Amplification and overexpression of genes in chromosome region 17p1.2-p12 in human osteocarcinoma
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Chapter 4:

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Overexpression through amplification of genes in chromosome region 17p11.2~p12 in high-grade osteosarcoma

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Abstract

Osteosarcomas are malignant tumors of the bone that are characterized by complex genetic changes, including loss and amplification of chromosome regions. Region 17p11.2~p12 is frequently found to be amplified in this tumor, suggesting the presence of an oncogene (or oncogenes) important in osteosarcoma tumorigenesis. We had previously determined amplification profiles for this region. Reasoning that amplification of a causative oncogene in a tumor should result in increased expression of that gene, we have now determined the expression status of genes and expressed sequence tags (ESTs) in 17p11.2~p12. We constructed a 17p11.2~p12-specific macroarray containing 40 genes and 21 ESTs from this region, which was used for expression profiling of 11 osteosarcoma samples (9 tumors and 2 cell lines) and of normal human osteoblasts. Compared to normal osteoblasts, genes with at least threefold increased expression were considered to be overexpressed in the tumor. Genes PMP22 and COPS3, EST AA126939 (encoding part of the hypothetical protein FLJ20343), and two anonymous ESTs (AA918483 and R02360) were found to be most consistently overexpressed after amplification. By real-time reverse transcriptase polymerase chain reaction, we could confirm the overexpression status of PMP22 and COPS3 but not of FLJ20343. We conclude that PMP22 and COPS3, and possibly also the three ESTs, are candidate amplification targets in 17p11.2~p12 in osteosarcoma.
Overexpression through amplification of genes in chromosome region 17p11.2–p12 in high-grade osteosarcoma

1. Introduction

Osteosarcomas are highly malignant tumors of bone, mostly affecting children and young adults [1]. These tumors are characterized by complex genetic changes, including loss and amplification of chromosome regions containing genes that are supposed to be involved in osteosarcoma tumorigenesis (for review, see reference [2]). Loss of heterozygosity is frequently detected at chromosome regions 13q14 and 17p13, which contain the retinoblastoma and p53 tumor suppressor genes, respectively [3]. By comparative genomic hybridization (CGH), frequent gains or amplifications have been found for regions on chromosome arms 1q, 3q, 6p, 8q, 12q, 14q, 17p, Xp, and Xq [4–6]. For most of these regions, however, the amplification targets (i.e., the putative oncogenes) remain to be identified. Identification of these oncogenes might increase our understanding of how osteosarcomas develop and progress to malignancy.

One of the regions frequently found to be amplified in osteosarcomas is 17p11.2–p12. CGH studies revealed amplification of this region in 13–29% of high-grade osteosarcomas [4–6]. By using semiquantitative microsatellite analysis, other researchers [7] and our group [8] have recently determined the amplification status of markers and candidate genes in this region in more detail. The amplification profiles proved to be complex, often involving several independently amplified regions. We noted relatively frequent involvement in the amplifications for a region in 17p12 containing marker D17S2041, for a region in 17p11.2 with candidate gene PMP22, and for another region in 17p11.2 containing candidate genes ADORA2B, COPS3, DRG2, TOP3A, and MAPK7. In a parallel study, we found infrequent but high-level amplifications in the 17p11.2–p12 region in malignant gliomas [9].

To be important in tumorigenesis, amplification of a gene should result in overexpression of that gene. Therefore, to identify the true amplification targets in 17p11.2–p12 in osteosarcomas, we determined the expression status of all genes in that region and combined this information with the amplification status of these genes, which we deduced from the established amplification profiles. We constructed a 17p11.2–p12-specific macroarray containing 40 genes and 21 expressed sequence tags (ESTs), which was used for expression profiling of 11 osteosarcoma samples with known amplification profiles and of normal osteoblasts. We found genes PMP22 and COPS3 and three ESTs to be most consistently overexpressed after amplification in the tumor samples. By quantitative real-time reverse transcriptase polymerase reaction (RT-PCR), we could confirm the overexpression status of PMP22 and COPS3.
2. Materials and methods

2.1. Samples

Nine osteosarcoma tumors, two newly established osteosarcoma cell lines (Os2 and Os6), and normal human osteoblasts were used in this study. For the osteosarcoma tumors, data on tumor location and type of sample have been given previously [8]. Cell line Os2 (passage number 11) was derived from the biopsy of a juxta cortical osteosarcoma, and Os6 (passage number 8) was derived from a biopsy of an osteosarcoma of the knee [10]. All osteosarcomas were high-grade tumors and contained at least 70% of tumor cells. Tumor sample 66a is another part of tumor 66, which was analyzed for the presence of amplifications in 17p11.2~p12 in a previous study [8]. Normal human osteoblasts derived from a 37-year-old Caucasian donor were cultured according to the instructions of the supplier (Cambrex Bioproducts Europe, Verviers, Belgium). Genomic DNAs were extracted using proteinase K and chloroform and RNAs were isolated using Trizol reagent (Invitrogen, Breda, The Netherlands) according to standard procedures [11].

2.2. Amplification status of 17p11.2~p12

The amplification status of most of the tumors has been determined previously [8]. Additional amplification analyses were performed for tumor 66a and for cell lines Os2 and Os6. Microsatellite markers and candidate genes from 17p11.2~p12 were used to assess the amplification status of this region, as described previously [8]. Only normalized amplification levels of four and higher were considered to represent significant amplifications. In tumor 66a, MAPK7 in 17p11.2 and D17S804 and D17S969 in 17p12 proved to be significantly amplified. In cell line Os2, only D17S805 in 17p11.2 was found to be amplified, while none of the markers proved to be amplified in cell line Os6 (data not shown).

2.3. Construction and preparation of a 17p11.2~p12-specific cDNA macroarray

To generate a 17p11.2~p12-specific cDNA macroarray, we selected, on the basis of the information available in GeneMap’99 (http://www.ncbi.nlm.nih.gov/genemap), the cedar map (ftp://www.cedar.genetics.soton.ac.uk/pub/chrom17/gmap), and mapviewer (http://www.ncbi.nlm.nih.gov/mapview/), genes and ESTs located between D17S805 in 17p11.2 and D17S804 in 17p12. One hundred thirty-three clones containing plasmids with cDNA inserts of these genes and ESTs were taken from the ResGen library (ftp://www.ftp.resgen.com/pub/sv_libraries/ RG_Hs_seq_ver_060101.txt). DNAs were isolated using the Plus Miniprep DNA purification System (Promega Benelux, Leiden, The Netherlands) and cDNA inserts were PCR-amplified using TIGR primers (TIGR-forward: 5' -GTTTTCCCCAGTCACGACGTGG-3; TIGR-reverse: 5' -TGAGCGGATAAACATTTACACAG-3). The PCR products were sequenced using the BigDye Terminator Cycle sequencing kit (Perkin Elmer Biosystems, Oosterhout, The Netherlands) and the TIGR-reverse primer. To confirm their
identity and location, the derived cDNA sequences were blasted to the original sequence of the clone and to the Human Genome Working Draft sequence, freeze June 2002 (http://www.genome.ucsc.edu/). Seventy-one clones appeared to be located in the region between markers D17S804 and D17S805. Of the other clones, 42 were located on chromosome 17, but not between these markers, and 20 clones were located on other chromosomes. (Full information for all clones is available upon request.) All 133 clones were spotted onto nylon filters (N-Hybond; Amersham Biosciences, Roosendaal, The Netherlands). Before spotting, the PCR products were diluted 1:1 with spotting buffer containing 15% sucrose and 0.01% cresol red. All clones were spotted in triplicate by an in-house-made arrayer using split-pin technology. Before use, filter-bounded DNAs were denatured in 0.5 mol/L NaCl/0.5 mol/L NaOH, renatured in 0.5 mol/L Tris-HCl, pH 7.5/1.5 mol/L NaCl, washed in 0.5x standard saline citrate (SSC), and cross-linked with UV at 0.2 J/m².

2.4. Probe generation and hybridization to the filters

Seven milligrams of each total RNA was labeled with \(^{33}\text{P}\) -dATP by oligo(dT)-primed polymerization using SuperScript II Reverse Transcriptase (Invitrogen). After labeling, 5 \(\mu\)g of COT1 DNA, 5 \(\mu\)g of yeast tRNA, and 5 \(\mu\)g of poly d(A) were added to prevent nonspecific binding. The probe was boiled for 5 minutes before adding to the filters. These were prehybridized in hybridization mix for at least 2 hours. Hybridization was performed for 72 hours at 65°C in 5x standard saline citrate (SSC), 5x Denhardt's, and 0.5% sodium dodecyl sulfate (SDS). Filters were washed once with 2x SSC; 0.1% SDS for 1 hour; and twice with 0.2x SSC, 0.1% SDS for 1 hour.

2.5. Data analysis

Hybridization signals on the filters were visualized with a Fuji BAS 1800 Imager (Raytest Benelux, Tilburg, The Netherlands) and their intensity was quantified with AIDA software (Raytest Benelux). After regional background subtraction, the signal intensity of each spot was normalized for labeling and hybridization efficiencies by dividing the signal intensity of that spot by the mean signal intensity of all spots on the filter. Next, we calculated the mean signal intensity for the three spots of the same clone. Clones with a mean signal intensity of less than 10% of the mean signal intensity of all spots on the filter were excluded for further analysis. The normalized mean signal intensity of each clone for the tumor sample was compared with the normalized mean signal intensity of that clone for the normal osteoblast sample. Clones were considered to be overexpressed in the tumor sample when the normalized mean signal intensity for the tumor sample was at least three times more than that for the normal osteoblast sample.
2.6. Quantitative real-time RT-PCR

Expression levels of PMP22, COPS3, FLJ20343, and TOP3A were also determined by quantitative real-time RT-PCR. For this purpose, 5 μg of total RNA was reverse-transcribed by the SuperScript II enzyme (Invitrogen) according to standard procedures. All PCR were performed in the light cycler using the LightCycler-Fast Start DNA Master SYBR Green I kit (Roche, Indianapolis, IN). Expression levels of the studied genes were normalized to the expression level of the glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH). All PCR were done in duplicate. Primers for cDNA amplification were designed to span an intron to prevent amplification of genomic DNA contamination. Amplifications were performed in a total volume of 10 μL with 30 μmol/L cDNA, 5 μmol/L of each primer, and 5 mmol/L MgCl2 for GAPDH and COPS3, 3 mmol/L MgCl22 for PMP22 and FLJ20343, and 6 mmol/L MgCl2 for TOP3A. Primer sequences, positions in their respective cDNA, and product lengths are given in Table 1. PCR conditions were 1 minute at 96°C, followed by 40 cycles of 10 seconds at 96°C, 10 seconds at the annealing temperature (56°C for GAPDH and 60°C for the other genes), and 10 seconds at 72°C.

Table 1 Primer sequences for quantitative real-time RT-PCR of candidate and reference genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>Position in cDNA</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMP22</td>
<td>5' TCTGGCAGAACTGTAGCACCTC 3'</td>
<td>227-248</td>
<td>117</td>
</tr>
<tr>
<td></td>
<td>5' TGCTGAAGATGATGCAGAGAG 3'</td>
<td>343-323</td>
<td></td>
</tr>
<tr>
<td>COPS3</td>
<td>5' GTCGCTGAGCTGGTCTGAGCT 3'</td>
<td>1013-1033</td>
<td>119</td>
</tr>
<tr>
<td></td>
<td>5' GGTATCATGGAATCTGACCATACC 3'</td>
<td>1131-1107</td>
<td></td>
</tr>
<tr>
<td>FLJ20343</td>
<td>5' GCCGTCACAGGCACAAG 3'</td>
<td>1137-1154</td>
<td>102</td>
</tr>
<tr>
<td></td>
<td>5' GCCAGGTCACTCATGACGAC 3'</td>
<td>1238-1219</td>
<td></td>
</tr>
<tr>
<td>TOP3A</td>
<td>5' AGAGTGTCGCTCAGCTGTGGTCTG 3'</td>
<td>2278-2296</td>
<td>125</td>
</tr>
<tr>
<td></td>
<td>5' GCATCGCGCTTAACATTTTAAC 3'</td>
<td>2402-2380</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>5' TGAGCACAGGCTGTCCTC 3'</td>
<td>906-923</td>
<td>123</td>
</tr>
<tr>
<td></td>
<td>5' AATTCGTTGTCATCAGGAAAT 3'</td>
<td>1028-1006</td>
<td></td>
</tr>
</tbody>
</table>
3. Results

To identify 17p11.2~p12 genes that were overexpressed through amplification, we determined the expression status of these genes in osteosarcoma tumors and cell lines with known amplification profiles for that region. High-quality RNA was available for eight osteosarcoma tumors for which we had determined the amplification profiles in a previous study [8]. Additional high-quality RNA was available for tumor 66a and osteosarcoma cell lines Os2 and Os6 [10], for which amplification profiles were established (see Materials and methods for details). To determine the expression status of genes and ESTs in 17p11.2~p12, we constructed a cDNA macroarray for that region. This macroarray included 71 sequence- and location-verified clones, representing 40 different genes and 21 ESTs, from the region between marker D17S805 in 17p11.2 and marker D17S804 in 17p12. Among these were PMP22, ADORA2B, COPS3, DRG2, and MAPK7, which we previously found to be frequently amplified in osteosarcomas [8]. No representative clone was available for TOP3A, which was also found to be amplified in a high percentage of the osteosarcomas analyzed. The macroarray filters were hybridized with 32P-labeled cDNA generated from the osteosarcoma tumors or cell lines and from normal osteoblasts. The hybridization signal for each clone was quantified and normalized for labeling and hybridization efficiencies. A gene was considered to be overexpressed when the normalized hybridization signal of the corresponding clone in the osteosarcoma sample was at least threefold higher than in the normal osteoblast sample. Table 2 lists the genes, in order of decreasing frequency of overexpression, that were found to be overexpressed in at least 4 of the 11 osteosarcoma samples. The amplification status of each gene in the tumor samples, as inferred from the previously and newly established amplification profile for each tumor, is also shown in this table. We conclude from columns O/A and -O/A in this table that genes PMP22 (represented by clones H28091 and R2690), as well as COPS3 and ESTs AA918483, AA126939, and R02360, were most consistently overexpressed after amplification. For NT5M (encoding mitochondrial 5'-nucleotidase), the correlation between amplification and overexpression was less consistent because this gene was amplified without overexpression in two cases (column -O/A). Except for COPS3, the genes with frequent overexpression after amplification were also overexpressed in cases without amplification, suggesting that their expression can be upregulated by other mechanisms (column O/-A).
### Table 2
Expression level and amplification status of genes and ESTs in osteosarcoma tumors and cell lines

<table>
<thead>
<tr>
<th>Gene</th>
<th>Nr</th>
<th>Tumor samples</th>
<th>Correlation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>15</td>
<td>21</td>
</tr>
<tr>
<td>PMP22</td>
<td>H28091</td>
<td>na</td>
<td>n</td>
</tr>
<tr>
<td>PMP22</td>
<td>R2690</td>
<td>0.6</td>
<td>n</td>
</tr>
<tr>
<td>EST</td>
<td>AA918483</td>
<td>4.2</td>
<td>n</td>
</tr>
<tr>
<td>EST</td>
<td>AA126939</td>
<td>2.0</td>
<td>n</td>
</tr>
<tr>
<td>EST</td>
<td>R02360</td>
<td>11.3</td>
<td>y</td>
</tr>
<tr>
<td>EST</td>
<td>A1002006</td>
<td>2.8</td>
<td>n</td>
</tr>
<tr>
<td>EST</td>
<td>N53453</td>
<td>5.3</td>
<td>n</td>
</tr>
<tr>
<td>NT5M</td>
<td>AA1446188</td>
<td>3.2</td>
<td>y</td>
</tr>
<tr>
<td>EST</td>
<td>R80675</td>
<td>3.0</td>
<td>n</td>
</tr>
<tr>
<td>EST</td>
<td>W06250</td>
<td>2.2</td>
<td>y</td>
</tr>
<tr>
<td>EST</td>
<td>AA192163</td>
<td>3.0</td>
<td>n</td>
</tr>
<tr>
<td>COP53</td>
<td>AA155640</td>
<td>5.2</td>
<td>y</td>
</tr>
</tbody>
</table>

Names of genes and accession numbers of the clones, by which they were represented on the macroarray, are indicated on the left. Osteosarcoma sample codes (15-109 for tumors, Os2, and Os6 for cell lines) are shown at the top. For each gene, the expression level in a tumor sample, as determined by macroarray analysis, is given in the left column and the amplification status, as inferred from the amplification profile of that tumor sample, is given in the right column. Overexpressed genes, with normalized expression levels at least threefold higher in the tumor sample than in normal osteoblasts, are marked in boldface. na indicates not analyzed because of very low signal intensity on the macroarray. For the amplification status, y indicates amplification level >4, and n indicates amplification level <4. The correlation between overexpression and amplification is analyzed in the columns on the right. Abbreviations: O/A, number of samples with overexpression and amplification of a gene; O/-A, number of samples with overexpression but no amplification of a gene; -O/A, number of samples with amplification but no overexpression of a gene; T, total number of samples for which information regarding overexpression and amplification status of a gene was available.
We wished to validate the expression status of PMP22, COPS3, EST AA918483, EST AA126939, and EST R02360 in the tumor samples by an independent method using quantitative real-time RT-PCR. Exon-based primers for intron-spanning cDNA amplification could not be designed for ESTs AA918483 and R02360, however, because the corresponding genes have not yet been identified. EST AA126969 is part of the hypothetical gene FLJ20343, for which a genomic structure has been proposed (Human Genome Working Draft, freeze June 2002). Therefore, we could only validate the expression status of PMP22, COPS3, and FLJ20343. We also determined the expression status of TOP3A, which was found to be frequently amplified in osteosarcomas but had no representative clone available for the macroarray. The expression levels for the studied genes were normalized to the expression level for the housekeeping gene GAPDH. A gene was considered overexpressed in an osteosarcoma sample when the normalized expression level was at least threefold higher than in the normal osteoblast sample. The expression level for TOP3A proved to be very low in both normal osteoblasts and osteosarcoma samples, thereby precluding a reliable determination of its overexpression status in the latter (data not shown). The normalized expression levels of the other genes, together with the normalized expression levels of these genes, as determined by macroarray analysis, are shown in Fig. 1. Although there were quantitative differences between the two methods regarding the levels of PMP22 expression, in eight of nine osteosarcoma samples, the overexpression status of this gene could be confirmed by RT-PCR, as determined by macroarray analysis (Fig. 1A). Likewise, the overexpression status of COPS3, as determined by macroarray analysis, correlated with the RT-PCR assay in six of the seven informative cases (Fig. 1B). The frequent overexpression of FLJ20434 that we detected by macroarray analysis, however, could not confirmed by RT-PCR (Fig. 1C).
Fig. 1. Validation of the overexpression status of PMP22, COPS3, and FLJ20434, as determined by macroarray analysis, by quantitative real-time RT-PCR. (Top) Graphic display; (bottom) quantification of expression levels. Quantitative real time RT-PCR was performed for each of the osteosarcoma tumors (15-109) and cell lines (Os2 and Os6). For each tumor sample, the left bar indicates normalized expression level relative to normal osteoblasts of PMP22 (A), COPS3 (B), or FLJ20434 (C), as determined by RT-PCR. The right bar indicates normalized expression level relative to normal osteoblasts of these genes, as determined by macroarray analysis. Na, not analyzed because of very low signal intensity on the macroarray; Nd, not determined because of insufficient availability of high-quality RNA.
4. Discussion

To identify in 17p11.2-p12 the genes whose overexpression through amplification might causally be related to osteosarcoma tumorigenesis, we investigated the expression status of 61 genes (40 genes and 21 ESTs) by using a region-specific macroarray. Eleven genes were found to be overexpressed in at least 4 of the 11 investigated osteosarcoma samples (Table 2). All other genes were overexpressed at considerably lower frequencies and, for that reason, were not given further consideration as candidate genes whose overexpression is causally related to osteosarcoma tumorigenesis. The latter included ADORA2B, DRG2, and MAPK7, which we had previously identified as being frequently involved in the amplifications in 17p11.2-p12 [8]. By quantitative real-time RT-PCR, the expression level of TOP3A in the osteosarcoma samples and in normal osteoblasts proved to be very low. Although we previously found this gene to be involved in the amplifications in 17p11.2-p12 at the highest frequency (i.e., in 72% of cases [8]), its very low expression in the osteosarcoma samples makes its function as a true amplification target in this region less likely. By comparing the expression level and amplification status of each of the 11 genes in the 11 osteosarcoma samples, we found PMP22, ESTs AA918483, EST AA126939, EST R02360, and COPS3 to be most consistently overexpressed after amplification (Table 2). Overexpression of PMP22 was detected by two different clones on the macroarray (H28091 and R2690) and could be confirmed by RT-PCR (Fig. 1A). In our previous studies, PMP22 proved to be frequently amplified in osteosarcomas (50% of cases) and amplified at high-level in a malignant glioma [8,9] Amplification of PMP22 was earlier reported in the rhabdomyosarcoma cell line RH30 and in glioma cell line SF763 [12]. In our study, PMP22 was overexpressed in eight of the examined osteosarcoma samples. In four of these cases, this overexpression was associated with amplification of the gene. In the four other cases, overexpression occurred without amplification, suggesting that it was induced by other mechanisms. More importantly, there were no cases in which PMP22 was amplified without overexpression (Table 2). The frequent amplification, overexpression, and the consistent correlation of amplification and overexpression makes PMP22 a serious amplification target (i.e., candidate oncogene) in the 17p11.2-p12 region in osteosarcoma. Very little information is available on the expression status of PMP22 in other human tumors. Expression of GAS3, which is another name for PMP22, was found to be lower in two malignant fibrous histiocytoma cell lines than in normal fibroblasts [13]. In the mouse, down-regulation of PMP22 expression has been reported in urethane-induced lung tumors [14]. Although the precise functions of PMP22 remain to be established, it is clear that it may have diverse functions in different tissues. In neural tissues, PMP22 function is merely related to myelin formation because both duplication and deletion of PMP22 are linked to hereditary demyelinating diseases Charcot-Marie-Tooth disease type 1A [15] and hereditary neuropathy with liability to pressure palsies [16], respectively. In non-neural tissues, PMP22 function is associated with more common cellular functions such as cell growth regulation. The latter is
supported by the observation that *PMP22/GAS3* expression was increased when NIH 3T3 fibroblasts were arrested in the cell cycle after serum starvation or at confluence [17]. In the two malignant fibrous histiocytoma cell lines, however, increased *PMP22/GAS3* expression could not be detected after serum starvation [13]. These contradictory results and the amplification and increased expression of *PMP22* in osteosarcomas and gliomas that we and others observed suggest that the effect of changes in *PMP22* expression on cell growth might be different between normal and tumor cells and between tumor cells of different types.

In our earlier study [8], we reported frequent amplification of *COPS3* in osteosarcomas (56% of cases). In this study, *COPS3* was found to be overexpressed in 4 of 11 osteosarcoma samples and, in each case, overexpression was associated with amplification of the gene. In one case, however, amplification did not result in overexpression (Table 2). The overexpression status of *COPS3* could be confirmed by RT-PCR (Fig. 1B) *COPS3* encodes subunit 3 of the COP9 signalsome [18,19]. This protein complex is involved in signal transduction and in ubiquitin-dependent proteolysis in the cell. Among other functions, the COP9 signalsome has kinase activity that may increase the half-life of the protooncogene product Jun [20]. One may speculate that an increased amount of COP9 signalsome, induced by overexpression of one of its subunits (*COPS3*), could stimulate tumorigenesis by increasing Jun stabilization. Alternatively, because no cases were found in which overexpression occurred without amplification, it cannot be excluded that *COPS3* amplification and overexpression resulted from coamplification with a nearby and as yet unidentified target gene.

Three ESTs, AA918483, AA126939, and R02360, were found to be frequently overexpressed and, with only one exception, overexpressed in case of amplification in the investigated osteosarcoma samples (Table 2). The overexpression of EST AA126939, however, could not be confirmed by quantitative RT-PCR (Fig. 1C). This EST contains the 3'-end of the hypothetical gene *FLJ20434*, for which a genomic organization has been proposed (Human Genome Working Draft, freeze June 2002). On the basis of its hypothetical intron–exon structure, we selected primers for cDNA amplification. One explanation for the discrepancy between the macroarray and RT-PCR data might be that the proposed intron–exon structure for *FLJ20434* is incorrect. Another reason might be that alternative transcript variants may exist for this gene and that a minor transcript was analyzed by RT-PCR that was not upregulated in osteosarcoma. The overexpression status of ESTs AA918483 and R02360 could not be evaluated by RT-PCR because of the lack of information about the genomic structure of the corresponding genes, which remains to be identified.
In conclusion, we identified *PMP22*, *COPS3*, EST AA126939 (encoding part of the hypothetical protein FLJ20343), and two anonymous ESTs (AA918483 and R02360) as candidate genes whose amplification and overexpression might be causally related to osteosarcoma tumorigenesis. Additional studies, including identification of the genes corresponding to the ESTs and functional analyses of the gene products, have to be done to assess their possible significance in the development and progression of osteosarcoma.

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