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Comparative Genomic Hybridization Analysis of Human Sarcomas: I. Occurrence of Genomic Imbalances and Identification of a Novel Major Amplicon at 1q21–q22 in Soft Tissue Sarcomas

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Comparative genomic hybridization (CGH) was recently developed as a tool to survey entire genomes for variations in DNA sequence copy numbers. We have applied this technique to detect and map amplified regions in 54 soft tissue sarcomas. Aberrations were detected by visual analysis of hybridizations or contrast-enhanced digital images, followed by quantitative digital ratio imaging of the aberrant chromosomes. Several tumors showed increased DNA sequence copy number at 12q14, as expected. However, CGH analysis detected amplification of 12q14 also in some tumors where neither MDM2 nor CDK4 was amplified, suggesting that another as yet unknown gene(s) may drive amplification of this region in sarcomas. Furthermore, a novel recurring amplicon was detected at 1q21–q22. DNA amplifications coinciding with this segment were as frequent as those observed for 12q14, indicating that 1q21–q22-linked gene(s) may also play an important role in the development and/or progression of human soft tissue sarcomas. *Genes Chromosom Cancer 14:8–14 (1995).*   © 1995 Wiley-Liss, Inc.

INTRODUCTION

The term soft tissue sarcoma (STS) is used to describe a heterogeneous group of malignant tumors of various supportive tissues. These tumors account for 1% of all human malignancies and include leiomyosarcomas, liposarcomas, angiosarcomas, rhabdomyosarcomas, and the most common form, malignant fibrous histiocytomas (MFH; Enzinger and Weiss, 1988). Cytogenetic studies have revealed structural anomalies in several STS subgroups (Mitelman, 1991; Sandberg and Bridge, 1994). In fact, most of the sarcoma types have been shown to be characterized by recurrent specific chromosomal translocations (Sreekantaiah et al., 1994). Cytogenetic studies of many STS, e.g., MFH and well-differentiated liposarcomas (WDLPS), have demonstrated the occurrence of double minutes, ring chromosomes, and large rod-shaped markers generally associated with gene amplifications (Meltzer et al., 1986; Dal-Cin et al., 1993; Pedeutour et al., 1994).

Overexpression of cellular oncogenes may be an important factor in the development of malignant tumors (Slamon et al., 1989; Dati et al., 1991). Such overexpression can be brought about by various mechanisms, including gene amplification. In soft tissue sarcomas, amplification of the *MYC* oncogene has been reported by several investigators (Ozaki et al., 1993; Barrios et al., 1994). Also, putative oncogenes in the 12q13–q14 chromosome region are amplified in a substantial number of cases (Oliner et al., 1992; Forus et al., 1993; Leach

et al., 1993; Smith et al., 1993). Because the mdm2 protein probably inactivates the tumor suppressor protein p53, a selective growth advantage via 12q13–q14 amplifications was first attributed to increased copy numbers and expression levels of the *MDM2* gene (Momand et al., 1992; Oliner et al., 1992). However, more recent studies have shown that *MDM2* is not always included in the amplicons, suggesting that alternative selection mechanisms may be involved (Forus et al., 1993, 1994).

Comparative genomic hybridization (CGH) allows the detection of DNA-sequence copy number variations in tumor cells and overcomes the need for specific probes or previous knowledge of the presence and nature of chromosomal aberrations (Kallioniemi et al., 1992, 1994b). We have used this technique to map regions of amplification in 54 soft tissue sarcomas. Our studies revealed amplification of various chromosomal regions not previously associated with the development of these tumors, including a recurring novel amplicon at 1q21–q22.

MATERIALS AND METHODS

Specimens

Tumor material was obtained from 45 patients with sarcomas of various subtypes and from nine

different sarcoma samples grown subcutaneously as xenografts in nude mice (Table 1). The xenografts have been passaged several times, and some of them have existed as xenograft lines for years. The tissues were cut into small pieces, frozen in liquid nitrogen immediately after surgery, and stored at −70°C. Blood samples obtained from 10 healthy men and women were used as controls. Peripheral blood from a healthy 46,XX woman was used as a reference in all the experiments.

Preparation of DNA

Genomic DNA from tumor tissues and peripheral blood cells was isolated by standard methods as described previously (Forus et al., 1993).

Comparative Genomic In Situ Hybridization

We used a commercially available nick-translation system (Gibco Life Technologies) to label genomic DNAs with digoxygenin-11-dUTP (Boehringer Mannheim) for tumor DNA and biotin-14-dATP (Gibco) for reference DNA. DNA fragment lengths can be increased by adjusting the ratio of DNase to DNA polymerase in the nick-translation reaction (Kallioniemi et al., 1994b). Currently, we add 6 units extra of the DNA polymerase I large fragment (Klenow). Subsequently, equal amounts (250–400 ng) of each of the labeled probes and fifty-told excess of Cot-1 DNA were coprecipitated using ethanol. The DNA was dissolved in 12 μl of hybridization buffer (50% v/v formamide, 10% w/v dextran sulphate, 2 × SSC, 1% v/v Tween-20, pH 7), denatured at 80°C for 10 min, and preannealed at 37°C for 30 min. Finally, this mixture was used for hybridization to denatured normal metaphase spreads.

Before hybridization, slides were denatured for 3 min at 70–74°C in 70% formamide/2 × SSC, followed by proteinase K digestion for 7.5 min at room temperature (0.1 μg/ml proteinase K in 20 mM Tris-HCl/2 mM CaCl). This treatment will maximize denaturation and probe penetration but allows the banding pattern necessary for chromosome identification to remain intact (Kallioniemi et al., 1994b). Hybridization was carried out under a coverslip in a moist chamber for 2–4 days.

After hybridization, the slides were washed according to regular fluorescence in situ hybridization (FISH) protocols (Pinkel et al., 1988). Immunohistochemical detection of the hybridizing probes was carried out as follows. For detection of biotin molecules, we used fluorescein isothiocyanate (FITC)-conjugated avidin (dilution 1:500; Vector Laboratories), followed by two amplifica-

tion steps using rabbit anti-FITC (dilution 1:250; Dakopats) and FITC-conjugated mouse anti-rabbit (dilution 1:100; Jackson Immunoresearch). Digoxygenin-labeled DNA was visualized after incubations with rhodamine-conjugated sheep anti-digoxygenin (dilution 1:20; Boehringer Mannheim) and Texas red-conjugated donkey anti-sheep (dilution 1:50; Jackson Immunoresearch). Afterwards, the preparations were counterstained with an antifade solution supplemented with 0.5 mg/ml 4,6-diamino-2-phenylindole (DAPI).

Evaluation of CGH Results

For visual examination of chromosome preparations, we used a Zeiss Axiophot epifluorescence microscope, equipped with appropriate filters for visualization of FITC, Texas red, and DAPI fluorescence. Digital images were recorded and processed as described previously (Suijkerbuijk et al., 1994a,b). Photographs were made from the computer screen on Kodak EPP 100 Plus color slide film using a Polaroid Quick Print.

Results of CGH experiments were analyzed using the Comparative Genomic Hybridization applications within the BDS-image FISH software (Oncor). These applications allow a pseudocolor reproduction of fluorescence ratios of Texas red (tumor DNA hybridization pattern) and fluorescein (normal reference DNA hybridization pattern) along the chromosomes. Aberrations were first screened based on visual analysis of hybridization or contrast-enhanced digital images. For each sample, at least 10 metaphase spreads were studied. Increases that were not systematically present in all metaphases or that were seen in only one chromatid or in only one of the chromosome homologs were considered nonspecific and were excluded from the analysis. Chromosome homologs showing regions of consistent overrepresentation of tumor DNA were subjected to further digital analysis, allowing the generation of fluorescence intensity profiles for each fluorochrome along the chromosome. The average green to red ratio was equilibrated to 1 in regions of normal sequence copy numbers. Higher ratios (peaks in the red profile) were interpreted as amplifications. Identification of the individual chromosomes and localization of the amplified regions were based on computer images obtained from the DAPI-banded metaphase chromosomes. To exclude the possibility of misinterpreting variations in pericentromeric and heterochromatic regions as amplifications (Kallioniemi et al., 1994b), we also analyzed DNA obtained from 10 healthy individuals. Regions of interindividual

TABLE I. Classification of the 54 Human Sarcomas Analyzed by CGH[a]

Tumor samples	No. of samples	Samples with amplifications
Fibrosarcoma	I	I
Leiomyosarcoma	12 (3)	3
Liposarcoma	22 (2)	10
Malignant fibrous histiocytoma	16 (2)	10
Malignant schwannoma	3 (2)	2

[a]For each subgroup, the numbers of sarcomas examined and the samples with amplification of any chromosomal region are shown. The numbers of xenografts in each subgroup are indicated in parentheses.

variability detected in this way were excluded from analysis.

RESULTS

Regions of increased sequence copy numbers were detected in 1 of 1 fibrosarcoma, in 3 of 12 leiomyosarcomas (25%), in 10 of 22 liposarcomas (45%), in 10 of 16 MFHs (62%), and in 2 of 3 malignant schwannomas (67%, Table 1), involving a total of 25 different chromosomal subregions (Fig. 1). As indicated in Table 2, the different amplicons varied considerably in size. Also, the number of amplified regions detected for each individual sample was variable.

In total, 13 of the 54 soft tissue tumors (24%) showed amplifications at 12q14. The amplicons were somewhat variable in size, however, and in some cases included, besides 12q14, parts of band 12q13 or 12q15. Also, other regions of chromosome 12 were amplified, although less frequently. Amplicons covering 12q21, 12q21–q22, or 12q22 were detected in three cases. Amplification of the 12q24 band was detected in two MFH (MFH25 and MFH43) and one malignant schwannoma (MS2×). The 12q21–q22 amplicon has previously been associated with WDLPS (Suijkerbuijk et al., 1994a). Our present observations, however, suggest that this amplification may not be specific for WDLPS only; it was also detected in one fibrosarcoma (FS1), and one malignant schwannoma (MS8/MS8x) (Table 2).

A region on the long arm of chromosome 1, 1q21–q22, also showed frequent increases in sequence copy numbers (Fig. 2). Although the different amplicons coinciding with this segment were somewhat variable in size, they showed a minimal region of overlap at 1q21–q22. Amplification of this segment was observed in a total of 14

TABLE 2. Distribution of Amplified Sequences in 25 Individual Sarcomas[a]

Sample	Regional copy number gain	Whole arms/chromosomes
FS1	1p31, 2q37, 4p16, **12q14,** 12q21	
LMS2	**1q21–q23,** 13q31	
LMS14	**1q21–q23,** 16p11–p12	
LMS15	**1q21–q23**	
LS2 (wd)	**1q21–q22, 12q14**	
LS3x (pl)	**1q21–q22**	1p, 3
LS6 (wd)	**1q21–q22,** 1q25, 1q32, **12q14**	
LS9 (wd)	**1q21–q22, 12q14**	4
LS11 (wd)	**12q13–q14**	1
LS13 (wd)	**1q21–q22,** 1q25	
LS21 (wd)	**1q21–q22, 12q14–q15**	
LS22 (wd)	**12q14,** 12q21–q22	
LS28/LS28x (pl/rc)	6qter, 11q13–q14, **12q14–q15,** 15q21–q22	
LS4501 (wd)	**1q21–q22,** 1q25, **12q14–15,** 12q22	
MFH3x	7q21–q31, 11q14, **12q14**	8q
MFH8		1p
MFH15 (pl)	8q22, 11q12	
MFH17	7q21–qter, 8p11–p12	1q
MFH19	**1q21–q22/23,** 3q26	
MHF21 (pl)	14q24–qter, 15cen–q15, 15q22–q26	1p, 7, 9q, 18, 20
MFH36	**1pter–q22,** 2q21, 8q24, 13q34	6p
MFH40	**1q21–q22,** 15q12–q13, 17p11–p12, 18p11.2, 18q21	7
MFH43	**12q13–14,** 12q24	4
MS2x	**12q14,** 12q24	
MS8/MS8x	**1q21–q22, 12q14,** 12q22	

[a]FS, fibrosarcoma; LMS, leiomyosarcoma; LS, liposarcoma; MFH, malignant fibrous histiocytoma; MS, malignant schwannoma. An x indicates a xenograft. For some of the LS and MFH, the subgroups are given in parentheses; wd, well differentiated; pl, pleomorphic; rc, round cell. The most frequently amplified regions are shown in boldface. In two cases, LS28/28x and MS8/8x, both primary and xenograft materials were analyzed. The amplification patterns were equal.

soft tissue sarcomas (26%), i.e., at the same frequency as the 12q14 amplicon. The 1q21–q22 amplicon was detected in all (3 of 3) leiomyosarcomas with regional sequence copy number variations and in 3 of 14 osteosarcomas (Forus et al., 1995). A second amplicon on chromosome 1 (1q25) was observed in 3 of 22 (14%) of the liposarcomas but not in any of the other soft tissue tumors examined.

DISCUSSION

Previous studies have convincingly demonstrated the usefulness of the CGH technique in the detection and mapping of DNA-sequence copy number changes in solid tumors (Kallioniemi et al.,
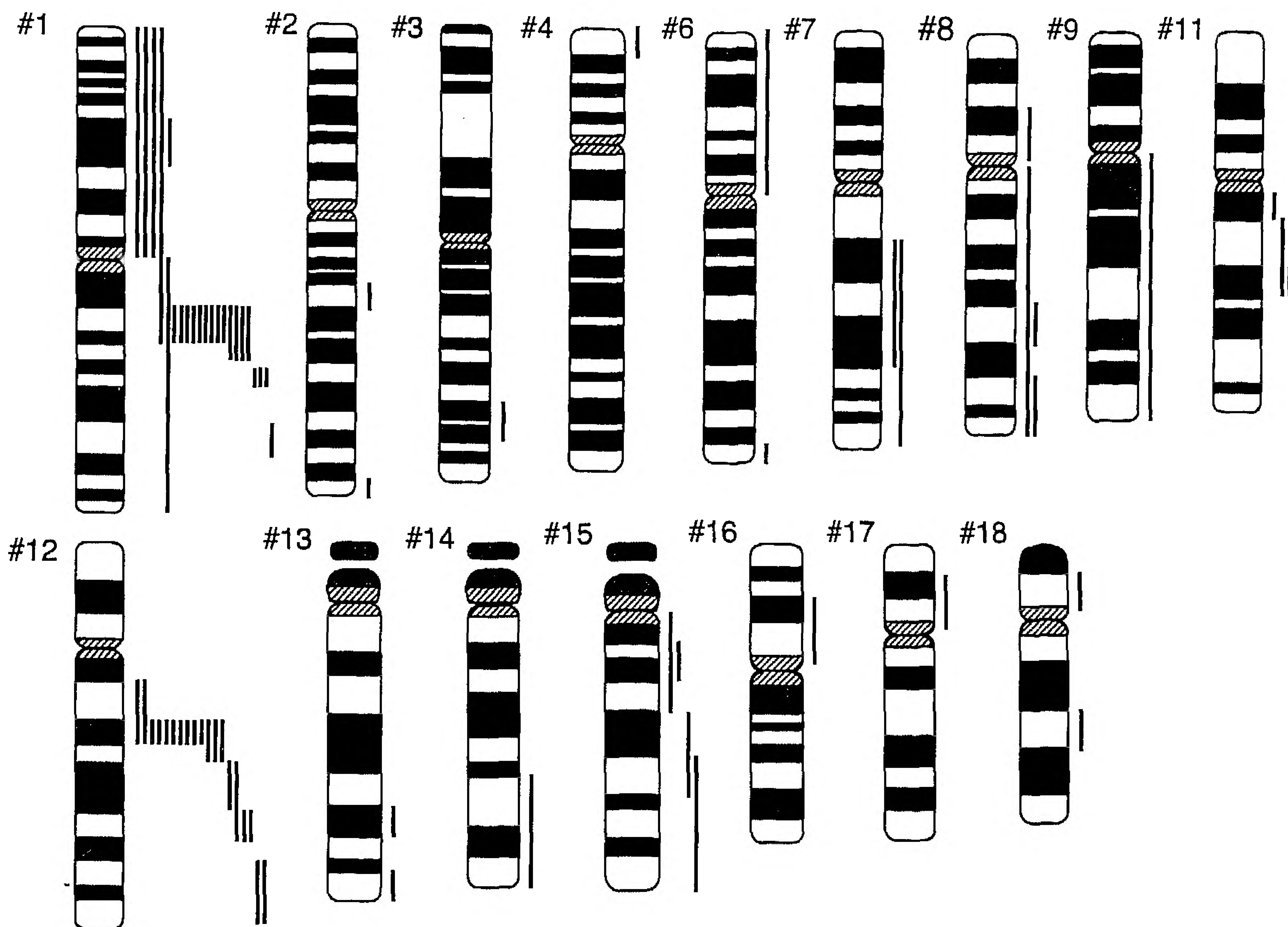
Figure I. Chromosomal localization of DNA-sequence copy number increases in 54 soft tissue sarcomas as revealed by CGH. Only those chromosomes in which amplified regions were detected are represented. A vertical bar indicates an amplified region detected in a given tumor sample. The chromosomal band location of the amplicons was determined on the basis of DAPI banding patterns. Since high-resolution subband localization was not possible, the amplified regions may be smaller than indicated.

1992, 1994a; Suijkerbuijk et al., 1994a). CGH provides information not only on the overall occurrence of deletions or amplifications in a given tumor but also on the size of the affected region (Cher et al., 1994; Kallioniemi et al., 1994a).

Using CGH, we have detected and mapped regions of amplification in 54 soft tissue sarcomas of various (sub)types. As a control, DNA extracted from peripheral blood samples of 10 healthy individuals was tested against the reference DNA. Regions surrounding some of the centromeres showed an increased copy number in some of the samples. However, ratio changes at these sites could be detected in only a minority of metaphases. Moreover, they never coincided with the chromosomal regions we determined as amplified in the tumors (not shown).

Fifty-three of the fifty-four STS had previously been examined by Southern blot analysis for amplifications of the 12q14-linked genes *MDM2, CDK4, SAS, CHOP, GLI*, and *A2MR* (Forus et al.,

1993, 1994). The present CGH analyses revealed increased DNA-sequence copy numbers in all of the seven cases with known amplifications (LS6, LS11, LS21, LS22, LS28/LS28x, FS1, and MS8/MS8x; Table 2), as expected. We also detected amplifications involving 12q14 in six additional cases, three liposarcomas (LS2, LS9, and LS4501), two malignant fibrous histiocytomas (MFH3x and MFH23), and one malignant schwannoma (MS2x). Only one of these, LS4501, was not included in the previous study (Forus et al., 1993).

In principle, Southern blot analyses are more sensitive than CGH (Kallioniemi et al., 1994a,b), and it is unlikely that in the previous studies (Forus et al., 1993, 1994) amplifications of *MDM2, CDK4*, or other 12q14-linked genes studied were missed. A role as a selective driver in these amplicons was previously assigned to the *MDM2* or *CDK4* gene (Oliner et al., 1992; Forus et al., 1993, 1994; Khatib et al., 1993). However, the present observations suggest that alternative explanations must
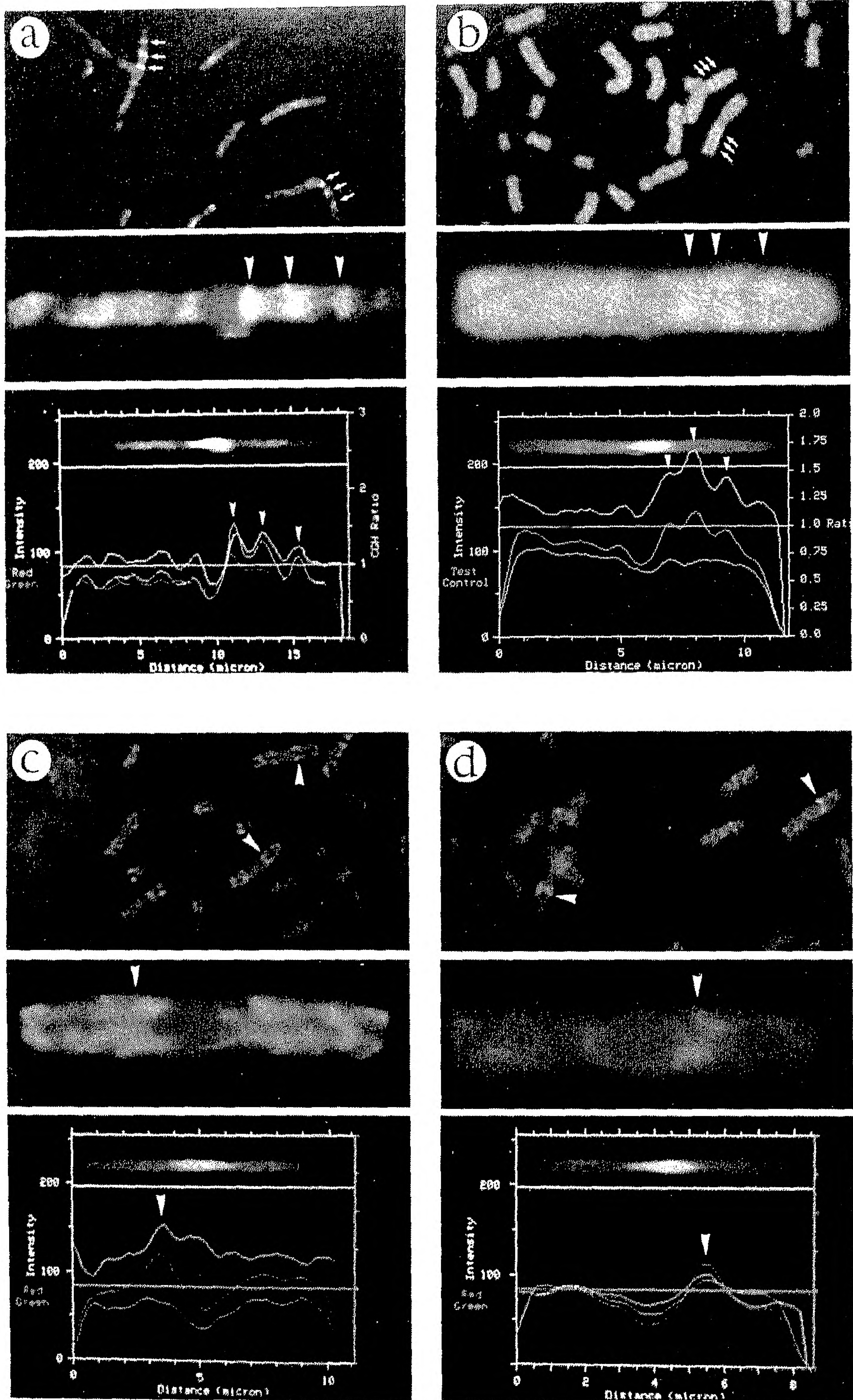
Figure 2. Hybridization of DNA from three different liposarcoma samples (in red) and normal female reference DNA (in green) to normal metaphase chromosomes. a,b: Two different metaphases hybridized with DNA from LS6. The three peaks in the lower panel (arrowheads) mark the 1q21–q22, 1q25, and 1q32 amplicons. Digital images of a partial metaphase spread including the two chromosome 1 homologs (upper panels) and a single straightened copy of chromosome 1 (middle panels) are shown. The amplicons are marked by arrows (upper panels) and arrowheads (middle panels), respectively. The lower panels show inte-gration of fluorescence profiles along the chromosome 1 homolog depicted in the middle panels. The measured intensity profiles are depicted in red (tumor DNA) and green (normal DNA) lines, respectively. The blue lines represent the CGH profile, i.e., the red-to-green fluorescence ratios along the whole chromosome length. c: A metaphase hybridized with DNA from LS3x, showing overrepresentation of the whole 1p arm and the 1q21–q22 segment (arrowheads). d: A metaphase hybridized with DNA from LS21, showing the 1q21–q22 amplicon (arrowheads).

be considered, at least for a subset of tumors. In this light, amplification of more than one gene could be important, e.g., *MDM2*, because its gene product can inactivate p53 (Momand et al., 1992; Oliner et al., 1992), and *CDK4*, because of its possible role in the regulation of pRB activity (Hinds et al., 1992). However, the simplest explanation might be that the 12q14 amplicons are selected by a common mechanism driven by an as yet hypothetical gene, as suggested previously (Forus et al., 1993). Our future studies will be aimed at the identification of such a gene(s).

Interestingly, the CGH analyses also revealed a novel major region of amplification in human sarcomas, namely, 1q21–q22 (Fig. 2). Increased sequence copy numbers coinciding with the 1q21–q22 segment were observed in a total of 14 soft tissue sarcomas (26%; Table 2) and were also detected in 3 of 14 osteosarcomas (Forus et al., 1995). Interestingly, the 1q21-22 amplicon was not observed in breast cancer (Kallioniemi et al., 1994a), prostate cancer (Cher et al., 1994), or malignant gliomas (Schröck et al., 1994), nor has it previously been associated with the development or progression of human sarcomas. Conceivably, the 1q21–q22 region harbors genes important for human sarcoma development and/or progression. Several potentially interesting genes have been mapped to this region, including the octamer-binding transcription factor *OTF1* (1q22–q25) and several members of the S-100 family of calcium-binding proteins, e.g., CACY and CAPL (1q21–q25; Engelkamp et al., 1993; Dracopoli et al., 1994). Notably, overexpression of the *CACY* gene has been associated with metastatic behavior of human melanomas (Weterman et al., 1992), and amplification of the gene has been detected in some melanoma cell lines.

The most frequently observed amplicons, 1q21–q22 and 12q14, were unevenly distributed among the different STS subgroups. We detected both amplicons in six samples, including five well-differentiated liposarcomas (LS2, LS6, LS9, LS4501, and LS21). Fifteen additional samples showed amplification of either 1q21–q22 or 12q14. However, there was no clear correlation between the presence of either 1q21–q22 or 12q14 or both amplicons and any known clinical parameters (not shown). Therefore, it is difficult to establish the biological consequences of the various combinations. The overall occurrence of DNA amplification was considerably lower in leiomyosarcomas (25%) than in other STS subgroups, i.e., liposarcomas (47%) and MFH (59%), showing that other

(genetic) mechanisms may be more important for the former group of sarcomas. However, the three leiomyosarcomas with detectable amplifications (LMS2x, LMS14, and LMS15; Table 2) all showed involvement of 1q21–q22, whereas neither the previous molecular analysis of 12q14-linked (onco-)genes (Forus et al., 1993, 1994) nor the CGH analysis described here detected 12q14 amplifications. These observations indicate that a gene in 1q21–q22 may play an important role in the development of at least a subset of leiomyosarcomas.

Our CGH analyses revealed other amplified regions as well. However, most of them were also detected in other tumor types (Cher et al., 1994; Kallioniemi et al., 1994a) or were present at relatively low frequencies (in one or two of the samples). Amplified regions observed in a large number of tumors are more likely to harbor relevant oncogenes, since one expects genes of importance for malignant development to be repeatedly activated (or aberrated). Thus, we consider these latter amplifications less significant for the processes leading to STS.

Although the CGH technique is very well suited for the detection and mapping of DNA-sequence copy number changes, especially amplifications, one should be aware of its limitations. For example, CGH may fail to detect low-level copy number changes involving small chromosomal regions. CGH analysis of breast cancer cell lines with known amplifications of *ERBB2* and *BCL1*, for example, revealed increased sequence copy numbers at the respective loci (17q12 and 11q13) in only two of three of the cases (Kallioniemi et al., 1994a). All high-level amplifications (5–15-fold) were detected, whereas those at lower levels (2–5-fold) were missed. In fact, it was later reported that the total length of amplified DNA has to be at least 2 Mb for detection by CGH (Kallioniemi et al., 1994b, and references therein). Thus, the actual amplification frequencies, especially for the smaller amplicons, may be significantly higher than those reported here. A more complete overview of genetic alterations in a given panel of tumors can be obtained by combining CGH analysis with interphase FISH and molecular analysis using specific probes targeting the regions highlighted by CGH. Such studies are currently in progress.

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