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# Isolation of Osteosarcoma-Associated Amplified DNA Sequences Using Representational Difference Analysis

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Comparative genomic hybridization analysis of a primary osteosarcoma and its metastasis revealed two regions of DNA amplification, one at 17p11.2-12 and one at 19q12-13. Subsequent representational difference analysis of the primary tumor resulted in the isolation of two distinct tumor-amplified DNA fragments originating from chromosome 19. A YAC clone corresponding to one of the two isolated DNA fragments was used for fluorescence in situ hybridization on normal human lymphocyte metaphases and tumor-derived nuclei. This resulted in the localization of this YAC to 19q12-13.1 and confirmed the amplification status of the isolated fragment in the tumors. The availability of such RDA-isolated sequences may be instrumental in the search for genes relevant for tumor development. *Genes Chromosomes Cancer* 20:196-200, 1997.

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Cytogenetic studies of osteosarcomas have revealed highly aneuploid and extremely complex karyotypes with numerous abnormalities, including homogeneously staining regions, double minutes, and supernumerary ring chromosomes (Fletcher et al., 1994; Sandberg and Bridge, 1994; Heim and Mitelman, 1995). Osteosarcoma-associated chromosomal alterations include loss or rearrangement of chromosome bands 13q14 and 17p13, harboring the *RBI* and *TP53* tumor suppressor genes, respectively (Toguschida et al., 1988, 1989; Scheffer et al., 1991). Homogeneously staining regions, double minutes, and/or ring chromosomes appear to be associated with gene amplification, as has been demonstrated, for instance, for the *MYC* oncogene on chromosome 8 (Ozaki et al., 1993) and the *MDM2* gene on chromosome 12 (Ladanyi et al., 1993). Previously, it has been shown that the technique of comparative genomic hybridization (CGH; Kallioniemi et al., 1992) is suitable for the detection and mapping of (sub)chromosomal losses, gains, and amplifications in osteosarcomas (Forus et al., 1995b; Tarkkanen et al., 1995; Geurts van Kessel et al., 1997).

The isolation of DNA fragments that are deleted, rearranged or amplified in tumors can be achieved using the more recently developed technique of representational difference analysis (RDA; Lisitsyn et al., 1993, 1995; Lisitsyn, 1995). This technique is based on subtractive hybridization of two DNA populations (tester and driver), followed by specific polymerase chain reaction (PCR) of the differences

(tester-specific) between the DNA populations. To gain further insight into the molecular genetic changes that are relevant for the development of osteosarcomas, we have combined CGH and RDA for the analysis of a primary osteosarcoma and its metastasis.

An osteosarcoma sample (T87) was obtained from the right distal femur of a 15-year-old female patient after chemotherapy. Histopathologic examination showed a high-grade medullary osteoblastic, partly fibroblastic osteosarcoma with hyalinization and abundant osteoid formation. No cytogenetic data could be obtained from this tumor. Two years later, the same patient developed a metastasis (T95) in the right ilium, which showed the same histopathologic characteristics as the primary tumor. Cytogenetic analysis revealed several clonal structural abnormalities, including marker chromosomes: 65,X,-X,-X,-1,del(1)(p31),add(1)(p36),-2,-2,-2,-3,add(3)(p26),del(4)(p15)×2,add(4)(p16),-5,-6,add(6)(p25),-7,-8,-9,-10,del(10)(p11),-11,-12,-13,-14,-15,-16,-16,-18,del(18)(p12),-19,-20,-21,-22,+21mar [2]. At least four of the marker chromosomes and the abnormal chromosomes add(3)(p26) and add(6)(p25) were clearly recognized in four to six other metaphases.

To characterize the genomic alterations in more detail, we performed CGH as described previously

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Received 15 January 1997; Accepted 18 March 1997

by Forus et al. (1995a,b). DNAs extracted from the primary tumor and its metastasis were compared to reference DNA isolated from peripheral blood cells of normal control persons. Two sites of overt amplifications could easily be recognized in both the primary tumor and its metastasis, one at 17p11.2-12 and one at 19q12-13 (Fig. 1A). A minimum of eight target chromosome homologues was assayed for imbalances using arbitrarily set thresholds for gains and losses of chromosomal (sub)regions, 1.25 and 0.75, respectively (du Manoir et al., 1993, 1995). As was seen before, two regions of gain were detected in both tumors, one on 17p, with the highest ratio value at 17p11.2-12, and one on 19q, with the highest ratio value at 19q12-13 (Fig. 1B). Complete suppression of heterochromatic regions of critical chromosomes (1, 9, 16, and 19; Du Manoir et al., 1995) was observed, indicating that the signal of gain on 19q12-13 is genuine. Other copy number alterations could not be detected in the tumor DNAs under the stringency conditions applied. Although in this study no cytogenetic data of the primary tumor was available, a genetic comparison of the primary tumor and its metastasis based on CGH results was still possible. We conclude that no CGH-detectable changes appear to have occurred after metastasis of the primary tumor.

Using whole chromosome 17 and 19 painting probes, we performed fluorescence in situ hybridization (FISH) on metastasis-derived metaphase spreads to localize these sequences within the tumor genome. The paints, detected in different colors, showed hybridization to at least five marker chromosomes (not shown). This indicates that extra material of chromosomes 17 and 19 is cryptically present in these marker chromosomes, which is in agreement with the CGH findings.

With the observed highly abnormal karyotype and the DNA amplifications detected by CGH, we set out to isolate aberrant, in particular, amplified, DNA fragments from the primary tumor via RDA. In the case of amplifications, the isolation of target sequences is based on their relative abundance in the tester, which leads to kinetic enrichment during the RDA procedure, and not on their absence in the driver (Lisitsyn 1995; Lisitsyn et al., 1995). RDA was performed essentially as described by Lisitsyn et al. (1993, 1995) using representations of primary tumor DNA (the tester) and of a DNA pool of five unrelated control persons (the driver). Representations were made by *Bgl*II cleavage of genomic DNA and subsequent PCR amplification using its corresponding anchor primers (Lisitsyn et al., 1993). Agarose gel electrophoresis of difference products

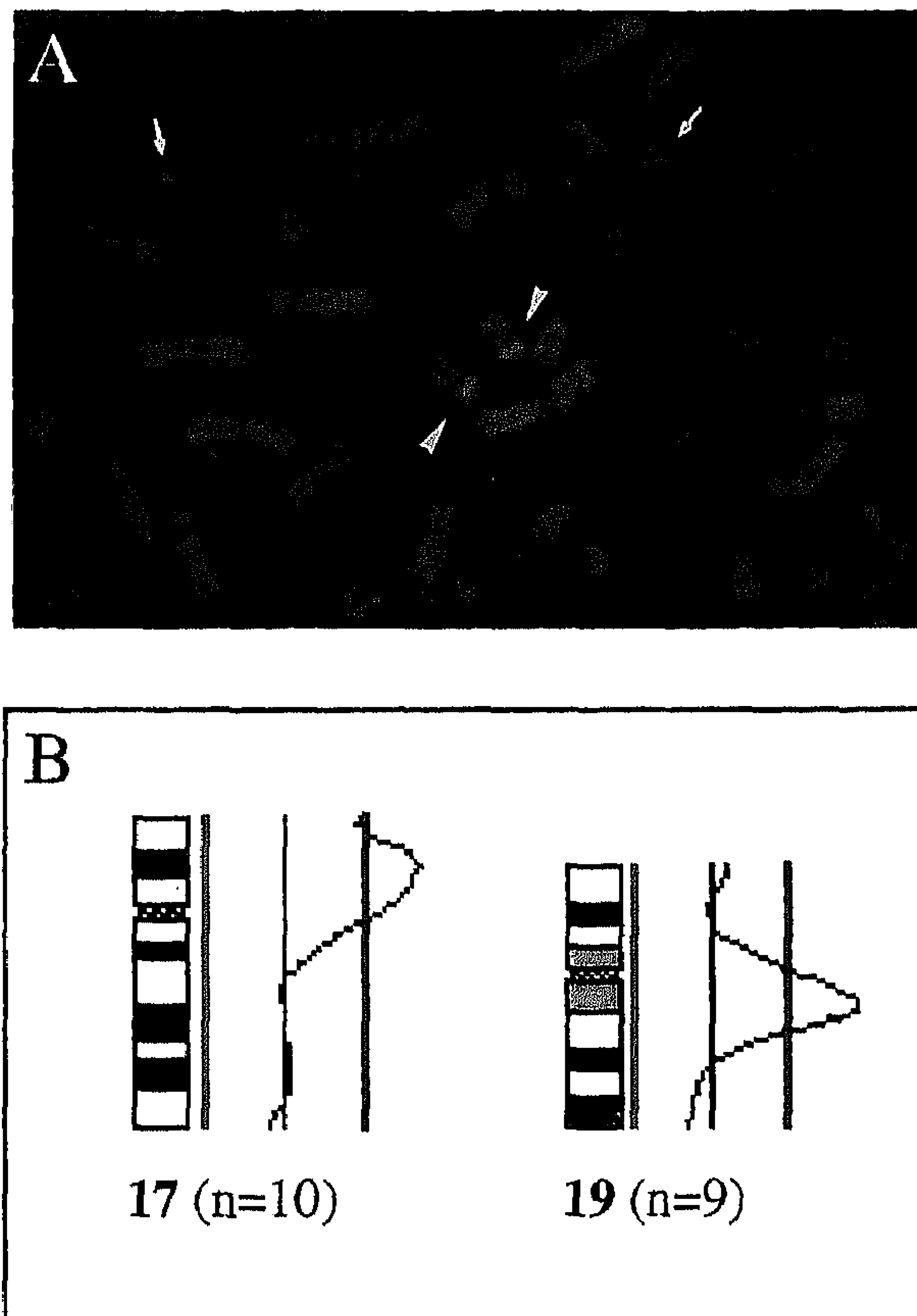


Figure 1. **A:** CGH result showing amplifications on 17p11.2-12 (arrowheads) and 19q12-13 (arrows) as encountered in both the primary tumor and its metastasis. Shown is a partial normal male metaphase spread after cohybridization with reference (red) and test (tumor T87; green) DNAs as probes. The chromosomes are counterstained in blue. **B:** Schematic representation of the average fluorescence ratio profiles of chromosomes 17 and 19 after CGH with T87 DNA as test probe. Profiles are plotted to the right of the chromosomes. The vertical lines reflect the lower threshold (left), the ratio value of 1 (middle), and the upper threshold, respectively. Two peaks of overrepresentation can be seen at 17p and 19q.

after one, two, and three rounds of subtractive hybridization and PCR amplification is shown in Figure 2. Five clear bands, varying in size from 300 to 500 bp, could be observed in the round 3 difference product, whereas background levels were reduced considerably. These five bands were isolated out of gel and cloned into plasmids. Subsequently, the cloned fragments were used as probes on Southern blots containing tester and driver (representation and total genomic) DNAs. Two distinct clones (clone 18 of 471 bp and clone 38 of 330 bp, derived from bands 5 and 2, respectively) showed more intense hybridization signals in the tester lanes than in the driver lanes, indicating that these fragments represent amplified sequences as present in the original tumor (Fig. 3). Also in the metastatic tumor, these sequences appeared to be highly amplified (not shown). Other clones, derived from all five bands, showed similar intensities in

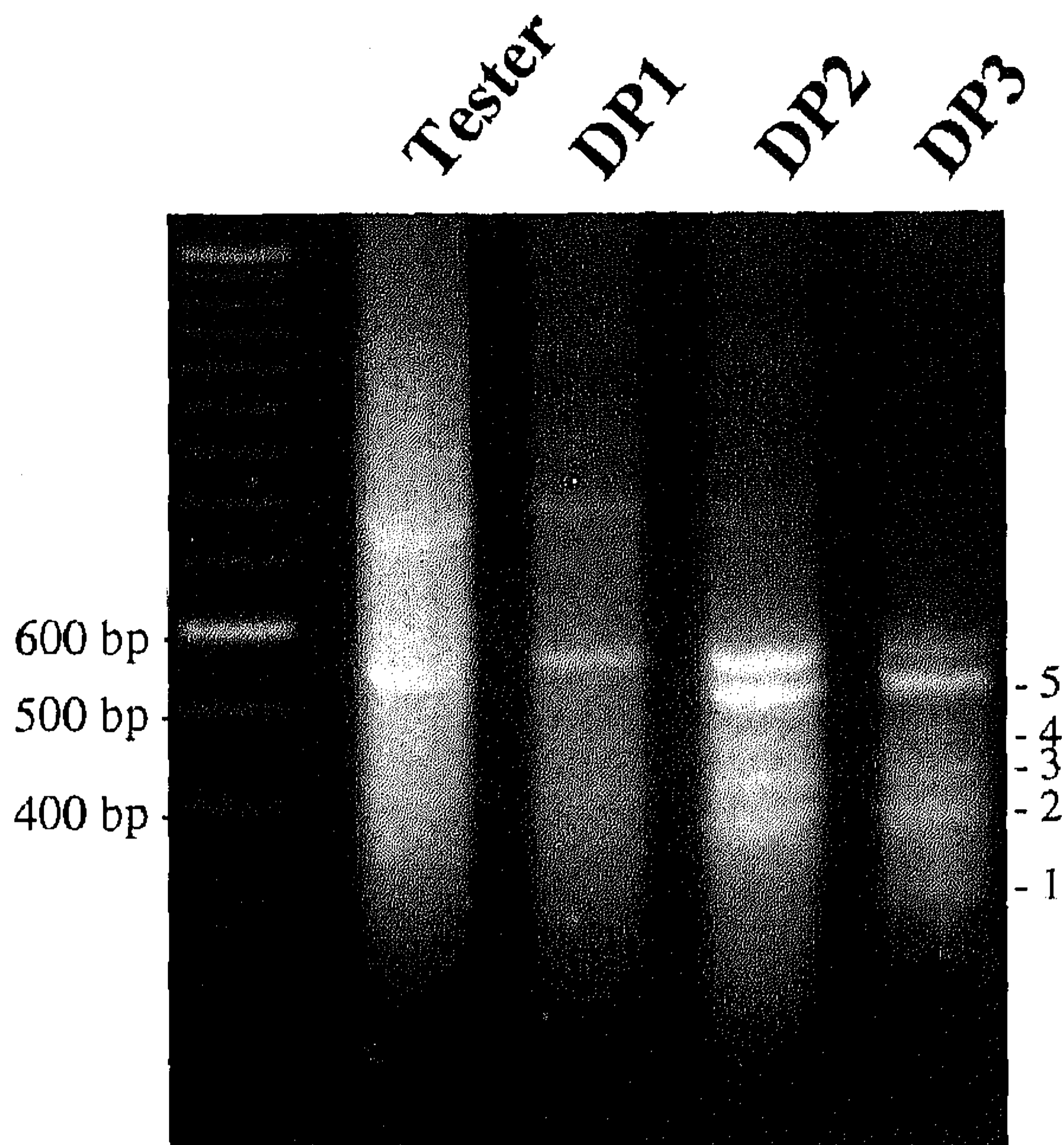


Figure 2. RDA results of tumor T87 as tester and a pool of five normal DNAs as driver. Tester: *Bgl*III representation of tumor DNA. DP1, DP2, and DP3: Difference products after first, second, and third round of hybridization/amplification, respectively. In DP3, five bands appear (1–5), which represent target sequences enriched from the tumor DNA.

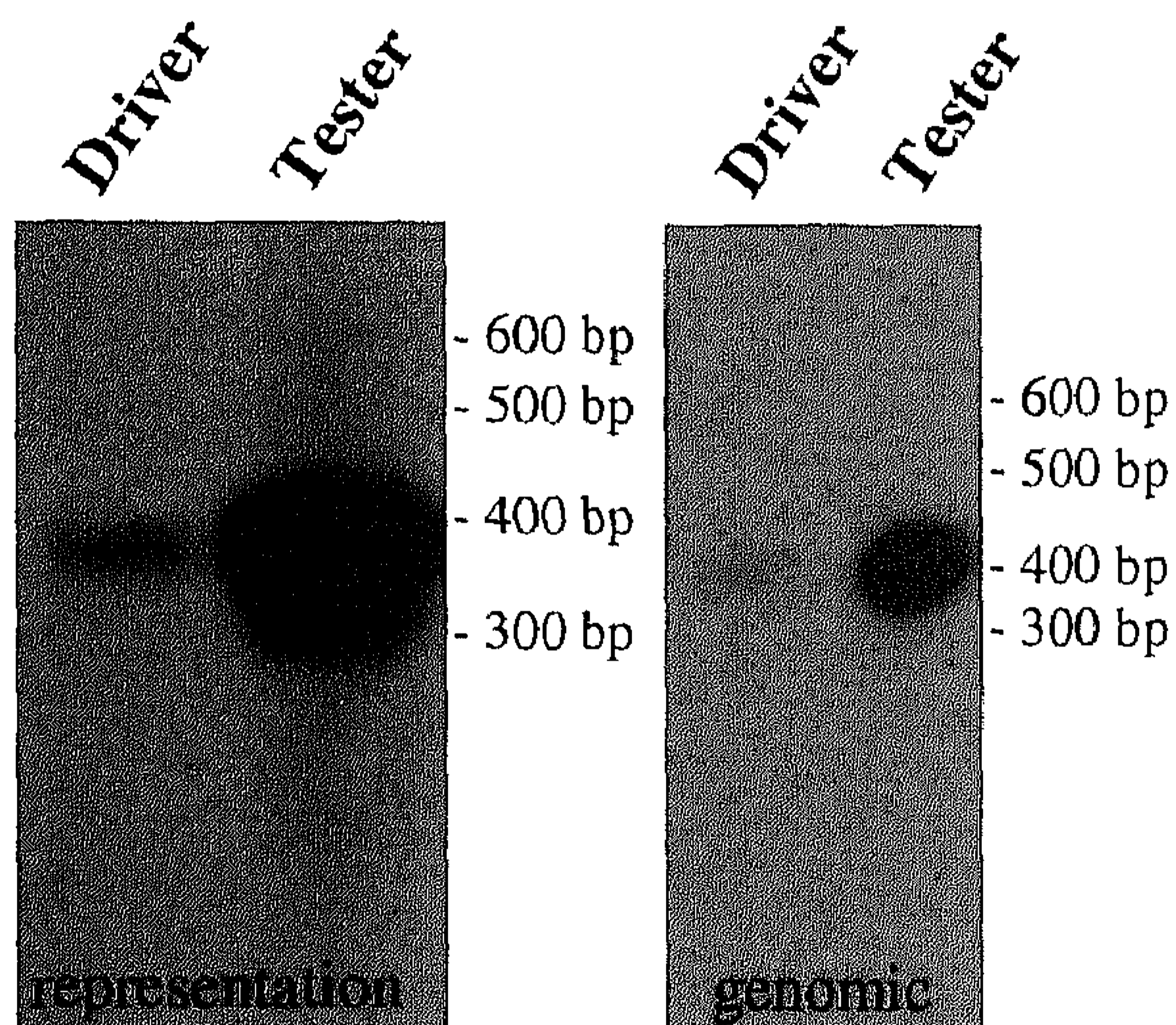


Figure 3. Hybridization of clone 38 to Southern blots containing *Bgl*III-representation DNAs and *Bgl*III-cleaved total genomic DNAs of driver (normal) and tester (tumor T87). Clone 38 sequences are overrepresented in the tester DNA compared to the driver DNA.

tester and driver lanes on Southern blots, indicating that they represent sequences that have escaped the RDA subtraction.

Clones 18 and 38 were sequenced and, subsequently, compared to known sequences in the Genbank and EMBL databases. No relevant homologies could be found for either. To establish the

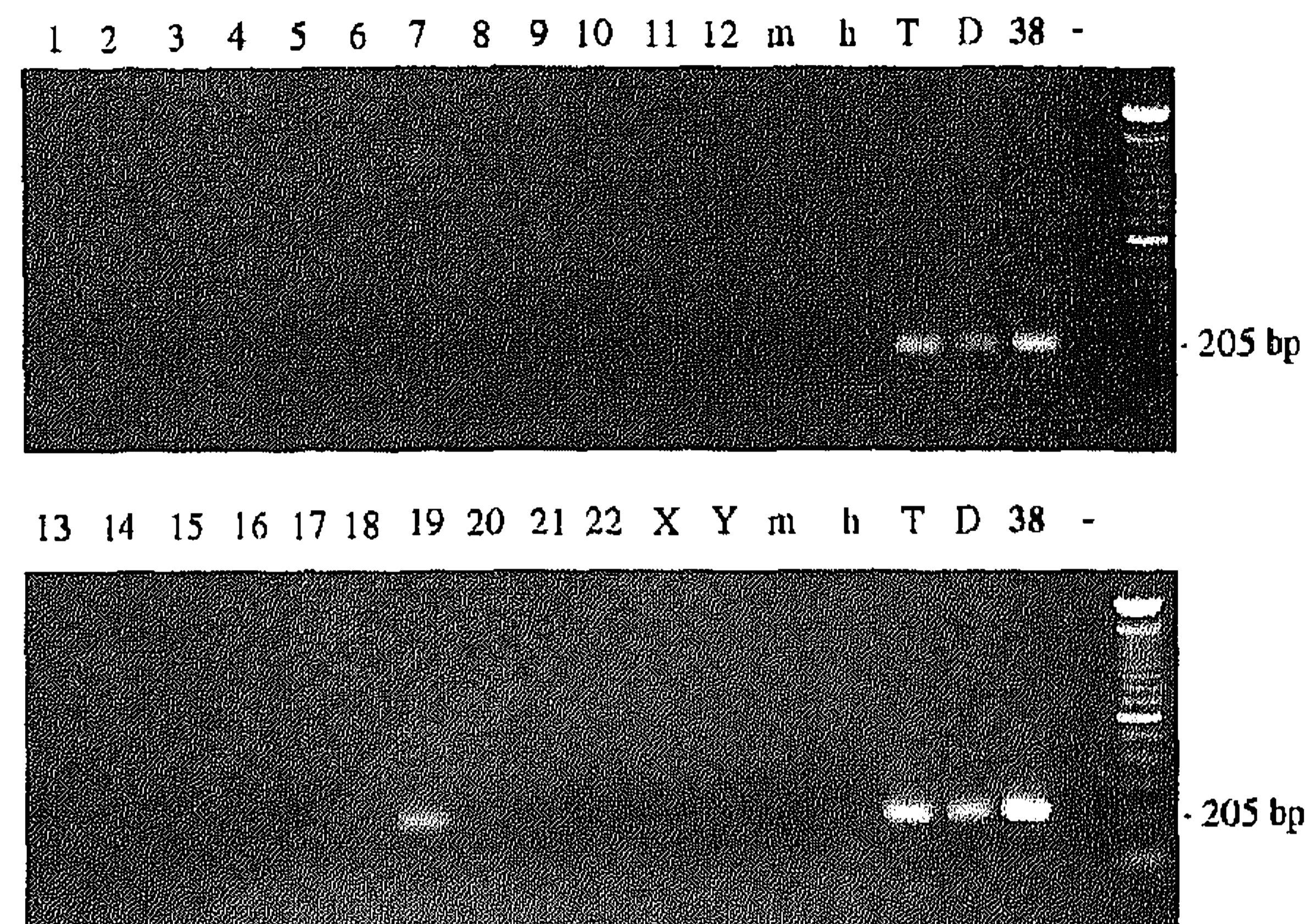


Figure 4. Chromosomal localization of clone 38 by PCR analysis of monochromosomal somatic cell hybrid lines (1–22, X, and Y). Mouse (m), hamster (h), and no (–) DNAs were used as negative controls, whereas tester (T), driver (D), and clone 38 (38) DNAs were used as positive controls. A PCR product was revealed only in the chromosome 19-containing cell hybrid, demonstrating that clone 38 maps to this chromosome.

chromosomal localization of these clones via PCR analysis of monochromosomal somatic cell hybrid lines, we used primer sets designed from both clones (18FOR: 5'AGTGTGTACCTGGCAGC-GGC-3' and 18REV: 5'TCTCATATGCCTGGGT-GATGGA-3'; 38FOR: 5'T-TCAGCATGATCCCA-ATCAAAA-3' and 38REV: 5'ATAAACCCAG-CACAATTAGCC-3'). PCR products of expected sizes (355 bp with the 18FOR/REV primer set and 205 bp with the 38FOR/REV primer set) were observed only in the chromosome 19-containing somatic cell hybrid, indicating that both clones map to this human chromosome (Fig. 4).

To reconfirm the chromosomal origin of clones 18 and 38 by visualization with FISH, a human YAC library (Anand et al., 1990; YAC plugs from UK HGMP Resource Centre) was screened by PCR with the FOR/REV primer sets. One clone 38-positive YAC (19B-A6) of approximately 400 kb was isolated. FISH analysis with this YAC on high-resolution normal human metaphase spreads revealed positive signals at 19q12-13.1 only, which is in line with the localization of the corresponding DNA fragment (clone 38) on chromosome 19. In addition, YAC 19B-A6 maps to a region exactly coinciding with the region of amplification as observed by CGH (Fig. 5).

Subsequently, YAC 19B-A6 was used for interphase FISH analysis of the metastasis. In all nuclei examined (over 50), four separate clusters of multiple copy signals could be observed. Examples of tumor nuclei exhibiting these clusters of amplification are shown in Figure 6. For comparison, two YAC signals on a normal lymphocyte nucleus are

also shown. These results confirm that the corresponding sequences are amplified in the osteosarcoma studied.

Although it has not been reported before that the 19q12-13 region is frequently amplified in osteosarcomas, it has recently been found that it is a hot spot for structural abnormalities in these tumors

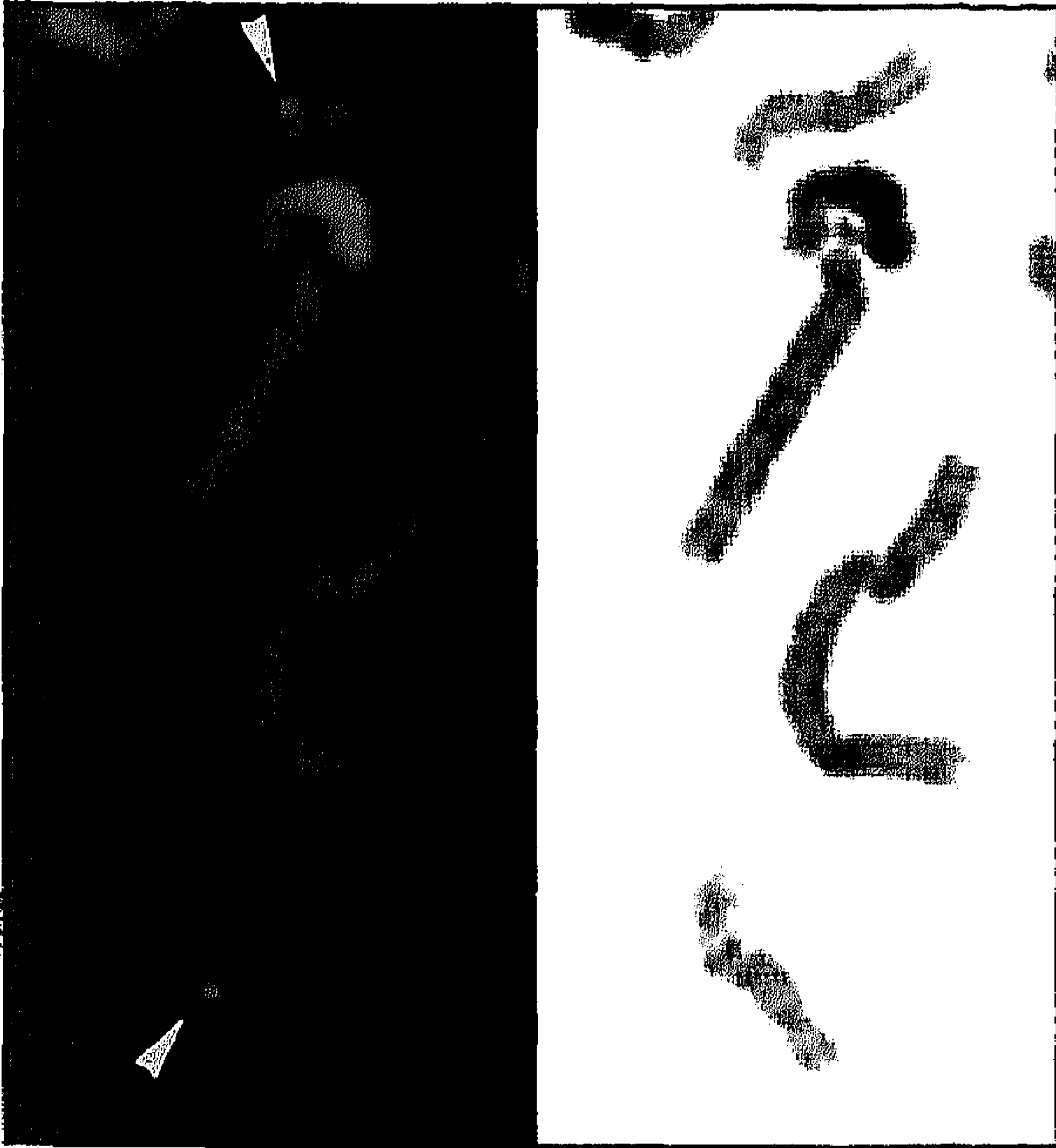


Figure 5. Chromosomal localization of YAC 19B-A6 by FISH on normal high-resolution metaphase chromosomes. A specific hybridization signal (green) can be seen at 19q12-13.1 (arrowheads, left). Chromosomes are counterstained in red (left). Chromosomal identification is performed using computer-inverted DAPI-banding (right).

(Bridge et al., 1997). In addition, this 19q segment has occasionally been shown to be amplified in ovarian cancers (Cheng et al., 1992; Thompson et al., 1996). At least four candidate genes are located in this region: 1) *AKT2*, a gene encoding a Ser/Thr kinase (Cheng et al., 1992); 2) *ERCC2*, a DNA repair gene (Thompson et al., 1996); 3) *BCL3*, encoding a DNA binding inhibitor of transcription factor NF- $\kappa$ B (Hatada et al., 1992); and 4) *TGFB*, which is involved in the differentiation of mesenchymal cells to chondrocytes and osteoblasts (Fuji et al., 1986). However, the role of these genes in the pathogenesis of osteosarcomas remains to be established, and it should be kept in mind that other candidate genes might be located within the 19q12-13 region as well. Although the clone 18 and 38 sequences did not exhibit open reading frames or relevant homologies with known sequences, they may be located close to such a gene or genes. No fragments from the amplification region on chromosome 17 were isolated in this RDA experiment, which may be explained by the fact that success depends on the restriction enzyme used and the presence of its restriction sites within the amplified segment. The 17p11.2-12 region may also harbor genes that are relevant for osteosarcoma development. This suggestion is based on our present and the previously reported CGH studies on osteosarcomas, indicating a frequent high-copy amplification of this region in these tumors. Currently, we are isolating DNA fragments from this

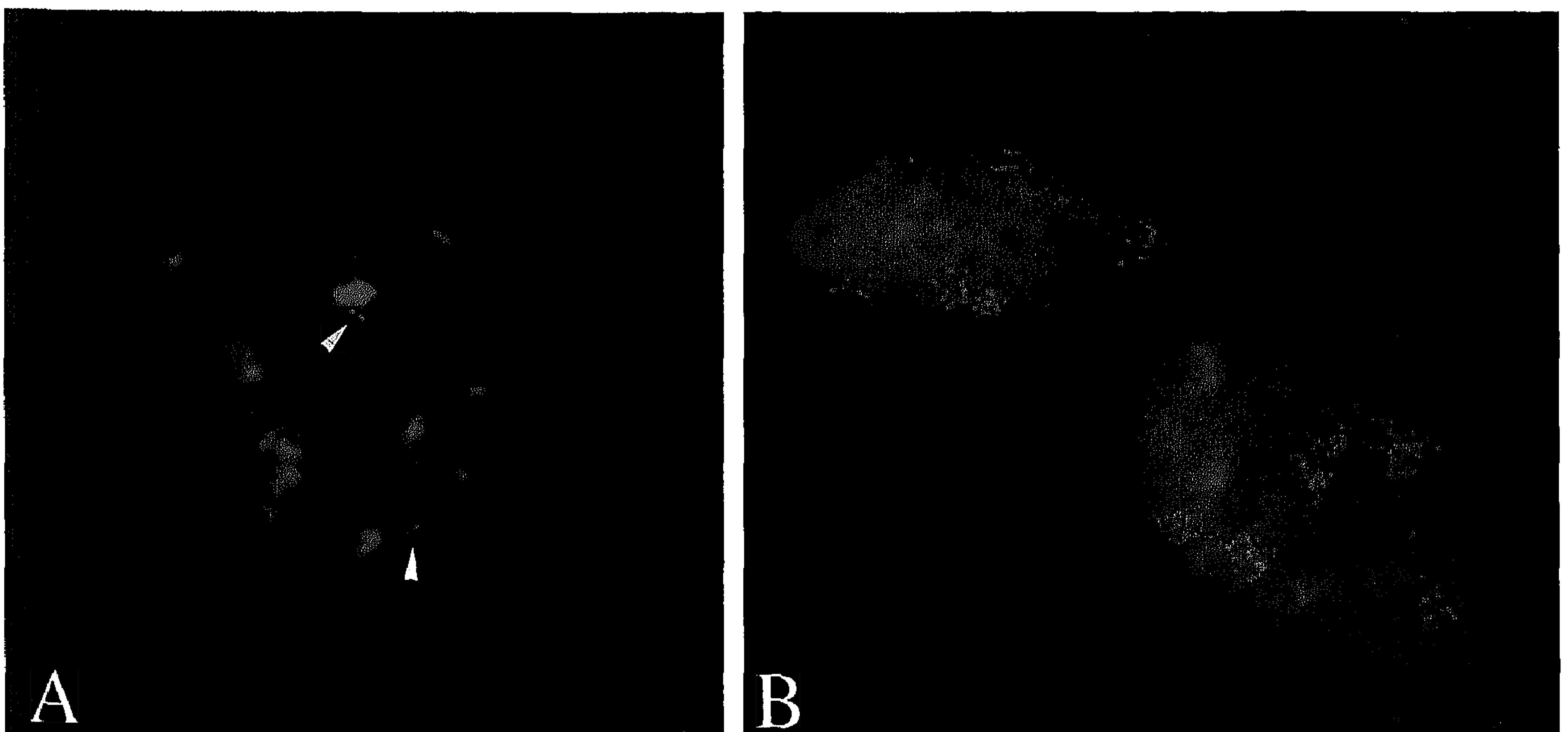


Figure 6. FISH analysis of normal lymphocyte and tumor T95-derived nuclei using YAC 19B-A6 as a probe (green). Multiple signals can be seen in the tumor nuclei (B) compared to the two signals (arrowheads) in the normal situation (A). Nuclei are counterstained in red.

region. These fragments and the chromosome 19-derived sequences reported here may be instrumental in the identification and isolation of relevant (onco)genes.

### ACKNOWLEDGMENTS

The authors thank Angelique Siepman, Hanneke Mieloo, Bert Janssen, and the Tumor Cytogenetics group for expert technical assistance. Daniel Olde Weghuis, Marian Weterman, Diederik de Bruijn, Wolfgang Berger, and Anne Forus are acknowledged for advice and support.

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