7/7	Six samples with −22
(B) Minimal recurrent regions altered in leiomyosarcomas (n = 12)						
1p36.32-p36.21	Loss	RP1-37J18	RP4-636F13	7.9	4/12	
1q21.1-q23.2	Gain	RP11-277L2	RP11-550P17	9.9	5/12	Three samples with +1q
1q23.2-q23.3	Gain	RP11-517F10	RP11-404F10	0.5	4/12	Three samples with +1q
1q23.3-q25.1	Gain	RP11-572K18	RP5-1198E17	11.3	5/12	Three samples with +1q
2p25.1-p21	Loss	RP11-83M8	RP11-27C22	35.9	7/12	Five samples with −2p
2p14-p13.1	Loss	RP11-263L17	RP11-1P9	8.8	6/12	Five samples with −2p
2q24.1-q31.2	Loss	RP11-552E1	RP11-34K3	41.8	6/12	Four samples with −2q
2q37.1-q37.2	Loss	RP11-52C8	RP11-556H17	9.4	7/12	Four samples with −2q
3p12.3-p12.1	Gain	RP11-11L10	RP11-447J13	12.0	5/12	Two samples with +3
4q31.3-qtel	Loss	RP11-259G7	CTC-963K6	36.2	4/12	
5p13.2-pcen	Gain	CTD-2291F22	RP11-269M20	14.7	6/12	Five samples with +5p
6p25.2-p22.3	Loss	RP1-136B1	RP11-289M23	21.2	5/12	Two samples with −6q
6q14.1-q23.3	Loss	RP11-173D14	RP11-95M15	58.6	4/12	Two samples with −6q
7p22.3-p13	Loss	RP11-510K8	RP4-647J21	43.4	4/12	Three samples with −7p
9q21.13-q31.3	Gain	RP11-563H8	RP11-202G18	39.8	5/12	Four samples with +9q
10q21.3	Loss	RP11-161L14	RP11-778O10	0.8	9/12	Five samples with −10
11p15.5-p15.4	Loss	RP11-295K3	RP11-438N5	2.4	4/12	Two samples with −11
11q22.1-q24.1	Loss	RP11-49M9	RP11-166D19	21.9	5/12	Two samples with −11
12p11.22-p11.21	Gain	RP11-59L15	RP11-388G12	2.3	4/12	Three samples with +12p
13q14.2-q14.3	Loss	RP11-305D15	RP11-40A8	2.7	9/12	Five samples with −13
14q12-q21.1	Gain	RP11-30H9	RP11-138H18	9.1	5/12	Three samples with +14q
14q21.2-q21.3	Gain	RP11-565J15	RP11-58E21	3.6	5/12	Three samples with +14q
14q31.3-q32.2	Gain	RP11-300J18	RP11-76E12	9.7	5/12	Three samples with +14q
15q11.2-q12	Gain	RP11-289D12	RP11-446P9	3.5	5/12	Three samples with +15q
15q25.1-q26.3	Gain	RP11-285A1	RP11-262P8	21.2	4/12	Three samples with +15q
16q21.2-q22.1	Loss	RP11-452G23	RP11-354N7	20.2	6/12	Four samples with −16q
17p13.2-p13.1	Loss	RP11-243K12	RP11-186B7	1.4	6/12	
17p11.2	Amplification	RP11-524F11	RP1-162E17	1.9	6/12	
18q11.2-qtel	Loss	RP11-535A5	CTC-964M9	57.9	4/12	Four samples with −18q
20q11.21-q13.33	Gain	RP3-324O17	RP11-358D14	32.1	4/12	Four samples with +20q
21q21.1-q22.11	Loss	RP1-152M24	RP1-245P17	18.4	5/12	Three samples with −21q
22q13.1-q13.33	Loss	CTA-228A9	CTA-722E9	11.4	5/12	

*Aberration detected in only two samples by ACE. Additional sample annotated manually, small homozygous deletion.

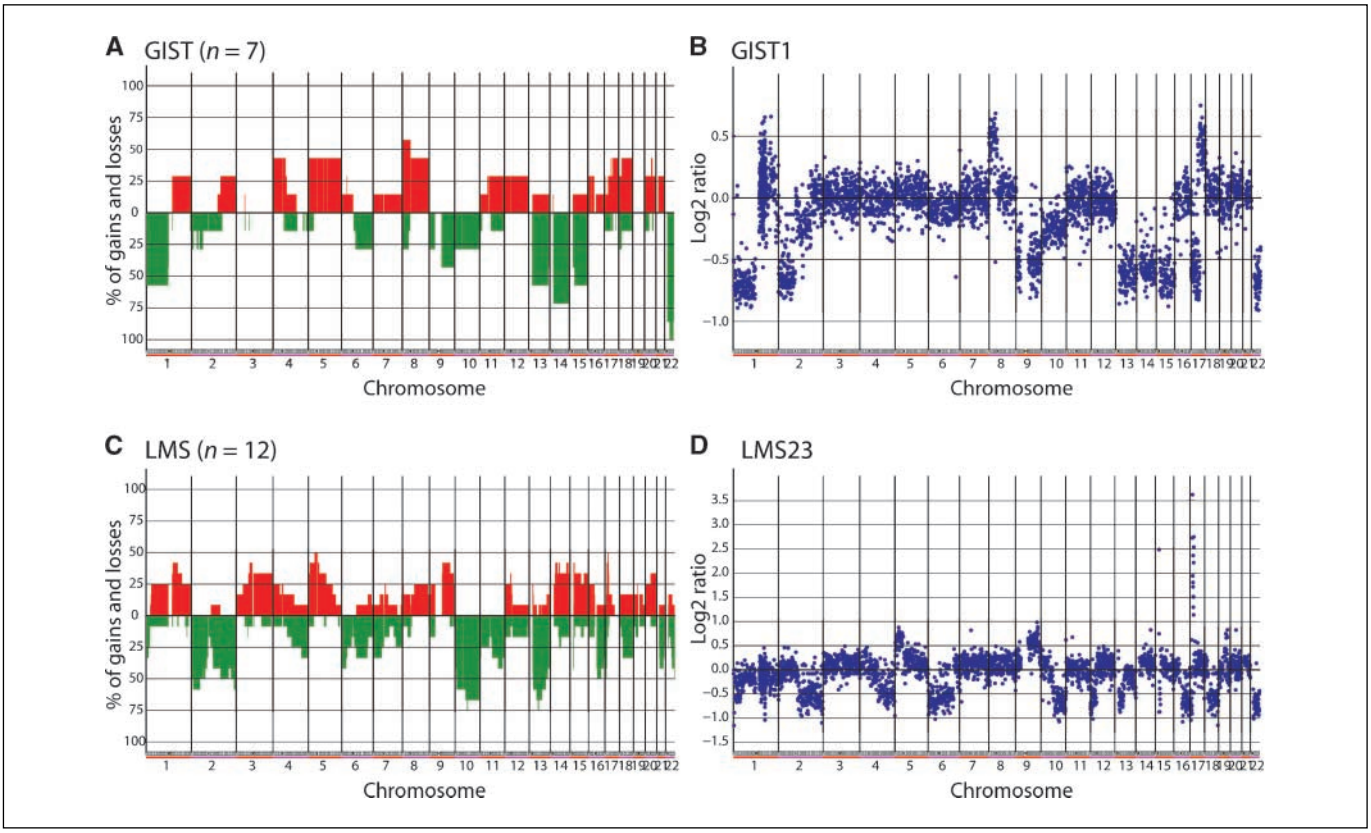

Figure 2. Genome-wide frequency plots of copy number alterations identified by ACE in (*A*) GISTs and (*C*) leiomyosarcomas. Representative whole-genome DNA copy number profile for (*B*) GISTs and (*D*) leiomyosarcomas. Log_2 ratios for each of the BAC and PAC clones are plotted according to chromosome position.

of chromosome 1 was also a target of frequent gains; five samples showed increased copy number of a large region of 1q, and three recurrent regions at 1q21.1-q23.2 (9.9 Mb), 1q23.2-q23.3 (0.5 Mb), and 1q23.3-q25.1 (11.3 Mb) could be delimited. Increased copy number of chromosome 14 was observed in 6 of 12 samples, 4 of them involving almost the complete chromosome. In five samples, smaller regions of gain at 14q12-q21.1 (9.1 Mb), 14q21.2-q21.3 (3.6 Mb), and 14q31.3-q32.2 (9.7 Mb) were observed. A number of other recurrent amplified regions were identified in leiomyosarcomas at 3p12.3-p12.1 (12 Mb), 5p13.2-pcen (14.7 Mb), 9q21.13-q31.3 (39.8 Mb), 12p11.22-p11.21 (2.3 Mb), 15q11.1-q12 (3.5 Mb), 15q25.1-q26.3 (21.2 Mb), and 20q11.21-q13.33 (32.1 Mb); all were altered in >30% of the samples (see Table 2*B*). A frequency plot of gains and losses for leiomyosarcomas is shown in Fig. 2*C*, as well as a representative ratio plot for this type of tumor (LMS23; Fig. 2*D*). Genome-wide ratio plots for all leiomyosarcomas are shown in Supplementary Fig. S1*B*.

A list of all recurrent aberrations for GISTs and leiomyosarcomas is shown in Table 2*A* and *B*. A detailed description of all defined regions of gain and loss from the ACE analysis is presented in Supplementary Table S1*A* and *B*.

Characterization of the 17p13-p11 amplification in leiomyosarcomas. A region in 17p13.1-p11.2 showed the highest level of amplification in the leiomyosarcoma samples. The region encompassed 12.7 Mb and was represented by 23 BACs and PACs, starting with RP11-404G1 and ending with RP11-121A13. Six of the 12 leiomyosarcomas showed increased copy number of all or parts of this region, with LMS1, -7, -21, and -23 showing particularly high levels of amplification. Figure 3*A* shows the copy number of chromosome 17 for the six leiomyosarcoma samples with increased copy number.

As part of an ongoing study, gene expression was analyzed in a panel of soft tissue sarcomas using cDNA microarrays.[11] In the current study, in order to narrow the list of candidate target genes for this amplification, expression levels in six of the leiomyosarcomas analyzed by array CGH and one additional sample (LMS29) were investigated. According to Ensembl, 172 genes are located within the amplified region. Probes for 70 of these genes were present on the cDNA microarray. The expression levels of genes determined to have a value in at least four leiomyosarcomas (43 of 70 genes) are shown in Fig. 3*B*.

Four genes located within the amplified region showed increased expression relative to the median for soft tissue sarcoma (log_2 ratio >1) in three or more of the seven leiomyosarcomas analyzed. The expression levels of these genes in the seven leiomyosarcomas are shown in Fig. 3*C*. Microfibrillar-associated protein 4 (*MFAP4*) was overexpressed in four leiomyosarcomas, whereas aurora kinase B (*AURKB*) and sterol regulatory element binding transcription factor 1 (*SREBF1*) were overexpressed in three leiomyosarcomas. In addition, one gene with unknown function, *FLJ10847*, showed increased expression in three leiomyosarcomas.

[11] P. Francis, HM. Namløs, C. Müller, P. Edén, J. Fernebro, J-M. Berner, B. Bjerkehagen, M. Akerman, A. Isinger, J. Engellau, A. Rydholm, O. Myklebost, M. Nilbert, unpublished data.

SREBF1 showed the highest level of expression; its expression was >16-fold higher in LMS1 and LMS3 compared with the median for soft tissue sarcoma. All genes, except *AURKB*, were located within the minimal recurrent region of amplification in 17p11.2 identified after ACE analysis.

Copy number changes distinguishing GISTs from leiomyosarcomas. SAM was used to identify genomic regions that can differentiate leiomyosarcomas and GISTs by means of DNA copy number changes. SAM uses a modified *t* test to identify genes or genomic clones in a microarray data set whose alteration significantly differs between groups. Using a two-class unpaired design and a false discovery rate of 1%, 238 genomic clones that were significantly different between leiomyosarcomas and GISTs were identified. The 238 clones identified six primary chromosomal regions in 1p, 9q, 14q (two segments), 15q, and 22q, all more frequently deleted in GISTs.

Eighty-nine of 100 genomic clones (89%) between 1p36.11 and p13.1 were identified by SAM to be frequently deleted in GISTs compared with leiomyosarcomas. On chromosome 9, 51 of 94 clones (54%) between 9q21.11 and 9q34.3 were identified. Two chromosomal segments were identified in 14q; 14q11.2-q23.2 and 14q31.3-q32.33 were deleted in GISTs, 29 of 42 (69%) and 19 of 20 (95%) clones, respectively. Five of 20 clones (25%) in 15q24.3-q26.3 were identified by SAM, in addition to 40 of 44 clones (91%) in 22q11.32-13.31. Only 5 of the 238 significant clones did not map to any of the six regions mentioned. Figure 4 shows the genomic areas that were significantly different in copy number between GISTs and leiomyosarcomas. The complete list of selected clones is presented in Supplementary Table S2.

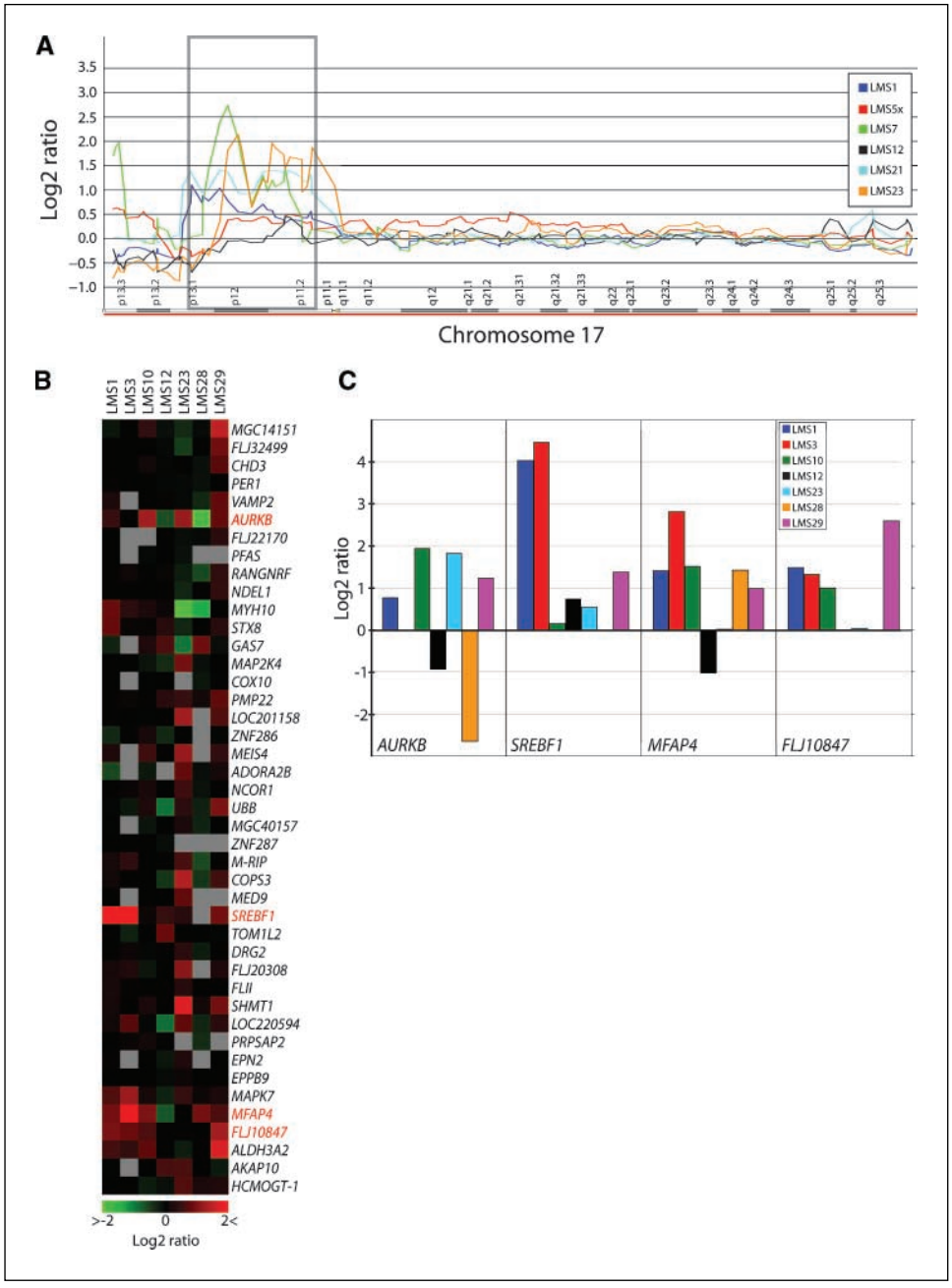

Figure 3. *A,* DNA copy number profile of chromosome 17 for six leiomyosarcomas showing copy number aberrations in 17p. Log_2 ratio for each of the genomic clones is plotted according to chromosome position using a "moving average smoother" with a three-clone window. The gray square highlights a region of 17p frequently gained in these tumors. *B,* heat map for expression of 43 genes located within the recurrent region of amplification in 17p13.1-p11.2 (in chromosomal order). *Red,* increases in expression level; *green*, decreases in expression level. *Gray boxes,* missing values. *C,* plot of expression for four candidate genes across seven leiomyosarcomas.

Discussion

We have used array CGH to compare DNA copy number changes in two groups of mesenchymal malignancies, GISTs and leiomyosarcomas. The initial aim of the study was to determine recurrent copy number aberrations in leiomyosarcomas and identify novel candidate proto-oncogenes and/or tumor suppressor genes. Pathologic review of the leiomyosarcomas revealed two distinct tumor entities based on the current classification standard. Seven of the initial 18 samples were reclassified as GISTs, which until recently, were difficult to distinguish from smooth muscle malignancies like leiomyosarcomas. Samples belonging to the biobank used in this study have been collected during the past 20 years and classified at the time of diagnosis.

Loss of DNA material was as frequent as gain in the GIST samples, seven minimal recurrent regions of loss were determined, as well as seven regions of gain. All chromosomes showed different levels of aberration, and chromosomes 3 and 22 were the least and most altered, respectively. GISTs tend to have simple karyotypes, losses of 14q and 22q are the most common cytogenetic findings, followed by loss of 1p, 9p, or 11p (10, 22–27). In our study, the two most frequent aberrations observed in GISTs were losses of 22q and chromosome 14. These alterations, together with loss of 1p, are believed to be early events in tumor development because they are found in benign as well as malignant and metastatic GISTs (8, 26). With our limited number of samples, we were able to identify a minimal recurrent region of loss between 14q11.2 and q32.33. This segment includes the two segments previously identified by conventional CGH and microsatellite analysis (9).

Gain of 5p and 20q was observed in three of seven GISTs, and previous reports have indicated that these regions are associated with aggressive and metastatic behavior (8). The same study also showed that gain of 8q and 17q and loss of 13q were frequently observed in aggressive and metastatic GISTs. In our panel, recurrent gain of 17q and loss of 13q were observed in three and four of seven GISTs, respectively. Three samples showed gain of 8q, but the only minimal recurrent aberration in chromosome 8 was situated in 8p23.3-cen (gained in four of seven samples). All tumor samples showing alterations in chromosomes 8, 13, and/or 17 belonged to patients that developed metastasis, and all these patients, except one, died of the disease.

Loss of heterozygosity of 9p and loss of the $p16^{ink4A}$ locus have also been associated with aggressive GISTs (11, 28). Our analysis revealed that three of seven samples showed loss in 9p21.3, in which the $p16^{ink4A}$ locus resides. Two tumor samples were classified as high risk and one as low risk, and all three patients developed metastasis and died of cancer.

Eighteen recurrent regions of loss and 14 of gain were identified in our leiomyosarcoma panel. The presence of a large number of alterations per tumor has been correlated with high-grade tumors (7), which also characterizes our tumor panel. The most frequent aberrations observed were losses of 10q and 13q in 9 of 12 samples. Deletion of 10q was recently associated with aggressive tumor behavior and is frequently found in large tumors and tumors that metastasize (29). The minimal recurrent deleted area in 10q21.3 contains only the gene *CTNNA3*, a cadherin-associated protein possibly involved in the organization of the actin cytoskeleton and in cell adhesion (30, 31). CTNNA3, like other α-catenins, inhibits Wnt signaling and expression of T cell transcription factor target genes (30). The *CTNNA3* locus has also recently been identified as a common fragile site (32), which might play a role in its frequent deletion. Deletion of *CTNNA3*, a gene associated with cell adhesion, would be a likely target during early steps of the metastatic process.

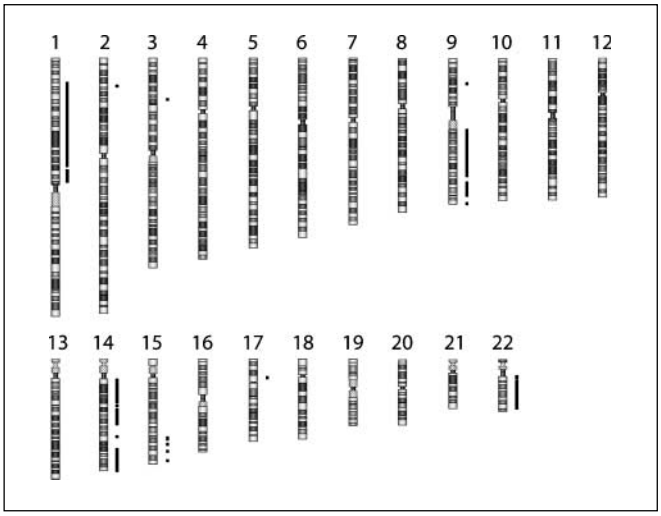

Figure 4. Graphical representation of genomic clones identified by SAM to be significantly different between GISTs and leiomyosarcomas. *Black dots,* chromosomal position of the 238 identified genomic clones.

A 2.7 Mb minimal recurrent region of loss between 13q14.2 and q14.3 was also identified; this chromosomal segment contains the known tumor suppressor gene *RB1*. Deletion of chromosome 13 in leiomyosarcomas has been previously identified in a number of conventional CGH studies (3, 4, 7). Loss of 13q has been found in all stages and grades of leiomyosarcomas (7), indicating that inactivation of a tumor suppressor gene on this chromosome arm may be an early event in the development of leiomyosarcoma. Additional evidence supporting RB1 as a target for inactivation comes from negative RB1 immunostaining of tumors showing deletion of this region (3), as well as generally frequent alteration of RB1 in soft tissue sarcomas (33).

Deletion of segments of 10q, 2p, and 12p, as well as gains in 1q and 17p, have been shown to be more frequent in aggressive, high grade, and recurrent leiomyosarcomas (7). Our analysis found deletions of chromosome 2 to be among the most frequent alterations in leiomyosarcomas, multiple recurrent regions were identified on the short and long arm of this chromosome. No highly recurrent deletion in 12p was observed in our study. Multiple areas of gain were identified in 1q, among them 1q21.1-q23.2 and 1q23.3-q25. Alteration of 1q21 was initially identified and characterized in sarcomas by our group (34, 35). Further analysis identified three novel candidate genes for the 1q21 amplicon (36). One of these, *PPIAL4*, previously termed *COAS2*, is located within the minimal region of aberration in 1q21 observed in leiomyosarcomas.

Alterations on the short arm of chromosome 17 were also seen in more than half of the leiomyosarcoma samples, and a 12.7 Mb region in 17p13.1-p11.2 showed the highest level of amplification. Six of the 12 leiomyosarcomas showed increased copy number of all or part of this region (see Fig. 3*A*), with four samples showing particularly high levels of amplification. The ACE analysis identified a minimal recurrent region within 17p11.2, in addition to a region of loss in 17p13.2-p13.1. In previous CGH studies, frequent high-level amplification of 17p has been observed in leiomyosarcomas (3–5, 7).

In order to identify candidate genes for this amplification, we investigated the expression level of genes present in this region.

In an ongoing expression profiling study, 70 of the 172 genes located within 17p13.1-p11.2 have been analyzed in a panel of soft tissue sarcomas.[11] The expression profiling of seven leiomyosarcomas, six of them analyzed here by array CGH, revealed four genes with >2-fold greater expression in at least three of the seven leiomyosarcomas compared with the median for soft tissue sarcoma (see Fig. 3*B* and *C*). Three of the genes encode proteins with known function, whereas the function of the fourth protein, encoded by *FLJ10847*, is undescribed. The *MFAP4* gene was overexpressed in four tumors, whereas *SREBF1, FLJ10847*, and *AURKB* were overexpressed in three tumors. All genes except *AURKB* were located within the minimal recurrent region of amplification in 17p11.2 that was identified after ACE analysis.

MFAP4 is an extracellular matrix protein possibly involved in calcium-dependent cell adhesion or intercellular interactions, and it has been shown to stimulate *ex vivo* expansion of hematopoietic stem cells (37). Based on these findings, MFAP4 could play a role in tumor growth.

SREBF1 is a transcriptional activator that regulates the transcription of genes for sterol biosynthesis and the low-density lipoprotein receptor gene. *SREBF1* has been shown to be up-regulated in prostate cancer during progression to androgen independence (38), and participates in the transcriptional regulation of proliferation-associated fatty acid synthesis in colorectal cancer (39), making *SREBF1* a possible growth-promoting gene. *SREBF1*, as well as *FLJ10847*, was also shown to be highly expressed in leiomyosarcomas in another microarray study (40).

AURKB is a member of the aurora subfamily of serine/threonine protein kinases. It plays an essential role in chromosome segregation and cytokinesis (41). Overexpression of *AURKB* is observed in a variety of tumors and has been correlated with malignancy and cell proliferation in prostate cancer (42), level of genetic instability in primary non–small cell lung carcinomas (43), and histological malignancy and clinical outcome in high-grade gliomas (44). *AURKB* has also been shown to be involved in Ras-mediated cell transformation (45).

High-level amplification of 17p12-p11.2 is also common in osteosarcomas, malignant bone tumors (46, 47). Several studies have reported recurrent amplification of the genes *PMP22, COPS3, TOP3A*, and *MAPK7* in osteosarcomas (48, 49). *COPS3* and *PMP22* have also been shown to be frequently overexpressed (48, 50), with *COPS3* consistently overexpressed in osteosarcomas with amplification of the gene. In our panel of leiomyosarcomas, *MAPK7* was overexpressed ($\log_2$ ratio >1) in two tumor samples, whereas *COPS3* and *PMP22* were overexpressed in only one sample each (see Fig. 3*B*). Expression of *TOP3A* was undetectable in all the leiomyosarcomas.

Based on their functions and association with cancer, several of the genes found to be overexpressed may be interesting candidate targets for the 17p13.1-p11.2 amplification in leiomyosarcomas. Altered expression of these genes may play a role in the development and/or progression of leiomyosarcoma. Judging from the frequency of overexpression in our data, *MFAP4* seems to be the most likely candidate, although it is possible that genes not represented on our expression array are also of importance in leiomyosarcoma biology.

In order to investigate whether there are regions that can differentiate leiomyosarcomas and GISTs by means of DNA copy number, we used statistical analysis in SAM. Six regions in 1p, 9q, 14q (two segments), 15q, and 22q were identified to have a significantly lower copy number in GISTs compared with leiomyosarcomas. Although some of the leiomyosarcoma samples also showed loss of these regions, it was to a much lower extent than what is observed within GISTs.

In summary, our study shows that array CGH can be used to differentiate histologically similar tumors such as GISTs and leiomyosarcomas, although further validation is required on a larger tumor set. The implementation of whole-genome amplification protocols is bringing array CGH closer to clinical use, enabling the analysis of small numbers of cells such as those obtained from thin-needle biopsies. Thus, array CGH has the potential to play an important role in differentiating and classifying tumors.

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