GENES, CHROMOSOMES & CANCER 14:15–21 (1995)

Comparative Genomic Hybridization Analysis of Human Sarcomas: II. Identification of Novel Amplicons at 6p and 17p in Osteosarcomas

Anne Forus, Daniël Olde Weghuis, Dominique Smeets, Øystein Fodstad, Ola Myklebost, and Ad Geurts van Kessel

Department of Human Genetics, University Hospital, Nijmegen, The Netherlands (A.F., D.O.W., D.S., A.G.v.K.); Department of Tumor Biology, The Norwegian Radium Hospital, Oslo, Norway (A.F., Ø.F., O.M.)

Using comparative genomic hybridization (CGH), we have identified and mapped regions of DNA amplification in primary and metastatic osteosarcomas. Samples were obtained from four patients and ten independent xenografts. Sixty-four percent of the tumors showed increased DNA-sequence copy numbers, affecting 23 different chromosomal sites. Most of these regions were not previously associated with the development and/or progression of these tumors. Amplicons originating from 1q21–q23, 6p, 8q23–qter, and 17p11–p12 were observed most frequently. The 6p and 17p11–p12 amplicons seem to be specific for osteosarcomas, indicating that these regions may harbor genes relevant for the development of these tumors. *Genes Chromosom Cancer 14:15–21 (1995).*

INTRODUCTION

Osteosarcoma is the most common malignant tumor of bone in adolescents. Classical osteosarcomas are high-grade malignant tumors with a poor prognosis, and 10–20% of the patients have detectable metastases at diagnosis. Little is known about the cytogenetic changes in these tumors, or how such alterations may relate to tumor initiation or progression. However, several reports have demonstrated highly aneuploid and extremely complex karyotypes with numerous cytogenetic abnormalities, including ring chromosomes and double minutes (Mertens et al., 1993; Tarkkanen et al., 1993; Fletcher et al., 1994; Sandberg and Bridge, 1994). The most frequent observations include loss of chromosomes 13 and 17 or of the 13q14 and 17p13 bands (Hoogerwerf et al., 1994; Sandberg and Bridge, 1994).

Accordingly, the most important genetic alterations in osteosarcomas may involve the tumor suppressor genes *TP53* (17p13) and *RB1* (13q14). Abnormalities of the *TP53* gene—loss, rearrangement, mutation, and deletion—have indeed been observed in a large fraction of the osteosarcomas studied (Miller et al., 1990; Mulligan et al., 1990; Andreassen et al., 1993). Other studies have shown that osteosarcomas frequently exhibit structural anomalies of the retinoblastoma gene *RB1* (Toguschida et al., 1988), and patients with hereditary retinoblastoma frequently develop osteosarcoma. It was recently shown that loss of heterozygosity (LOH) or other DNA alterations at the *RB1* locus in primary osteosarcoma are associated with a poor clinical outcome (Wadayama et al., 1994). Thus, functional inactivation of these genes, by either dominant or recessive modes, could play a major role in the development of these cancers.

Recently, the protooncogene *MDM2*, which encodes a protein that binds to and probably inactivates p53 (Momand et al., 1992; Oliner et al., 1992), was found to be amplified in a number of sarcomas (Oliner et al., 1992; Forus et al., 1993; Ladanyi et al., 1993; Leach et al., 1993). In osteosarcomas, *MDM2* amplification was detected in about 30% of recurrent and metastatic lesions, but not in primary tumors (Ladanyi et al., 1993), and it was suggested that *MDM2* amplification may be associated with metastatic disease and concurrent poor prognosis. However, we detected no *MDM2* amplifications in our osteosarcoma panel, although more than one-third of the samples were obtained from metastatic lesions (Forus et al., 1993; Fløerenes et al., 1994). Clearly, our current knowledge of the occurrence, nature, and role of specific genetic changes in the development and progression of these tumors is still far from complete.

Here we have applied comparative genomic hybridization (CGH), a recently developed method to survey entire genomes for DNA-sequence copy number variations (Kallioniemi et al., 1992; Du Manoir et al., 1993; Suijkerbuijk et al., 1994a), to elucidate some of these questions. In doing so, we were able to identify and map 23 regions of DNA amplification in 14 primary and metastatic human osteosarcomas.

Received December 14, 1994; accepted March 9, 1995.

Address reprint requests to Anne Forus, Department of Tumor Biology, The Norwegian Radium Hospital, 0310 Oslo, Norway.

MATERIALS AND METHODS

Specimens

Tumor tissues were obtained from four patients with osteosarcoma (three primary and one metastatic lesion) and from ten independent xenografts grown subcutaneously in nude mice. All the tumors were highly malignant (grade IV). Seven of the xenografts were established from primary tumors, the rest from metastatic lesions. The xenografts have been passaged several times, and some of them have existed as xenograft lines for years. The tumor tissues were frozen in liquid nitrogen immediately after surgery and stored at −70°C. DNA isolated from blood from a healthy woman (46,XX) was used as reference.

Preparation of DNA

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

Comparative Genomic In Situ Hybridization

CGH experiments and the evaluation of the results were performed as described previously (Forus et al., 1995). The analysis was based on visual assessment, followed by profile quantitation of the aberrant chromosomes.

RESULTS

DNA extracted from frozen tissue was used for CGH analysis. Based on the previously described criteria for interpretation of CGH results (Kallioniemi et al., 1994b; Suijkerbuijk et al., 1994b; Forus et al., 1995), we detected regions of increased DNA-sequence copy numbers in nine of the 14 (64%) primary and metastatic lesions examined. In Figure 1, the chromosomal localization of the various amplicons is depicted. The regions involved in each individual case are provided in Table 1. As indicated, the size of the different amplicons varied considerably, ranging from very small DNA segments to whole chromosome arms. Usually, multiple regions were amplified in a single specimen. Regions of interindividual variance (i.e., heterochromatic) and pericentrometric regions were excluded from the analysis (Forus et al., 1995).

In five cases (OS4x, OS5x, OS9x, OS11x, and OS13x), CGH revealed gain of whole chromosomes or chromosome arms, and in three of these cases (OS5x, OS9x, and OS13x), 6p was involved (Fig. 2). In another sample (OS6x), amplification of the 6p11–p21.2/21.3 region was revealed. Extra copies of 5p and 10p were detected only once (OS5x and OS9x).

Regional copy number increases were seen repeatedly at 1q21–q23, 8q23–8qter, and 17p11–p12. The amplicons on 1q (OS4x, OS9x, and OS13x) were somewhat variable in size but showed a minimal region of overlap at 1q21–q23. The 8q23–8qter amplicon was observed in three cases (OS5x, OS11x, and OS13x), including one showing gain of the whole 8q arm (OS11x; Fig. 2). Amplification of 17p11–p12 was detected in a total of four cases (OS4x, OS5x, OS8x, and OS21; Fig. 2), again including one with amplification of the whole 17p arm (OS5x). In one sample (OS8x), 17p11–p12 was the only detectable amplicon.

Also, regions on 5q (OS9x and OS13x), 12q (OS6x and OS11x), 15q (OS5x, OS11x, and OS21), and 17q (OS4x and OS5x), as well as other chromosomal sites, showed copy-number increases. However, these latter anomalies occurred at relatively low frequencies (in only one or two samples). Overall, the present CGH analysis revealed high-level DNA amplifications at 23 different chromosomal sites in primary as well as metastatic osteosarcomas.

DISCUSSION

The observations reported here significantly extend our current knowledge on the genetic alterations that occur in human osteosarcomas. Previous molecular and cytogenetic analyses have demonstrated the occurrence of nonrandom loss of chromosome 13- and 17-derived regions during the development of osteosarcomas (Miller et al., 1990; Andreassen et al., 1993; Mertens et al., 1993; Tarkkanen et al., 1993; Fletcher et al., 1994; Sandberg and Bridge, 1994; Wadayama et al., 1994). However, most of the regional copy-number changes (amplicons) reported here were not detected previously, nor were any of them associated with the development and/or progression of these tumors.

Regions of increased DNA-sequence copy numbers were detected in primary as well as metastatic lesions, but our studies failed to show any overt correlation between the malignancy stage of individual tumors and the number of amplified regions observed in them (Table 1). However, amplified regions were detected in a substantially larger fraction of xenografts (eight of ten) than of primary tumors (one of four). Previous analyses of 12q13–q14 amplifications in a large panel of human sarcomas showed an equal distribution of such aberrations among xenografts and primary samples (Forus et al., 1993; Flørenes et al., 1994). Cases where

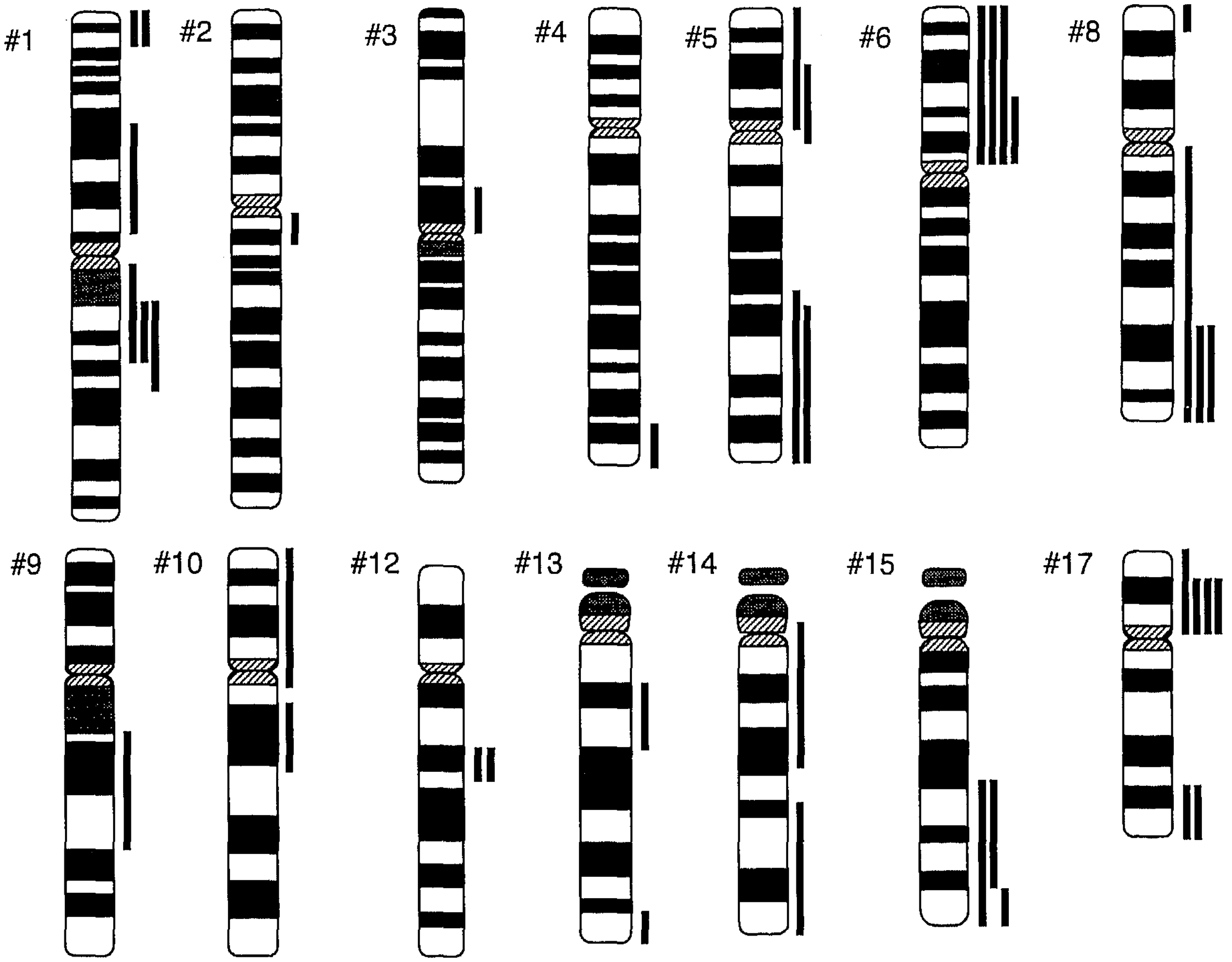

Figure 1. Chromosomal localization of DNA-sequence copy number increases in 14 osteosarcomas as detected by CGH. Only those chromosomes in which amplified regions were detected are represented. A vertical bar indicates an amplified region in a given tumor sample. The chromosomal band location of the amplicons was determined on the basis of DAPI banding pattern. Because high-resolution subband localization was not possible, the amplified regions may in fact be smaller than indicated.

both the xenograft and the primary sample were available consistently showed the same gene amplification status both by Southern blot analysis (Forus et al., 1993) and by CGH analysis (MS8/VMS8x and LS28/LS28x; Forus et al., 1995). Apparently, xenografting per se does not induce amplifications. However, xenografts are more easily established from aggressive tumors, which may be more likely to contain such aberrations.

The two samples (OS6x and OS11x; Table 1) with previously detected amplifications of *SAS* and *CDK4* (Forus et al., 1994) showed increased DNA-sequence copy numbers at 12q14, as expected. Gains of sequences were also observed at the distal parts of 5q, 15q, and 17q. Although the amplifications at 15q were highly variable in size, they overlap at 15q22–q25 (OS5x and OS11x) and 15q26–qter (OS5x and OS21). Thus, important genes in this context may be the protooncogenes *FES* and *FUR* at 15q25–qter (Donlon and Malcolm, 1991). There are no obvious candidate genes in the 5q22–q23–qter (OS9x and OS13x) and 17q24–q25 (OS4x and OS5x) regions (Solomon and Ledbetter, 1991; Washmut et al., 1991; Fain, 1992).

Gain of sequences from the long arm of chromosome 1 was detected in three of the 14 osteosarcomas (OS4x, OS9x, and OS13x; Table 1), with a minimal region of overlap at 1q21–q23. Amplification of this segment (1q21–q22) was detected also in 14/54 soft tissue sarcomas (Forus et al., 1995), indicating that sequences from this region may be related to the development of sarcomas in general, a possibility that has been discussed in more detail by Forus et al. (1995).

Four cases showed amplification of 6p sequences, with a minimal region of overlap at 6p11–

TABLE 1. Distribution of Amplified Regions in 14 Individual Osteosarcomas[a]

Tumors	Regional copy-number gains	Whole arms/chromosomes
Primary		
OS2x	13q34	
OS4x	**1q11–q23,** 2q11–q12, **17p11–p12,** 17q24–q25	7, 14, 22
OS5x	3p11–p12, **8q23–qter,** 10cen, 10q21, 13q13–q14, 14cen–q21, 14q23–qter, **15q22–qter,** 17q24–25	**6p,** 10p, **17p**
OS6x	4q34–qter, **6p11–21.2,** 12q14	
OS7x		
OS9x	1p13–p31, 1p36, **1q21–q25,** 5q22–qter, 9q13–q22	**5p, 6p,** 17
OS11x	12q14, **15q22–q25**	**8q**
OS21p	8p23, **15q26, 17p11–p12**	
OS29p		
OS31p		
Metastatic		
OS8x	**17p11–p12**	
OS12x		
OS13x	1p36, **1q21–q23,** 5cen–p14, 5q23–qter, **8q23–qter**	**6p**
OS17p		

[a]Samples were obtained from primary material (p) and xenografts (x). The most frequently amplified regions are shown in boldface.

p21.3. The protooncogene *PIM1* at 6p21 (Ziegler et al., 1991) is a possible target gene. Amplification of 6p has also been observed in breast cancer, although at lower frequencies (Kallioniemi et al., 1994a), and was detected only once in a panel of 54 soft tissue sarcomas (Forus et al., 1995). Since this event seems to be more frequent in osteosarcomas, it is possible that genes located in this region are involved in the development of these tumors.

The 8q23–qter amplicon was detected in three of 14 osteosarcomas (21%) and, most likely, involves the *MYC* locus. Amplifications and other aberrations of the *MYC* oncogene have been observed in various neoplasms but were thought to be relatively rare in osteosarcomas (Ladanyi et al., 1993). Our results indicate that *MYC* amplifications may be more frequent in our panel than in tumors analyzed previously, although it cannot be excluded that genes other than *MYC* are responsible. Insofar as putative transcription factors are among the major classes of oncogenes (Feldman and Yaar, 1991), the most interesting alternative candidates may be the two zinc-finger proteins ZNF7 and ZNF34 in band 8q24 (Donis-Keller and Buckle, 1991). It is conceivable that the target gene(s) of this amplicon plays a significant role(s) in a broader range of tumors; the 8q24 band is known also to be amplified frequently in breast and prostate cancer (Cher et al., 1994; Kallioniemi et al., 1994a).

Increased copy number of sequences at 17p11–p12 was detected in four of the 14 osteosarcomas (29%). Although regions distally in 17p appear to be frequently lost in this class of tumors (Miller et al., 1990; Mulligan et al., 1990; Andreassen et al., 1993; Fletcher et al., 1994; Sandberg and Bridge, 1994), amplification of proximal 17p has not been reported previously. The 17p11–p12 amplicon was detected in only one of 54 soft tissue tumors (Forus et al., 1995).

Most of the proposed models for amplification (McClintock, 1941; Toledo et al., 1992; Stark, 1993) are based on asymmetric distribution of genes during cell division. It has been suggested that mechanisms accounting for amplification will also lead to loss of heterozygosity (LOH; Stark, 1993). One might therefore speculate that the mechanism leading to increased copy numbers of the 17p11–p12 segment is related to LOH at the distal 17p region (e.g., of *TP53*). However, it is difficult to envisage how loss and gains in the same region could be simultaneously selected for. Among the tumors examined, LOH at the *TP53* locus was detected in the tumor with gain of the whole 17p arm (OS5x), and the remaining *TP53* allele was mutated (Andreassen et al., 1993). Data on the *TP53* status were not available for the samples with 17p11–p12 amplification (OS4x, OS8x, and OS21), but LOH involving *TP53* was found in other tumors without detectable gains of 17p re-

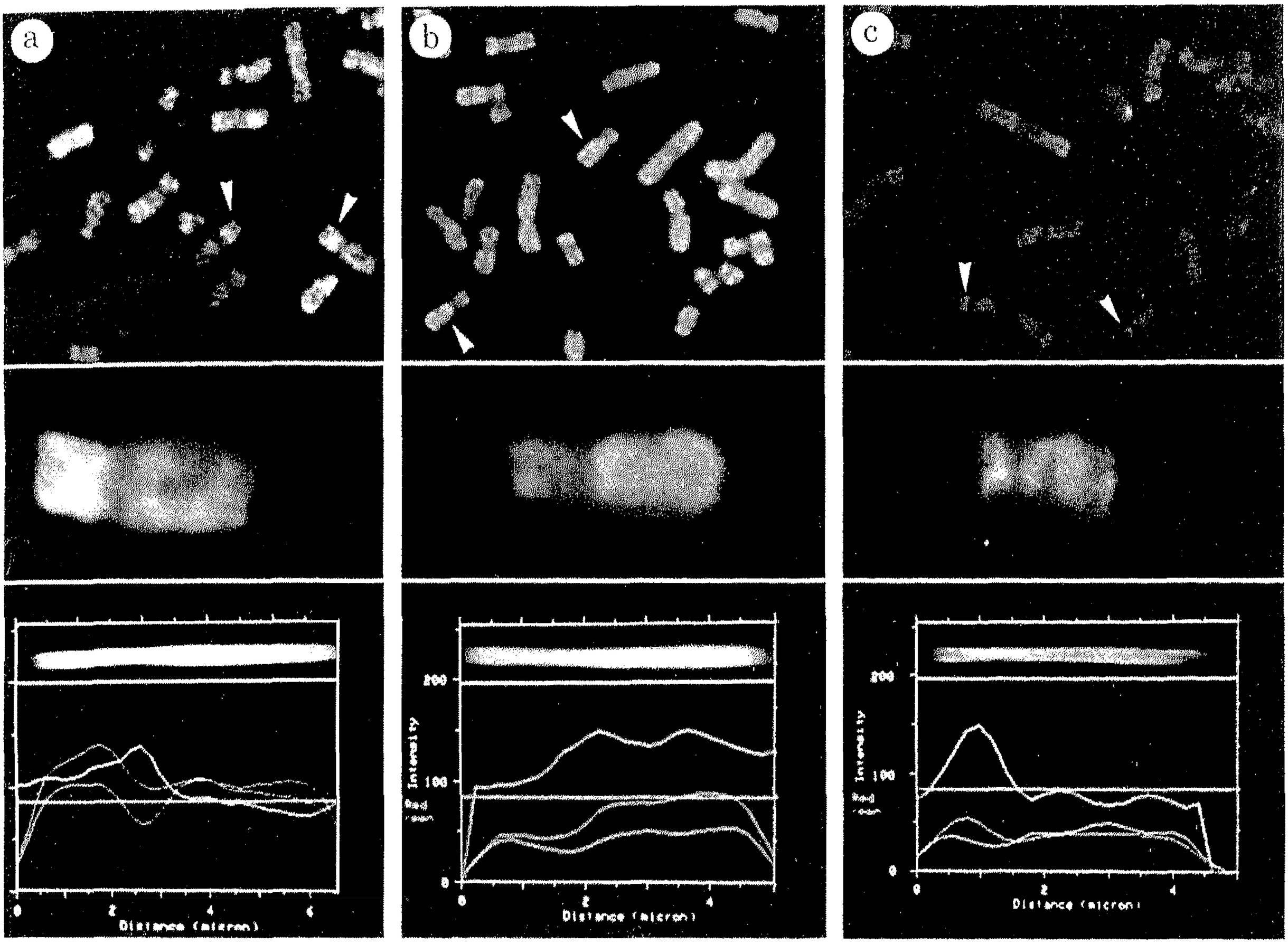

Figure 2. Image analysis of fluorescent DNA from various osteosarcomas (in red) and normal female reference DNA (in green) hybridized to normal metaphase chromosomes. **a–c** (top): CGH analysis using DNA from OS9x (a), OS11x (b), or OS21 (c). The amplicons on 6p (OS9x), 8q (OS11x), and 17p11–p12 (OS21) are indicated by arrowheads. Middle panels: Isolated images of chromosomes 6 (a), 8 (b), and 17 (c). Lower panels: Integration of fluorescence intensity along the chromosomes depicted in the middle panels. The measured intensity profiles are depicted by red (tumor DNA) and green (normal DNA) lines. The blue lines represent the CGH profiles, i.e., the red-to-green ratio along the whole chromosome length. The peaks indicate the respective amplifications. **d:** Partial metaphase cell hybridized to DNA from OS5x. **e:** DAPI banding pattern from the metaphase shown in d. Figure shows the amplifications on 3p11–p1, 6p, 8q23–qter, 10p.10cen, 10q21, 17p, and 17q24–q25. Chromosomes with amplifications are indicated by their numbers (e).

gions (OS12x and OS13x; Andreassen et al., 1993). We therefore conclude that LOH at 17p13 and amplification of the 17p11–p12 region probably are independent events in the development of osteosarcomas. So far, the 17p11–p12 segment does not seem to harbor obvious candidate (onco)genes as drivers for these DNA amplifications. However, a number of (anonymous) reference markers previously mapped to this region (Solomon and Ledbetter, 1991; Fain 1992) may be instrumental for positional cloning of putative target genes.

ACKNOWLEDGMENTS

The authors thank G.M. Maelandsmo, H.H. Ropers, R.J. Sinke, and R.F. Suijkerbuijk for advice and support and H. Mieloo for expert technical assistance. This work was supported by the Norwegian Research Council, the Norwegian Cancer Society, and the Dutch Cancer Society (Koningin Wilhelmina Fonds).

NOTE ADDED IN PROOF

Among the tumors with amplification of 8q, at least one was negative for *MYC* amplification, indicating that other genes on 8q may be responsible.

REFERENCES

Andreassen A, Øyjord T, Hovig E, Holm R, Florenes VA, Nesland JM, Myklebost O, Hødie J, Bruland OS, Børresen A-L, Fodstad Ø (1993) p53 abnormalities in different subtypes of human sarcomas. Cancer Res 53:468–471.

Cher ML, MacGrogan P, Bookstein R, Brown JA, Jenkins JB, Jensen RH (1994) Comparative genomic hybridization, allelic imbalance, and fluorescence in situ hybridization on chromosome 8 in prostate cancer. Genes Chromosom Cancer 11:153–162.

Donis-Keller H, Buckle V (1991) Report of the committee on the genetic constitution of chromosome 8. Cytogenet Cell Genet 58: 382–402.

Donlon TA, Malcolm S (1991) Report of the committee on the genetic constitution of chromosome 15. Cytogenet Cell Genet 58:624–642.

Du Manoir S, Speicher MR, Joos S, Schröck E, Popp S, Dohner H, Kovacs G, Robert-Micoud M, Lichter P, Cremer T (1993) Detection of complete and partial chromosome gains and losses by comparative genomic in situ hybridization. Hum Genet 90:590–610.

Fain PR (1992) Third international workshop on human chromosome 17 mapping. Cytogenet Cell Genet 60:178–186.

Feldman SR, Yaar M (1991) Oncogenes. Control genes. Arch Dermatol 127:707–711.

Fletcher JA, Gebhardt MC, Kozahevrich HP (1994) Cytogenetic aberrations in osteosarcomas. Nonrandom deletions, rings and double minute chromosomes. Cancer Genet Cytogenet 71:81–88.

Florenes VA, Maelandsmo GM, Forus A, Andreassen A, Myklebost O, Fodstad Ø (1994) *MDM2* gene amplification and transcript levels in human sarcomas—Relationship to p53 status. JNCI 86: 1297–1302.

Forus A, Florenes VA, Maelandsmo GM, Meltzer PS, Fodstad Ø, Myklebost O (1993) Mapping of amplification units in the q13–14 region of chromosome 12 in human sarcoma: Some amplica do not include *MDM2*. Cell Growth Differ 4:1065–1070.

Forus A, Schmitt J-M, Pedeutour F, Turc-Carel C, Meltzer PS, Fodstad Ø, Myklebost O (1994) Amplification of 12q13–14 in human sarcomas: Is there a common selective gene, or are different mechanisms important in different sets of tumors? Cytogenet Cell Genet 67:269.

Forus A, Olde Weghuis D, Smeets D, Fodstad Ø, Myklebost O, Geurts van Kessel A (1995) 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. Genes Chromosom Cancer, 14:15–21.

Hoogerwerf WA, Hawkins AL, Perlman EJ, Griffin CA (1994) Chromosome analysis of nine osteosarcomas. Genes Chromosom Cancer 9:88–92.

Kallioniemi A, Kallioniemi O-P, Sudar D, Rutovitz D, Gray JW, Waldman F, Pinkel D (1992) Comparative genomic hybridization for molecular cytogenetic analysis of solid tumors. Science 258: 818–821.

Kallioniemi A, Kallioniemi O-P, Piper J, Tanner M, Stokke T, Chen L, Smith HS, Pinkel D, Gray JW, Waldman FM (1994a) Detection and mapping of amplified DNA sequences in breast cancer by comparative genomic hybridization. Proc Natl Acad Sci USA 91:2156–2160.

Kallioniemi O-P, Kallioniemi A, Piper J, Isola J, Waldman FM, Gray JW, Pinkel D (1994b) Optimizing comparative genomic hybridization for analysis of DNA sequence copy number changes in solid tumors. Genes Chromosom Cancer 10:231–243.

Ladanyi M, Cha C, Lewis R, Jhanwar SC, Huvos AG, Healey JH (1993) *MDM2* gene amplification in metastatic osteosarcoma. Cancer Res 53:16–18.

Leach FS, Tokino T, Meltzer PS, Burrell M, Oliner JD, Smith S, Hill DE, Sidransky D, Kinzler KW, Vogelstein B (1993) *p53* Mutations and *MDM2* amplification in human soft tissue sarcoma. Cancer Res 53:2231–2234.

McClintock B (1941) The stability of broken ends of chromosomes in *Zea mays*. Genetics 26:234–282.

Mertens F, Mandahl N, Orndal C, Baldetorp B, Bauer HCF, Rydholm A, Wiebe T, Willen H, Åkerman M, Heim S, Mitelman F (1993) Cytogenetic findings in 33 osteosarcomas. Int J Cancer 55:44–50.

Miller CW, Aslo A, Tsay C, Slamon D, Ishizaki K, Toguschida J, Yamamuro T, Lampkin B, Koeffler HP (1990) Frequency and structure of *p53* rearrangements in human osteosarcoma. Cancer Res 50:7950–7954.

Momand J, Zambetti GP, Olson DC, George DL, Levine AJ (1992) The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. Cell 69:1237–1245.

Mulligan LM, Matlaschewski GJ, Scrable HJ, Cavenee WK (1990) Mechanism of p53 loss in human sarcoma. Proc Natl Acad Sci USA 87:5863–5867.

Oliner JD, Kinzler KW, Meltzer PS, George DL, Vogelstein B (1992) Amplification of a gene encoding a p53-associated protein in human sarcoma. Nature 358:80–83.

Sandberg AA, Bridge JA (1994) The Cytogenetics of Bone and Soft Tissue Tumors. Austin, TX: RG Landers Co.

Solomon E, Ledbetter (1991) Report of the committee on the genetic constitution of chromosome 17. Cytogenet Cell Genet 58: 686–738.

Stark G (1993) Regulation and mechanisms of mammalian gene amplification. Adv Cancer Res 61:87–113.

Suijkerbuijk RF, Olde Weghuis DEM, van den Berg M, Pedeutour F, Forus A, Myklebost O, Glier C, Turc-Carel Ç, Geurts van Kessel A (1994a) Comparative genomic hybridization as a tool to define two distinct chromosome 12-derived amplification units in well-differentiated liposarcomas. Genes Chromosom Cancer 9:292–295.

Suijkerbuijk RF, Sinke RJ, Olde Weghuis DEM, Roque L, Forus A, Stellink F, Sipeman A, van de Kaa C, Soares J, Geurts van Kessel A (1994b) Amplification of chromosome subregion 12p11.2–p12.1 in a metastasis of an i(12p)-negative seminoma: Relationship to tumor progression? Cancer Genet Cytogenet 78: 145–152.

Tarkkanen M, Kaipainen A, Karaharju E, Bohling T, Szymanska J, Helio H, Kivioja A, Elomaa I, Knuutila S (1993) Cytogenetic study of 249 consecutive patients examined for a bone tumor. Cancer Genet Cytogenet 68:1–21.

Toguschida J, Ishizaki K, Sasaki MS, Ikenaga M, Sugimoto M, Kotoura Y, Yamamuro T (1988) Chromosomal reorganization for the expression of recessive mutation of retinoblastoma susceptibility gene in development of osteosarcoma. Cancer Res 48: 3939–3943.

Toledo F, Roscouet D, Buttin G, Debatisse M (1992) Co-amplified markers alternate in megabase long chromosomal inverted repeats and clusters independently in interphase nuclei at early steps of mammalian gene amplification. EMBO J 11:2665–2673.

Wadayama B, Toguchida J, Shimizu T, Ishizaki K, Kotoura Y, Ya-

mamoto T (1994) Mutation spectrum of the retinoblastoma gene in osteosarcoma. Cancer Res 54:3042–3048.
Washmut JJ, Bishop DT, Westbrook CA (1991) Report of the committee on the genetic constitution of chromosome 5. Cytogenet Cell Genet 58:261–294.
Ziegler A, Field LL, Sakaguchi AY (1991) Report of the committee on the genetic constitution of chromosome 6. Cytogenet Cell Genet 58:295–336.