Advances in Brief

Quantitative Analysis of Circulating Tumor Cells in Peripheral Blood of Osteosarcoma Patients Using Osteoblast-specific Messenger RNA Markers: A Pilot Study

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Abstract

Metastasis is a major cause of mortality and morbidity in osteosarcoma (OS) patients. To monitor tumor dissemination, we assessed the circulating tumor burden in OS patients by semiquantitative reverse transcription-PCR using osteocalcin, osteonectin, osteopontin, and type I collagen (COLL) mRNAs as molecular markers. We distinguished levels of the mRNAs in peripheral blood between OS patients and healthy subjects using an OS-derived cell line (Saos-2) as a reference standard. We prospectively analyzed 40 peripheral blood samples from 11 OS patients at diagnosis and 29 healthy subjects. In all 29 (100%) healthy subjects, we detected osteocalcin, osteonectin, and osteopontin mRNAs that were most likely attributed to illegitimate transcription in normal hematopoietic cells. In contrast, we found low COLL mRNA levels in only 35% (10 of 29) of healthy subjects, but significantly higher COLL mRNA levels in 91% (10 of 11) of OS patients (P < 0.0001). The reverse transcription-PCR assay for COLL mRNA was sensitive down to the detection of 10 Saos-2 cells among 10⁶ normal peripheral blood nucleated cells. The upper limit of COLL mRNA determined among the healthy subjects was found exceeded by six OS patients. The substantially elevated COLL mRNA levels in peripheral blood seemed to originate from circulating malignant cells in these six OS patients, all of whom subsequently developed clinical metastases within 12 months of diagnosis (P = 0.002). Conversely, no metastases were detected in the remaining OS patients with normal COLL mRNA levels. Quantification of COLL mRNA may prove valuable for diagnosing OS micrometastasis and assessing prognosis.

Introduction

OS is potentially a fatal malignancy affecting predominantly children and young adults, where alterations of Rb, p53, mdm2, and myc, and erbB-2 overexpression have been identified (1–5). However, the etiology and molecular mechanisms of OS remain unclear. Metastasis that occurs early in the natural history of OS is a major cause of mortality and morbidity. Virtually all OS patients may develop subclinical micrometastasis at initial diagnosis. Using computerized tomography scans, about 20% of OS patients have clinically detectable lung metastasis at presentation with consequently poor prognosis (6). Despite surgical resection of the primary lesion, nearly 90% of OS patients develop metastasis/recurrence after operation (7). Early detection of micrometastasis, or the potential for metastasis/recurrence, may permit prognostication and early treatment (8–10).

In this regard, RT-PCR could be clinically valuable for the detection of micrometastasis or circulating malignant cells in OS patients.

OS produces osteoid and/or bone (11). We, therefore, selected molecular markers for OS based on the fact that OC, ON, OPN, and COLL mRNAs are differentially expressed in osteoblasts (12–15). OC is a bone matrix protein required for bone resorption, tissue remodeling, and extracellular matrix mineralization (12, 16). ON is a glycoprotein involved in extracellular matrix remodeling, cell adhesion, differentiation, and proliferation (17). Of interest, ON is also expressed in stromal myofibroblasts of carcinomas, at the interface between stromal cells and hepatocellular carcinoma cells, in melanoma cells, breast cancer, and colorectal cancer cells (18–22). Moreover, there is evidence suggesting that both ON and OPN are involved in angiogenesis and tumor progression (23, 24).

OPN is a bone matrix glycoprotein that modulates mineralization and bone resorption (14, 25, 26). Intriguingly, OPN mRNA is also expressed in human carcinomas, including breast, kidney, and endothelial cancers, where OPN may have adhesion/migration functions in promoting invasion and metastasis (27–30). In the circulation of patients with metastatic cancers, elevated OPN levels have been detected (31). Also, it has been demonstrated that tumor-derived OPN may enhance tumor growth and survival of metastases (32). COLL is another bone-specific marker gene, which encodes the major extracellular matrix component in bone (13).

In this prospective study, we evaluated whether OC, ON, OPN, and COLL mRNAs could be applied as molecular markers for detecting circulating tumor cells in peripheral blood of OS patients.

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3The abbreviations used are: OS, osteosarcoma; RT-PCR, reverse transcription PCR; OC, osteocalcin; ON, osteonectin; OPN, osteopontin; COLL, type I collagen; PBNC, peripheral blood nucleated cell; β-2M, β-2-microglobulin.
Quantitative Analysis of Circulating Osteosarcoma Cells

patients. Previous findings suggest that illegitimate transcription in normal hematopoietic cells may limit the specificity of RT-PCR (33, 34). To minimize this potential problem, we applied a quantitative approach for the precise assessment of the circulating tumor burden and, hence, the risk for metastasis/recurrence (8, 34). We, thus, developed a semiquantitative RT-PCR method for measuring levels of OC, ON, OPN, and COLL mRNAs in peripheral blood from OS patients and healthy subjects and correlated the mRNA levels with clinical outcomes of patients.

Materials and Methods

Peripheral Blood Samples from Patients and Controls. With informed consent and approval from the Ethics Committee of the Chinese University of Hong Kong, 40 peripheral blood samples were collected from 11 OS patients without clinically detectable metastases at diagnosis (median age, 21 yr; range, 12–37 yr; male:female ratio, 8:3) and 29 healthy volunteers between 19 and 40 yr of age. The diagnosis of all OS cases was histologically confirmed. The sites of primary OS were located in the femur, proximal tibia, proximal humerus, and pelvis. All of the patients were treated by surgery and chemotherapy and were followed up clinically for at least 12 months. The healthy subjects served as negative controls for semiquantitative RT-PCR.

PBNC Isolation, RNA Extraction, and DNase I Digestion. PBNCs were isolated by Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden) from 20 ml of citrated blood from the OS patients and healthy subjects studied. After washing in 30 ml of PBS and centrifugation at 1000 × g for 10 min, the cell pellet was resuspended in 1 ml of PBS. The number of PBNCs was counted in a hemocytometer. After centrifugation, the cell pellet was resuspended in 0.5 ml of guanidinium thiocyanate solution and total RNA was extracted by a single-step method (35).

Before RT-PCR, total RNA was treated with DNase I to remove contaminating genomic DNA. Digestion was conducted at 37°C for 1 h in the presence of 10 units of DNase I (Boehringer Mannheim, Mannheim, Germany), 10 mM MgCl₂, 0.1 mM DTT, and 50 mM Tris-HCl. After heat-inactivation, RNA was extracted using phenol-chloroform and then ethanol-precipitated.

Cell Culture. An OS-derived cell line, Saos-2 (American Type Culture Collection, Manassas, VA), was used to establish standard curves for measuring levels of the mRNA markers. The cell line was cultivated in DMEM added with penicillin, streptomycin, 2 mM glutamine, and 10% fetal bovine serum (Life Technologies, Inc., Gaithersburg, MD). The medium was changed every 3 days, and the cells were harvested when the growth was subconfluent. The total number of Saos-2 cells was counted in a hemocytometer.

Development of Standard Curves Using the Saos-2 Cell Line. To simulate the presence of OS cells in the circulation of OS patients, total RNA was first extracted from 10⁷ normal PBNCs and 10⁷ OS cells from the Saos-2 cell line. Aliquots of total RNA from 10⁶ normal PBNCs were mixed with Saos-2 total RNA, corresponding to 1, 10, 10², 10³, 10⁴, and 10⁵ tumor cells (based on the calculation of the average amount of RNA extracted per cell). The RNA mixtures were subjected to semiquantitative RT-PCR.

### Table 1

<table>
<thead>
<tr>
<th>Primer/probe</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>OCF</td>
<td>5′-TGCAGATGTCGACAGAAGGTCGAC-3′</td>
</tr>
<tr>
<td>OCR</td>
<td>5′-ATAGGCTCCTGACAGAAGGTCGAC-3′</td>
</tr>
<tr>
<td>ONF</td>
<td>5′-GATGCTTCTTCTTCTTCTTCTGCG-3′</td>
</tr>
<tr>
<td>ONR</td>
<td>5′-TTTTGCACTGTTGTTCTTGCAG-3′</td>
</tr>
<tr>
<td>OPNF</td>
<td>5′-TCCAGCGGCTGAGATATGCTGG-3′</td>
</tr>
<tr>
<td>OPNR</td>
<td>5′-TACAGGGGATTTCTAGAAGGCCGC-3′</td>
</tr>
<tr>
<td>COLFF</td>
<td>5′-GGTGTTGTTGTAGCTTTGTTTT-3′</td>
</tr>
<tr>
<td>COLLR</td>
<td>5′-CTTGGCTGGATGTTTCCAGGTT-3′</td>
</tr>
<tr>
<td>COLLP</td>
<td>5′-ATAGTGCACTTGGTTAGGGTCATCCAC-3′</td>
</tr>
<tr>
<td>β-2MF</td>
<td>5′-CCTGAAATGTTGCTATGCGTCTCGGTTTCATCCA-3′</td>
</tr>
<tr>
<td>β-2MR</td>
<td>5′-GAGGCACAACTTCGCTCACAGATCAAATGCGG-3′</td>
</tr>
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</table>

Semiquantitative RT-PCR and Southern Blot Analysis. Total RNA (1 μg) was denatured at 65°C for 2 min and annealed with 1 μg of random primers at 37°C for 10 min (34). RT was carried out in 1× reaction buffer [50 mM Tris-HCl (pH 8.3), 75 mM KCl, and 3 mM MgCl₂] with 10 mM DTT, 0.5 mM deoxynucleoside triphosphates, and 0.5 μl of RNase block (Stratagene, La Jolla, CA). cDNA was synthesized at 37°C for 1 h using 200 units of Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.). The reaction was stopped at 70°C for 7 min.

PCR amplification of OC, ON, OPN, and COLL cDNAs was conducted using gene-specific primers lying within different exons to give products of 199 bp, 225 bp, 298 bp, and 325 bp, correspondingly (Table 1). β-2M mRNA served as an internal control to ensure that an exact amount of high-integrity total RNA was reverse-transcribed to produce cDNA in each PCR (34, 36).

PCR was conducted in 1× PCR buffer [20 mM Tris-HCl (pH 8.4), 50 mM KCl, and 2.5 mM MgCl₂] added with 0.2 mM deoxynucleoside triphosphates; 30 pmol of sense and antisense primers for OC, ON, OPN, COLL, or β-2M cDNA; 3 μl of cDNA; and 2.5 units of Taq DNA polymerase (Life Technologies, Inc.; Ref. 34). The optimized thermal profile was initiated with a 5-min denaturation at 94°C, followed by 30 cycles of 94°C for 1 min, 61°C (β-2M), 69°C (OC/ON), 65°C (OPN), or 67°C (COLL) for 1 min and 72°C for 1 min, and a final extension at 72°C for 10 min. Aerosol-resistant pipette tips and separate areas were used for pre-PCR, PCR, and post-PCR procedures. Each sample was analyzed in duplicate. Saos-2 RNA standards and multiple water blanks were analyzed in parallel with peripheral blood samples for each set of PCR. PCR products were loaded onto 2% agarose gels and stained with ethidium bromide.

The gene-identity of the PCR product was verified by nonradioactive Southern blot analysis using a gene-specific oligonucleotide probe (Table 1), which was labeled at the 3′ end with digoxigenin (34, 36). Chemiluminescent detection was conducted using disodium 3-(4-methoxyisopropyl)-1,2-dioxetane-3,2'-dioxime (5′-chloro) tricyclo[3.3.1.13,7] decane-4-yl) phenyl phosphate (Boehringer Mannheim). By using imaging densitometry (BioRad, Hercules, CA), the amounts of PCR products for blood samples were quantified on the same Southern blot as the PCR products generated for establishing the Saos-2 standard curve.
Table 2  Levels of OC, ON, OPN, and COLL mRNAs in PBNCs from 29 healthy subjects

<table>
<thead>
<tr>
<th>mRNA levels</th>
<th>OC</th>
<th>ON</th>
<th>OPN</th>
<th>COLL</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT-PCR positivity</td>
<td>High</td>
<td>High</td>
<td>High</td>
<td>Low</td>
</tr>
<tr>
<td>% of positivity</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>35%</td>
</tr>
</tbody>
</table>

**Results**

**Levels of Osteoblast-specific mRNAs in PBNCs from Healthy Subjects.**  OC, ON, and OPN mRNAs were detected in PBNCs from all 29 (100%) healthy subjects at levels undistinguishable from those in the Saos-2 cell line (Table 2). In contrast, COLL mRNA levels, markedly lower than in Saos-2 cells, were obtained in only 35% (10 of 29) of healthy subjects (Fig. 1A). The frequency of COLL mRNA detection among the control subjects was much lower than that for OC, ON, and OPN mRNAs. We, therefore, determined the upper limit of COLL mRNA in the control group (mean + 3 SD) and set this as the reference range for distinguishing COLL mRNA levels in circulation between OS patients and healthy subjects.

**Linear Saos-2 Standard Curve for COLL mRNA Measurement.** A linear relationship was found between the amount of COLL PCR product and the level of Saos-2 total RNA used corresponding to 0.13 ng to 1.3 μg, over a range of 10–10⁶ Saos-2-RNA equivalents (correlation coefficient, 0.94; Fig. 2). The RT-PCR assay was consistently sensitive down to the detection of 10 Saos-2 cells among 10⁶ normal PBNCs.

**Quantification of COLL mRNA in Peripheral Blood from OS Patients and Association with Clinical Metastases.** In this prospective study, we detected variable levels of COLL mRNA in 20 ml of peripheral blood from 91% (10 of 11) of OS patients at diagnosis (Fig. 1B and Fig. 3). The COLL mRNA levels in the OS patients were significantly higher than in the 29 healthy subjects studied (Mann-Whitney U test, P < 0.0001). According to the Saos-2 standard curve, the mean COLL mRNA level in the control group (n = 29) was 767.5 Saos-2-RNA equivalents (pg; range, 0.1–5338 Saos-2-RNA equivalents; Fig. 3). The mean COLL mRNA level among the 11 OS patients was 36361.95 Saos-2-RNA equivalents (pg; range, 1.7–132373.3 Saos-2-RNA equivalents; Table 3). The upper limit of COLL mRNA (mean + 3 SD) in the control group (5338 Saos-2-RNA equivalents) was found exceeded by six OS patients (Fig. 3).

Of clinical relevance, all of the six OS patients with substantially raised COLL mRNA levels subsequently developed metastases within 12 months of diagnosis (Table 3). Two of the six patients with the highest COLL mRNA levels developed clinical metastases within 4–5 months of diagnosis, whereas the other four patients with similarly high COLL mRNA levels developed metastases within 6–10 months of diagnosis. In striking contrast, no metastases were detected in any of the remaining five OS patients with normal COLL mRNA levels (P = 0.002; Fisher’s exact test).

**Discussion**

In this first attempt to detect circulating malignant cells in peripheral blood of OS patients, we have developed semiquantitative RT-PCR for COLL mRNA using the Saos-2 cell line as a reference standard. As compared with healthy subjects, markedly elevated COLL mRNA levels in peripheral blood from OS patients at diagnosis were strongly associated with the subsequent development of clinical metastases (P = 0.002). On the other hand, the presence of OC, ON, and OPN mRNAs in PBNCs from all of the healthy subjects studied was most likely attributed to illegitimate transcription in normal hematopoietic cells. These results suggest that OC, ON, and OPN mRNAs are not specific markers for OS. Our present data are consistent with the fact that OC mRNA is expressed in peripheral blood platelets, bone marrow megakaryocytes, and multiple soft tissues such as aorta, liver, lung, kidney, and brain (37, 38). Moreover, ON mRNA has been detected in developing blood vessels, and its transcription can be induced by transforming growth factor β (23, 39, 40). Furthermore, OPN mRNA expression in macrophages has been documented, and its transcription is inducible by transforming growth factor β or during the activation of natural killer cells (19, 26, 29, 41).

Using RT-PCR with DNase I pretreatment, we demonstrated low levels of COLL mRNA in PBNCs in only 35% (10 of 29) of healthy subjects. Because the PCR product has the expected molecular size, the possibility of genomic DNA contamination can be ruled out. Because COLL mRNA is also expressed in skin (42, 43), the first aliquot of peripheral blood might have skin contamination caused by needle aspiration. We may be able to eliminate this kind of contamination by disregarding the first aliquot of blood and collecting subsequent aliquots for molecular analysis. However, the low levels of COLL mRNA detected in healthy subjects were also highly likely attributed to illegitimate transcription in normal hematopoietic cells. To minimize this potential problem, we have applied a quantitative approach for differentiating the COLL mRNA levels in peripheral blood between OS patients and healthy subjects. Our semiquantitative RT-PCR enables us to determine the upper limit of COLL mRNA among healthy subjects. Above this reference range, elevated COLL mRNA levels (up to ~25-fold) in 55% (6 of 11) of OS patients at diagnosis may genuinely reflect the presence of circulating malignant cells in complete concordance with the subsequent development of clinical metastases.

Unlike DNA alterations such as p53 and Rb mutations, which were inconsistently found in OS and micrometastasis (1, 2), markedly raised COLL mRNA levels were frequently detectable among the OS patients studied. Moreover, the semiquantitative RT-PCR assay that measures mRNA levels offers much higher sensitivity than mutation screening by DNA sequencing. As opposed to oncogene/tumor suppressor gene alterations that could also be found in a wide variety of other tumors, the advantage of using COLL mRNA as a marker for detecting OS micrometastasis is its relatively high osteoblast specificity.

For OS may have already metastasized at clinical presentation, early detection of micrometastasis is critical for permitting early chemotherapy or intensive adjuvant chemotherapy, which should be more effective against micrometastasis than clinically detectable metastasis (7). In our cohort, all of the six (100%) OS patients with substantially elevated COLL mRNA levels subsequently developed clinical metastases within 12
months of diagnosis. This strongly suggests that COLL mRNA may be applied as a prognostic marker to identify OS patients at diagnosis with a high risk of metastasis/recurrence; such disease progression could possibly be prevented by early treatment. Because the most frequent site of metastasis is the lung (6), the sputum from OS patients would possibly be another sample source to be tested for circulating OS cells using semiquantitative RT-PCR for COLL mRNA.

Furthermore, the COLL mRNA quantity in PBNCs may provide useful diagnostic information in conjunction with cross-sectional imaging results before histological confirmation can be made on surgically resected tumors/biopsies. Characterization of circulating OS cells detected might also contribute to the understanding of the OS pathogenesis. For further investigation, it is worthwhile to explore the diagnostic and prognostic significance of the COLL mRNA level in a larger series of OS patients with long-term follow-up.

As a potential prognostic factor, the COLL mRNA level in peripheral blood may be sequentially monitored to follow up OS patients without micrometastasis at diagnosis. For the five OS...
patients studied without evidence of micrometastasis, as reflected by normal COLL mRNA levels in PBNCs, the toxicity of chemotherapy might be avoided at an initial stage (7). During clinical follow-up, quantification of COLL mRNA in peripheral blood may help assess the patients’ response to therapies, which is crucial in determining the patients’ prognosis. The molecular approach of using COLL mRNA may possibly open up the prospect of monitoring OS in a noninvasive manner without the requirements of surgery and computerized imaging. As compared with these conventional methods, semiquantitative RT-PCR for COLL mRNA seems to be sufficiently sensitive, rapid, and reliable for evaluating histological and tumor response to treatments. Taken together, this novel mRNA marker could potentially help manage OS patients more effectively and, hence, improve the clinical outcome or guide the selection of therapies.

Acknowledgments

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References


Table 3 Patient characteristics and the association of COLL mRNA levels in PBNCs measured at initial diagnosis with clinical outcomes within 12 months of diagnosis

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex/Age</th>
<th>Site of primary OS</th>
<th>COLI mRNA level in PBNCs (Saois-2-RNA equivalents, pg)</th>
<th>Clinical evidence of metastasis (months after initial diagnosis)</th>
</tr>
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<tbody>
<tr>
<td>OS1</td>
<td>M/17</td>
<td>Femur</td>
<td>10,510.41</td>
<td>Yes (9)</td>
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<tr>
<td>OS2</td>
<td>F/12</td>
<td>Proximal tibia</td>
<td>3,367.62</td>
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<tr>
<td>OS3</td>
<td>M/16</td>
<td>Humerus</td>
<td>47,128.71</td>
<td>Yes (8)</td>
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<tr>
<td>OS4</td>
<td>M/15</td>
<td>Humerus</td>
<td>132,373.33</td>
<td>Yes (5)</td>
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<tr>
<td>OS5</td>
<td>M/18</td>
<td>Femur</td>
<td>40,640.46</td>
<td>Yes (6)</td>
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<td>OS6</td>
<td>M/25</td>
<td>Pelvis</td>
<td>40.20</td>
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<tr>
<td>OS7</td>
<td>M/21</td>
<td>Femur</td>
<td>49.80</td>
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<td>OS8</td>
<td>M/37</td>
<td>Humerus</td>
<td>37,631.50</td>
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<td>OS9</td>
<td>F/23</td>
<td>Proximal tibia</td>
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