Molecular and Cellular Pathobiology

Recurrent Focal Copy-Number Changes and Loss of Heterozygosity Implicate Two Noncoding RNAs and One Tumor Suppressor Gene at Chromosome 3q13.31 in Osteosarcoma

Ivan Pasic1,2,5, Adam Shlien3,5, Adam D. Durbin1,3,5, Dimitrios J. Stavropoulos6, Berivan Baskin6, Peter N. Ray6, Ana Novokmet7, and David Malkin2,3,4,5,7

Abstract

Osteosarcomas are copy number alteration (CNA)–rich malignant bone tumors. Using microarrays, fluorescence in situ hybridization, and quantitative PCR, we characterize a focal region of chr3q13.31 (osteo3q13.31) harboring CNAs in 80% of osteosarcomas. As such, osteo3q13.31 is the most altered region in osteosarcoma and contests the view that CNAs in osteosarcoma are nonrecurrent. Most (67%) osteo3q13.31 CNAs are deletions, with 75% of these monallelic and frequently accompanied by loss of heterozygosity (LOH) in flanking DNA. Notably, these CNAs often involve the noncoding RNAs LOC285194 and BC040587 and, in some cases, a tumor suppressor gene that encodes the limbic system–associated membrane protein (LSAMP). Ubiquitous changes occur in these genes in osteosarcoma, usually involving loss of expression. Underscoring their functional significance, expression of these genes is correlated with the presence of osteo3q13.31 CNAs. Focal osteo3q13.31 CNAs and LOH are also common in cell lines from other cancers, identifying osteo3q13.31 as a generalized candidate region for tumor suppressor genes. Osteo3q13.31 genes may function as a unit, given significant correlation in their expression despite the great genetic distances between them. In support of this notion, depletion of either LSAMP or LOC285194 promoted proliferation of normal osteoblasts by regulation of apoptotic and cell-cycle transcripts and also VEGF receptor 1. Moreover, genetic deletions of LOC285194 or BC040587 were also associated with poor survival of osteosarcoma patients. Our findings identify osteo3q13.31 as a novel region of cooperatively acting tumor suppressor genes. Cancer Res; 70(1); 160–71. ©2010 AACR.

Introduction

Osteosarcoma is the most common bone malignancy in children and adolescents (1), with a 65% 5-year survival (2). Although germline mutations in p53, Rb1, and RecQ4 have been implicated in genetic susceptibility to osteosarcoma (3–10), less is known about the etiology of sporadic osteosarcoma. Cytogenetically, osteosarcomas are highly complex (11–19). Gains and losses affect all chromosomes in osteosarcoma and are largely nonrecurrent, although some examples of recurrent copy number alterations (CNA) exist, including gains at 6p12, 8q24 and 17p12–p11 and losses at 13q14 and 17p13.1 (11–15, 17–19). Chromosome 6p12 contains the RUNX2 gene, which promotes terminal osteoblast differentiation (20). Elevated RUNX2 levels have been reported in osteosarcoma cells (21), providing an example of how integrative genome and functional analyses may help identify chromosomal regions important in tumorigenesis. Identification of these regions has been facilitated by the advent of high-resolution microarray technologies. This is highlighted in the recent identification of ALK as a familial neuroblastoma predisposition gene (22) and implication of CDKN2A in childhood acute lymphoblastic leukemia (23).

We characterize a region of chr3q13.31, designated osteo3q13.31, which harbors frequent focal CNAs and loss of heterozygosity (LOH) in primary osteosarcoma samples and cell lines from other malignancies. Most osteo3q13.31 CNAs involve the noncoding RNAs (ncRNA) LOC285194 and BC040587 and, sometimes, the limbic system–associated membrane protein (LSAMP) tumor suppressor (TS). A change in expression of osteo3q13.31 genes is ubiquitous in osteosarcoma and, in the case of LOC285194 and BC040587, reflects the presence of osteo3q13.31 CNAs. Distantly spaced osteo3q13.31 genes may function as a unit, as they display significant correlation in expression. In support of this notion, depletion of either LSAMP or LOC285194 promotes proliferation of normal osteoblasts through regulation of apoptotic,
cell cycle, and tumor-promoting transcripts. Furthermore, the presence of LOC285194 or BC040587 deletions in tumor DNA is associated with poor patient survival. Recently, two studies (24, 25) have implicated LSAMP as an osteosarcoma TS, although neither addressed the functional role of LSAMP in repressing tumorigenesis or the potential TS role of the osteo3q13.31 ncRNAs. Our findings implicate osteo3q13.31 as a novel universal TS region containing several cooperatively acting genes.

Materials and Methods

Subject recruitment. Tumor biopsy tissue on 20 children and adolescents with osteosarcoma, with paired blood on 19 patients, was obtained at the Hospital for Sick Children, Toronto. Tumor and paired blood DNA was obtained on 28 adolescents and adults under 31 y with osteosarcoma from the Interdisciplinary Health Research Team in Musculoskeletal Neoplasia Tumor Bank, Mount Sinai Hospital. Tumor biopsy tissue was obtained on one adolescent with osteosarcoma from the Cooperative Human Tissue Network. Samples were labeled OS01 through OS49. This study was approved by the Research Ethics Board at the Hospital for Sick Children.

Cells and reagents. U2OS, SAOS-2, HOS, MNN, and KHOS cells were purchased from the American Type Culture Collection; osteoblasts were from PromoCell; COOH-terminal myc/DDK-tagged LSAMP expression clone was from OriGene Technologies; and small interfering RNAs (siRNA) were from Ambion. Cell lines tested for low passage were from an adolescent with osteosarcoma from the Cooperative Human Tissue Network. Samples were labeled OS01 through OS49. This study was approved by the Research Ethics Board at the Hospital for Sick Children.

Sample processing. Samples were processed as performed by Shlien and colleagues (27). Pooled blood DNA from 80 controls (Roche) was used for real-time quantitative PCR. Reverse-transcriptase qPCR (RT-qPCR) was performed on OS01-OS08, OS10-OS17, OS20-OS28, OS30-31, OS33-OS48, HOS, K-HOS, MNN, SAOS-2, U2OS, and osteoblast RNA. Expression of LSAMP, LOC285194, and BC040587 was measured relative to that of TATA-binding protein, by the comparative C(T) method (30). A 1.5-fold expression change cutoff was used. Primer sequences are in Supplementary Table S2.

Fluorescence in situ hybridization (FISH). Fluorescence in situ hybridization (FISH) was performed using standard protocols. A commercially available RP11-956M14 bacterial artificial chromosome clone, covering the region of chromosome 3 between 117,799,751 and 117,974,624 bp, tagged with a green FITC tag, was used as a 3q13.31 probe. A chromosome 3 α-satellite (D3Z1) probe (MP Biomedicals), tagged with a red cyanine fluorescent tag, was used as a chromosome 3 centromeric probe. FISH probes were synthesized at The Centre for Applied Genomics.

Statistical analyses. Analyses were performed using PASW Statistics 17.0. Kaplan-Meier survival analysis was performed using Partek. Error bars represent SDs unless noted otherwise.

Antibodies. Anti-myc and anti–proliferating cell nuclear antigen antibodies were from Santa Cruz Biotechnology. Anti-vinculin antibody was from Millipore. Cleaved poly(ADP-ribose) polymerase-1 antibody was from Cell Signaling Technology.

LSAMP mutation screening. Primer sequences are in Supplementary Table S4.

Results

Osteo3q13.31: site of highly common focal CNAs and LOH in osteosarcoma. To identify novel CNAs in osteosarcoma, we analyzed DNA from primary tumor biopsies of 27 osteosarcoma patients by genome-wide oligonucleotide 6.0 microarrays. In 26 of these, we used qPCR to validate DNA calls, in addition to DNA from primary tumor biopsies of four osteosarcoma patients, which were analyzed by qPCR only. CNAs identified by microarrays are shown in Supplementary Fig. S1. The most common deletion was within chr3q13.31 (Fig. 1A, arrowhead). CNAs were observed in 70.4% (19 of 27) of samples analyzed by microarrays: 51.9% (14 of 27) exhibited CN loss at chr3q13.31 (Fig. 1B, samples with blue tracks only), 3.7% (1 of 27) exhibited CN gain (Fig. 1B, samples with red tracks only), whereas 14.8% (4 of 27) displayed complex changes involving regions of CN loss and gain (Fig. 1B, samples with blue and red tracks). Validating our approach, we detected previously reported amplifications at chr8q24.21 in 70.4% (19 of 27) and chr6p21.2-6p12.3 in 66.7% (18 of 27) of samples (Fig. 1A, arrowheads).

We next determined if CNAs at chr3q13.31 represented de novo events in tumor DNA or CN changes of germline CNVs by comparing CN of chr3q13.31 on microarrays between blood- and tumor-derived DNA of each patient. We identified
a 0.7-Mb region (Fig. 1C) with significantly ($\chi^2$, $P < 0.01$) lower CN in tumor (median CN, 1.5) than blood DNA (median CN, 2.1). In fact, whereas CN of this locus in blood DNA clustered tightly around 2, CN of the same locus in tumor DNA varied significantly. Therefore, chr3q13.31 CNAs in osteosarcoma represent de novo events in tumor DNA.

To determine the minimal region of overlap (MRO) of chr3q13.31 CNAs, we used significance testing for aberrant CN (STAC; ref. 31), a multiple testing–corrected permutation approach for identifying regions harboring CNAs more often than by chance. We identified a 0.5-Mb region between 117,8 and 118.3 Mb of chr3q13.31 (Fig. 1D) that showed a higher frequency of CNAs than the remainder of chr3q ($P < 0.05$) in tumor DNA. Importantly, this region corresponded closely to the 0.7-Mb stretch of chr3q13.31 that had significantly lower CN in tumor than in blood DNA of patients (Fig. 1C). A 10-kb MRO between 118,035,000 and 118,045,000 bp (Fig. 1B and D) was seen in 63% (17 of 27) of patients. Therefore, STAC confirms the focal and nonrandom nature of chr3q13.31 events.

To validate chr3q13.31 CNAs in osteosarcoma, we performed qPCR. Five pairs of probes were used, corresponding to the 0.7-Mb stretch of chr3q13.31 affected by CNA, with one pair centering over the MRO (Fig. 1B, *). As a positive control, we used HOS cells with a chr3q13.31 deletion (14). HOS cells showed biallelic loss at all locations tested (Table 1). CNAs were observed within chr3q13.31 in 80% (24 of 30) of osteosarcoma samples: 53.3% (16 of 30) exhibited chr3q13.31 CN loss (Table 1, samples with blue tracks only), 13.3% (4 of 30) exhibited CN gain (Table 1, samples with red tracks only),
whereas 13.3% (4 of 30) displayed complex changes including regions of CN loss and gain (Table 1, samples with blue and red tracks). qPCR confirmed our microarray findings in the majority of cases, with few exceptions (deletion in OS26; amplifications in OS08, OS11, and OS23; Table 1; Fig. 1B), possibly reflecting use of a different CN baseline in qPCR (blood DNA from 80 controls) and DNA microarray (patients’ own blood DNA). We named the region of chr3q13.31 affected by CNAs in osteosarcoma osteo3q13.31 to emphasize their focal nature, which contrasts the majority of osteosarcoma CNAs previously identified.

We next evaluated whether deletions at osteo3q13.31 were monoallelic or biallelic. Of 20 osteosarcoma patients with osteo3q13.31 deletions (Table 1), 75.0% (15 of 20) were monoallelic (Table 1; Fig. 2A), 15.0% (3 of 20) were biallelic (Table 1; Fig. 2B), and 10.0% (2 of 20) contained regions of monoallelic and biallelic CN loss (Table 1; Fig. 2C). DNA from OS14 contained a region of CN gain neighboring a region of biallelic deletion (Table 1; Fig. 2D). Allele-specific CN analysis revealed (Supplementary Fig. S2) that the observed CN gain was caused by unequal amplification of one allele, a situation functionally resembling loss of one allele through LOH. Therefore, monoallelic and biallelic deletions at osteo3q13.31 are both common and could, along with unequal amplifications, lead to loss of genetic information.

CNAs arise through mechanisms that can lead to LOH in flanking DNA (32–34). We therefore examined if osteo3q13.31 was affected by LOH. Of 27 osteosarcoma samples analyzed by microarrays, 33.3% (9 of 27) displayed LOH at osteo3q13.31 (Supplementary Fig. S3). LOH was common in tumors with biallelic osteo3q13.31 deletions where the deletion was flanked by LOH extending several megabases into chr3q (Fig.

### Table 1. qPCR analysis of osteo3q13.31 CNAs in osteosarcoma tumor DNA at five positions

<table>
<thead>
<tr>
<th>Sample</th>
<th>Position 117.668 Mb</th>
<th>Position 117.737 Mb</th>
<th>Position 117.927 Mb</th>
<th>Position 118.040 Mb</th>
<th>Position 118.355 Mb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>HOS</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>OS01</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>OS02</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>OS03</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>OS04</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>OS05</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>OS06</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>OS07</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>OS08</td>
<td>3</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>OS09</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>OS10</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>OS11</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>OS12</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>OS13</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>OS14</td>
<td>6</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>OS15</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>OS16</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>OS17</td>
<td>2</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>OS18</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>OS19</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>OS20</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>OS21</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>OS22</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>OS23</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>OS24</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>OS25</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>OS26</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>OS27</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>OS28</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>OS29</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>OS30</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>OS31</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>OS32</td>
<td>6</td>
<td>6</td>
<td>8</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

NOTE: Light blue, monoallelic deletions; dark blue, biallelic deletions; red, CN gain. HOS cells are included as a control. CN values are rounded to the nearest integer.
Figure 2. Osteo3q13.31 CNAs in osteosarcoma tumor DNA involve regions of monoallelic and biallelic CN loss, high-level CN gain, and LOH. Microarray analysis of tumor DNA from a patient with a monoallelic deletion without LOH (A), biallelic deletion with LOH (B), monoallelic and biallelic deletions with LOH (C), and complex changes involving regions of high-level CN gain and biallelic CN loss (D). CN of patient’s blood (blue dots) and tumor (red dots) DNA. Dots, smoothed CN for 15 (A) or 10 (B–D) markers. Gray tracks, LOH. Boxes, LSAMP, LOC285194, and BC040587 exons; connecting lines, introns.
LOH at osteo3q13.31 was also confirmed by DNA sequencing of an osteo3q13.31 gene, LSAMP, in 25% (3 of 12) of informative samples (Supplementary Table S5). Therefore, in addition to CN loss, LOH also leads to loss of genetic information at osteo3q13.31 in osteosarcoma.

To confirm microarray and qPCR findings, we performed FISH using the bacterial artificial chromosome probe RP11-956M14, covering the region near MRO at osteo3q13.31. As a control, we used early-passage osteoblasts. These were compared with three tumor biopsies: one without an osteo3q13.31 deletion (OS02), one with a monoallelic deletion (OS15), and one with a biallelic deletion (OS16). Metaphase and interphase FISH analyses confirmed that osteoblasts were diploid at osteo3q13.31 (Supplementary Table S6; Fig. 3A). Similarly, the majority of OS02 cells (66.7%) showed two osteo3q13.31 signals by interphase FISH (Supplementary Table S6; Fig. 3B). Interphase FISH of OS15 tumor revealed approximately equal proportion of cells with two osteo3q13.31 signals and cells with one signal (Supplementary Table S6; Fig. 3C). This agrees with the estimated CN of 1.4 for osteo3q13.31 in this sample by qPCR (Supplementary Table S6; Fig. 3D). Therefore, FISH results are consistent with the focal nature of deletions at osteo3q13.31 in osteosarcoma.

The osteo3q13.31 genes LOC285194, BC040587, and LSAMP commonly show loss of expression in primary osteosarcoma samples and cell lines. Having identified osteo3q13.31 as the site of common, nonrandom, and focal CNAs and LOH in osteosarcoma, we examined if loss of osteo3q13.31 genetic information had functional significance. CNAs and LOH at osteo3q13.31 overlap or map near one TS gene, LSAMP, and the ncRNAs LOC285194 and BC040587. We therefore examined the effect of osteo3q13.31 CNAs on LSAMP, LOC285194, and BC040587 expression by RT-qPCR. Expression was analyzed in 43 primary osteosarcoma tumor biopsies and five osteosarcoma cell lines, normalized to primary osteoblasts. All three genes are expressed in osteoblasts (Fig. 4A), suggesting a possible role in bone biology. Of 48 samples, 64.6% (31 of 48) showed decreased LSAMP expression (calculated as mean expression of exons 1–2 and 3–4) compared with osteoblasts (Fig. 4A, top, bold samples). In 18.8% (9 of 48), LSAMP expression was increased (Fig. 4A, top, italicized samples). In 16.7% (8 of 48), there was no difference in LSAMP expression compared with osteoblasts (Fig. 4A, top). LOC285194 expression was decreased compared with osteoblasts (Fig. 4A, top, bold samples). In 81.2% (39 of 48) of samples, LOC285194 expression was decreased compared with osteoblasts (Fig. 4A, middle, bold samples). The majority of these (62.5%, 30 of 48) had a large decrease, with residual LOC285194 RNA levels <10% of that in osteoblasts. Like LSAMP, LOC285194 expression was increased in some samples: 8.3% (4 of 48) showed higher expression than

---

**Figure 3.** FISH confirms focal monoallelic and biallelic osteo3q13.31 deletions in osteosarcoma. A, metaphase (center) and interphase (right) FISH of primary osteoblasts using chr3 centromere (red) and osteo3q13.31 (green) probes. Interphase FISH is also shown of cells from the osteo3q13.31-diploid tumor of OS02 (B), osteo3q13.31-haploid tumor of OS15 (C), and the OS16, which has a biallelic deletion of osteo3q13.31 (D).
Figure 4. The expression of osteo3q13.31 genes is reduced in primary osteosarcoma tumor samples and cell lines and often reflects the presence of osteo3q13.31 CNAs. Expression levels for 43 osteosarcoma tumor samples and five osteosarcoma cell lines were normalized to primary osteoblasts. Positive control: testis cDNA. Bold, decreased expression; italics, increased expression; regular font, expression levels similar to osteoblasts. Columns, mean; bars, SEM. A, RT-qPCR analysis of LSAMP expression (top) at exon 3 to 4 junction (open columns) and exon 1 to 2 junction (gray columns); LOC285194 expression (middle) at exon 1 (open columns) and exon 4 (gray columns); and BC040587 exon 2 expression (bottom). B, linear regression analysis for correlation between LOC285194 (top) or BC040587 (bottom) expression and CN of osteo3q13.31 CNA MRO at 118,040 kb. C, linear regression analysis for correlation between expression of LOC285194 and LSAMP (top), BC040587 and LSAMP (middle), and LOC285194 and BC040587 (bottom).
osteoblasts (Fig. 4A, middle, italicized samples). In 10.4% (5 of 48), there was no difference in LOC285194 expression compared with osteoblasts (Fig. 4A, middle). BC040587 expression was decreased in 77.1% (37 of 48) of samples (Fig. 4A, bottom, bold samples), increased in 10.4% (5 of 48; Fig. 4A, bottom, italicized samples), and unchanged in 12.5% (6 of 48; Fig. 4A, bottom). In summary, LSAMP expression was affected in 83.3% (40 of 48), LOC285194 expression in 89.6% (43 of 48), and BC040587 expression in 87.5% (42 of 48) of samples. Intriguingly, a change in expression of at least one osteo3q13.31 gene was evident in 100% (48 of 48) of samples, suggesting that dysregulation of osteo3q13.31 genes is ubiquitous in osteosarcoma.

To examine if changes in LSAMP and LOC285194 expression were caused by alternative splicing, we compared expression of LSAMP mRNA at the junction of exons 3 to 4 to that at the junction of exons 1 to 2, and expression of LOC285194 mRNA at exon 1 to that at exon 4. Linear regression analysis revealed (Spearman’s $\rho = 0.956, P = 3.7 \times 10^{-36}$) that expression of LSAMP exons 3 to 4 correlated well with expression of exons 1 to 2 (Supplementary Fig. S4A). Similarly, expression of LOC285194 exon 4 correlated well with exon 1 (Spearman’s $\rho = 0.886, P = 8.2 \times 10^{-42}$; Supplementary Fig. S4B), indicating that changes in LSAMP and LOC285194 expression in osteosarcoma were not caused by alternative splicing. Instead, changes in expression of LOC285194, BC040587, and, less often, LSAMP, could be explained by CNAs at osteo3q13.31. For example, OS22 DNA contains a deletion eliminating LOC285194, but not LSAMP or BC040587 (Fig. 1B), leading to complete loss of LOC285194, but not LSAMP or BC040587, expression (Fig. 4A). OS14 DNA contains a region of CN gain involving LSAMP, plus 100 kb upstream of LSAMP, followed by a region of biallelic CN loss eliminating both LOC285194 and BC040587 (Figs. 1B and 2D). This leads to a nearly 3-fold increase in LSAMP expression and loss of LOC285194 and BC040587 expression (Fig. 4A). OS16 DNA contains a large biallelic deletion eliminating all three osteo3q13.31 genes (Fig. 1B) and leads to loss of expression of all three (Fig. 4A). However, in some tumors, we detect changes in LSAMP, LOC285194, and BC040587 expression in the absence of osteo3q13.31 CNAs. For example, osteo3q13.31 CN-neutral samples OS03 and OS28 (Table 1; Fig. 1B) show a large reduction in LSAMP, LOC285194, and BC040587 expression (Fig. 4A). In contrast, OS15, which contains a monallelic deletion at osteo3q13.31 (Supplementary Table S6; Fig. 3C), shows a large increase in expression of all three osteo3q13.31 genes (Fig. 4A). We considered the possibility that in tumors without osteo3q13.31 CNAs, the apparent change in expression levels of osteo3q13.31 genes resulted from point mutations or small deletions. To test this possibility, we sequenced all seven exons of LSAMP in blood and tumor DNA of 29 patients. We did not detect LSAMP mutations in any samples (Supplementary Table S5). To assess the extent to which expression of osteo3q13.31 genes could be explained by CNAs, we calculated the correlation between expression of LSAMP, LOC285194, and BC040587 and CN of osteo3q13.31 at five different positions measured by qPCR (Fig. 1B). The expression of LSAMP did not show significant correlation with CN at any osteo3q13.31 site (data not shown). In contrast, LOC285194 expression correlated with osteo3q13.31 CN at positions 117,927 kb (Spearman’s $\rho = 0.493, P = 0.009$) and 118,040 kb (Spearman’s $\rho = 0.578, P = 0.002$; Fig. 4B, top). Likewise, BC040587 expression correlated with osteo3q13.31 CN at 117,927 kb (Spearman’s $\rho = 0.536, P = 0.004$) and 118,040 kb (Spearman’s $\rho = 0.592, P = 0.001$; Fig. 4B, bottom). Therefore, osteo3q13.31 CNAs account for changes in LOC285194 and BC040587 expression and, less often, LSAMP. LSAMP, LOC285194, and BC040587 map far apart at osteo3q13.31 (Fig. 1B), spanning 46 kb. As all three are under-expressed in osteosarcoma, we investigated if expression patterns of LSAMP, LOC285194, and BC040587 were correlated. Intriguingly, we found evidence for correlation between expression of all genes: LSAMP and LOC285194 (Spearman’s $\rho = 0.431, P = 5.01 \times 10^{-4}$; Fig. 4C, top), LSAMP and BC040587 (Spearman’s $\rho = 0.460, P = 8.16 \times 10^{-3}$; Fig. 4C, middle), and LOC285194 and BC040587 (Spearman’s $\rho = 0.653, P = 2.25 \times 10^{-4}$; Fig. 4C, bottom). Together, these data suggest that expression of all osteo3q13.31 genes follows a similar pattern in osteosarcoma; in the case of LOC285194 and BC040587, this relates to the presence of CNAs at osteo3q13.31.

**Focal osteo3q13.31 deletions and LOH are common in cell lines from various cancers.** As loss of expression of osteo3q13.31 genes is common in osteosarcoma, we hypothesized that they might be implicated in tumorigenesis in other cell types. We therefore searched public data on cell lines from the Catalogue of Somatic Mutations in Cancer panel for focal osteo3q13.31 CN loss and LOH (35). Interestingly, focal osteo3q13.31 biallelic deletions (Supplementary Fig. S5A) and LOH (Supplementary Fig. S5B) are common in cell lines from various cancers, including those of lung, autonomic and central nervous system, blood, endometrium, soft tissue, skin, gastrointestinal tract, urinary tract, and breast (Supplementary Table S7). Osteo3q13.31 may thus be important in tumorigenesis in various malignancies, including sarcomas and carcinomas.

**Depletion of LSAMP or LOC285194 promotes osteoblast proliferation through regulation of apoptotic and cell cycle transcripts and VEGF/VEGFR1.** As osteo3q13.31 CNAs sometimes involve LSAMP, which belongs to the family of genes implicated as TS in clear cell renal cell carcinoma, glioma, and ovarian carcinoma (36–38), we examined if LSAMP also acted as a TS in osteosarcoma. We did not detect any effect of LSAMP overexpression on proliferation and survival (Supplementary Fig. S6A and B), cell cycle progression (Supplementary Fig. S6C and D), or levels of endogenous apoptosis (Supplementary Fig. S6D) of HOS and U2OS osteosarcoma cell lines. We next investigated the effect of depletion of LSAMP, LOC285194, and BC040587 on normal osteoblasts. Depletion of LSAMP and LOC285194 by siRNA successfully reduced mRNA levels by 90% and 50%, respectively (Fig. 5A). In contrast, siRNA-mediated silencing of BC040587 was not effective (data not shown), consistent with reports of reduced susceptibility of ncRNAs to siRNA (39).

To examine the effect of LSAMP and LOC285194 depletion, we conducted MTT proliferation assays, modified Boyden chamber migration assays, and cell cycle analysis. Depletion of LSAMP and LOC285194 mRNA had no effect on osteoblast migration (data not shown). However, depletion of
LSAMP ($P = 0.007$) and LOC285194 ($P = 4.8 \times 10^{-4}$) both promoted cell growth in MTT assays (Fig. 5B). Cell cycle analysis showed a mild increase in the G1 population of LOC285194-depleted osteoblasts (Supplementary Fig. S7A) and an increase in the S-phase population of LSAMP-depleted osteoblasts (Supplementary Fig. S7B). This implicates these two genes as functional growth suppressors in osteoblasts in vitro.

Because both LSAMP and LOC285194 depletion promoted proliferation (Fig. 5B), with differing effects on the cell cycle (Supplementary Fig. S7), we examined changes induced by depletion of these genes on transcription of a panel of proliferation-associated genes. Cyclin D1, VEGF, and VEGFR1 were all upregulated by LOC285194 depletion (Fig. 5C). Consistent with the results of cell cycle analysis (Supplementary

---

**Figure 5.** In vitro and clinical evidence implicates LSAMP, LOC285194, and BC040587 as osteo3q13.31 TS genes. A, levels of LSAMP and LOC285194 mRNA were assessed in osteoblasts transfected with control, LSAMP, or LOC285194 siRNA. B, proliferation of osteoblasts in response to treatment with control, LSAMP, or LOC285194 siRNA was measured by MTT assays. Columns, mean of three independent experiments; bars, SEM. C, effect of LSAMP or LOC285194 siRNA on transcription of a panel of proliferation-associated genes in osteoblasts was measured by RT-qPCR. Columns, mean of three independent experiments; bars, SEM. D, survival of patients with LOC285194 deletions (left) or BC040587 deletions (right) in tumor DNA was compared with patients without LOC285194 or BC040587 deletions.
Fig. S7), depletion of LSAMP and LOC285194 had opposing effects on cyclin A2 and cyclin B1 expression, with these transcripts being induced by LSAMP siRNA and suppressed by LOC285194 siRNA, indicating that these genes may regulate proliferation by differing mechanisms. Finally, LOC285194 depletion suppressed expression of proapoptotic BCL2 and BimEL (Fig. 5C). Taken together, these data implicate LSAMP and LOC285194 as growth suppressors through regulation of apoptotic and cell cycle transcripts and VEGF/VEGFR1.

The presence of LOC285194 or BC040587 deletions in tumor DNA is associated with poor survival of osteosarcoma patients. Chromosomal aberrations involving LSAMP are associated with poor outcome in osteosarcoma (24, 25). To examine if deletions involving the ncRNAs LOC285194 or BC040587 also affect patient survival, we used Kaplan-Meier survival analysis to compare survival of patients with LOC285194 or BC040587 deletions in tumor DNA (based on microarray data) to those without deletions. Intriguingly, the presence of either LOC285194 deletions (log-rank P = 0.008; Fig. 5D, left) or BC040587 deletions (log-rank P = 0.01; Fig. 5D, right) in tumor DNA was associated with a dramatic decrease in survival. Therefore, LOC285194 and BC040587 ncRNAs, in addition to LSAMP, function as TS genes at osteo3q13.31.

Discussion

Osteosarcomas are cytogenetically complex malignancies (11–19). Because the prevailing view was that most osteosarcoma CNAs were nonrecurrent, we used microarrays, qPCR, and FISH to characterize highly recurrent CNAs within a region of chr3q13.31 that we term osteo3q13.31. Although osteosarcomas are characterized by marked aneuploidy, osteo3q13.31 CNAs are focal and include events clustered in a region <0.7 Mb in size. CNAs and LOH at chr3 in osteosarcoma had been reported previously (12, 14, 18, 19, 40); however, these studies have not provided insight into the roles of the involved genes in osteosarcomagenesis.

The observed CNAs represent de novo events in tumor DNA rather than CN changes of germline CNVs. The presence of focal osteo3q13.31 CNAs in as many as 80% of osteosarcoma samples strongly suggests a functional role for this region.

In samples with osteo3q13.31 deletions, both monoallelic and biallelic deletions are common and involve the genomic sequence of LOC285194 and BC040587 ncRNAs and, sometimes, the LSAMP locus. Using multiple probes, we show by RT-qPCR that expression of one or more osteo3q13.31 genes is nearly ubiquitously dysregulated in 48 primary osteosarcoma biopsies and cell lines. However, although changes in expression of LOC285194 and BC040587 are frequently accounted for by osteo3q13.31 CNAs, this is less often true for LSAMP. Changes in LSAMP expression are not caused by point mutations either, as sequencing of LSAMP in patient blood and tumor DNA only revealed a common silent polymorphism in exon 4. In addition to mutations in the coding sequence, changes involving distant elements can affect gene expression. In the case of osteo3q13.31 genes, changes at one such element, through CNAs, mutations, or epigenetic mechanisms, could lead to a change in expression levels. In support of this, we found a tight correlation between expression of LSAMP, LOC285194, and BC040587, which span 468 kb of DNA. One such element may reside near the osteo3q13.31 MRO, as the presence of genomic deletations at this site correlates well with loss of LOC285194 and BC040587 expression. LSAMP, by virtue of its more distant location, displays an expression pattern showing little correlation with the CN of the MRO. However, as in the case of LOC285194 and BC040587, its expression may be affected by events involving cis-regulatory elements outside its coding sequence such as promoter methylation (24, 36). Therefore, multiple mechanisms must exist to explain the behavior of osteo3q13.31 genes in osteosarcoma, CNAs being one that led us to study this region.

As loss of expression of osteo3q13.31 genes is frequent in osteosarcoma, we hypothesized they may be functionally important in tumorigenesis. In support of this idea, we found that cell lines from other malignancies contain regions of focal CN loss involving osteo3q13.31 genes. Whereas some of these CN changes may represent germline CNVs, most do not (Supplementary Table S6) as they are far more common than osteo3q13.31 CNVs, estimated to be present in <0.1% of healthy controls (41). LSAMP and a related gene have been implicated as TS in clear cell renal cell carcinoma (36), ovarian carcinoma (37), and glioma (38). During the preparation of this article, two groups (24, 25) reported deletions at osteo3q13.31 in osteosarcoma, implicating LSAMP as an osteosarcoma TS. However, these studies did not provide functional evidence or recognize the role of ncRNAs at osteo3q13.31. Supporting the notion that osteo3q13.31 genes act together, we report that deletion of either LSAMP or LOC285194 leads to increased proliferation of normal osteoblasts. Interestingly, although LSAMP and LOC285194 both promote growth and proliferation, they may act through different mechanisms because LSAMP depletion induces S-phase cyclin A2 and LOC285194 depletion suppresses the proapoptotic genes BCL2 and BimEL and induces the VEGF/VEGFR1 axis, previously implicated in osteosarcomagenesis (42). Furthermore, although depletion of LSAMP led to increased proliferation of normal osteoblasts, LSAMP overexpression had no effect on OSU or U2OS cells. These contrasting effects may indicate that loss of LSAMP and other osteo3q13.31 genes are early events in osteosarcoma, and restoration of LSAMP expression alone may not be sufficient to suppress proliferation of fully transformed cell lines carrying a significant burden of genomic instability.

Although chromosomal aberrations involving LSAMP have previously been associated with poor prognosis (24, 25), our work shows that the presence of LOC285194 or BC040587 deletions in tumor DNA is also associated with dramatically reduced survival of osteosarcoma patients. In fact, additional osteo3q13.31 TS properties may lie in interactions between osteo3q13.31 genes that, although far apart, show remarkable correlation in their expression. Dysregulation of LSAMP, LOC285194, and BC040587 expression may be a stepwise process in osteosarcoma, representing a novel example of “multiple hits” in tumorigenesis where expression of neighboring genes is altered sequentially. Monoallelic or biallelic loss, or
upregulation of a particular allele of one or a combination of osteo3q13.31 genes, may have a profound effect on osteosarcoma tumor biology. A challenge remains to determine the functional role for BCO40587 ncRNAs (individually or in a network with other osteo3q13.31 genes) as well as the potential value for osteo3q13.31 in diagnosis, prognosis, and evaluation of response to anticancer treatment. Given the high frequency, focal nature, nonrandom distribution, and universal presence of CNAs overlapping three osteo3q13.31 genes, of which at least two now seem to suppress cellular proliferation and survival, these data support a role for the loss of osteo3q13.31 TS unit as a driver in osteosarcomagenesis, perhaps with a similar role in other cancers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


Acknowledgments

We thank Drs. Eleftherios Diamandis, Stephen Meyn, and Jeremy Squire for the critical review of the manuscript, and Daniel Picard for the help with flow cytometry.

Grant Support

Canadian Institutes of Health Research (CIHR) and the Harry and Hannah Fisher Fund ( SickKids Foundation); CIHR M.D./Ph.D. Studentships (I. Pasic and A.D. Durbin); and a CIHR Doctoral Scholarship (A. Shlien).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received 5/26/09; revised 10/19/09; accepted 11/2/09; published online 1/4/10.
Recurrent Focal Copy-Number Changes and Loss of Heterozygosity Implicate Two Noncoding RNAs and One Tumor Suppressor Gene at Chromosome 3q13.31 in Osteosarcoma

Ivan Pasic, Adam Shlien, Adam D. Durbin, et al.

Cancer Res 2010;70:160-171.

Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/70/1/160

Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2010/01/04/0008-5472.CAN-09-1902.DC1

This article cites 37 articles, 15 of which you can access for free at:
http://cancerres.aacrjournals.org/content/70/1/160.full#ref-list-1

This article has been cited by 4 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/70/1/160.full#related-urls

Sign up to receive free email-alerts related to this article or journal.

To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.