An Expression Signature Classifies Chemotherapy-Resistant Pediatric Osteosarcoma

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Abstract

Osteosarcoma is the most common malignant bone tumor in children. Osteosarcoma patients who respond poorly to chemotherapy are at a higher risk of relapse and adverse outcome. Therefore, it was the aim of this study to identify prognostic factors at the time of diagnosis to characterize the genes predictive of poor survival outcome and to identify potential novel therapeutic targets. Expression profiling of 30 osteosarcoma diagnostic biopsy samples, 15 with inferior necrosis following induction chemotherapy (Huvos I/II) and 15 with superior necrosis following induction chemotherapy (Huvos III/IV), was conducted using Affymetrix U95Av2 oligonucleotide microarrays. One hundred and four genes were found to be statistically significant and highly differentially expressed between Huvos I/II and III/IV patients. Statistically significant genes were validated on a small independent cohort comprised of osteosarcoma xenograft tumor samples. Markers of Huvos I/II response predominantly were gene products involved in extracellular matrix (ECM) microenvironment remodeling and osteoclast differentiation. A striking finding was the significant decrease in osteoprotegerin, an osteoclastogenesis inhibitory factor. Additional genes involved in osteostegogenesis and bone resorption, which were statistically different, include annexin 2, SMAD, PLA2G2A, and TGFβ1. ECM remodeling genes include desmoplakin, SPARC/11, biglycan, and PECAM. Gene expression of select genes involved in tumor progression, ECM remodeling, and osteostegogenesis were validated via quantitative reverse transcription-PCR in an independent cohort. We propose that osteosarcoma tumor–driven changes in the bone microenvironment contribute to the chemotherapy-resistant phenotype and offer testable hypotheses to potentially enhance therapeutic response. (Cancer Res 2005; 65(5): 1748-54)

Introduction

Osteosarcoma is a primary malignant tumor of the bone. Osteosarcoma accounts for ~5% of childhood tumors in the United States and is the most common malignant bone tumor in children (1). Over the past three decades, advances in treatment have been responsible for improved limb salvage, reduced metastases, and overall higher survival rates (1). Multigent dose-intensive chemotherapy regimens have resulted in long-term disease-free survival rates of ~60% to ~76% in patients with localized disease. Osteosarcoma patients whose tumors respond poorly to chemotherapy are at a higher risk of relapse and adverse outcome. Therefore, it is imperative to identify prognostic factors at the time of diagnosis to detect chemotherapy-resistant tumors and to generate a modified treatment regimen.

The standard therapy regimen of high-grade osteosarcoma includes induction multiagent chemotherapy followed by surgical resection and postoperative chemotheraphy (2). Induction therapy allows for treatment of micrometastatic disease, tumor shrinkage, and decreased tumor vascularity, thus facilitating the surgical removal of the tumor (3). The percentage of necrotic tissue following induction chemotherapy is classified using the Huvos grading system, where the various levels of necrosis reflect the effectiveness of the given therapy (Table 1; ref. 4). Patients with <90% tumor necrosis following induction therapy are classified as inferior responders, or Huvos grade I/II (1). To date, aside from metastatic lesions at presentation, histologic response to chemotherapy is the most dependable and reproducible prognostic indicator of the probability of disease-free survival (1). The degree of necrosis following definitive surgery remains the only consistent prognostic factor in the majority of patients presenting with apparently localized disease. As treatment regimens have evolved over time, numerous clinical trials have attempted to increase the disease-free survival rate for poorly responding patients with intensified postoperative therapy. However, no survival benefit has been convincingly shown through the administration of more intensified therapy to poor responders. This conclusion has been reached by several independent clinical trials (5–7). This suggests that there may be an innate biological difference between responsive and nonresponsive tumors.

Although Huvos grading is a powerful predictor of survival, the prognostic marker is ineffective in altering the outcome of chemoresistant tumors, as this indicator can only be determined after therapy has already been given. For patients who display inferior histologic response to chemotherapy, it is crucial to determine the biological factors that drive lack of response at the time of diagnosis. The importance of this is twofold: First, to identify a genetic fingerprint that will distinguish patients as good or poor responders before therapy; second, to identify potential
target candidates to customize induction therapy. Therefore, it was the aim of this study to compare the gene expression profiles of biopsy samples obtained from responsive and nonresponsive patients with osteosarcoma to characterize the genes predictive of poor survival outcome and to identify potential novel therapeutic targets. The results of this study show that resistant tumors may have an increased ability to express osteoclastogenesis, tumor progression, and extracellular matrix (ECM) remodeling genes. We have validated these findings on a limited number of independent tumor samples and propose bisphosphonate analogues as potential adjuvant therapies in patients with chemotherapy-resistant osteosarcoma at the time of induction therapy.

Materials and Methods

Patient Samples. All tumor samples utilized in this study were obtained from patients treated with the same standard induction therapy of 10 weeks cisplatin, doxorubicin, and high-dose methotrexate. Thirty flash-frozen osteosarcoma specimens, including 15 Huvos grade I/II and 15 Huvos grade III/IV osteosarcoma tumors, were obtained from Memorial Sloan-Kettering Cancer Center (New York, NY) with informed consent from patients/guardians in accordance with a biology study approved by Memorial Hospital Institutional Review Board and stored at −80°C until ready for use in the gene expression profiling studies. Samples from each primary tumor were acquired at the time of the initial diagnostic biopsy. Three additional samples that had been maintained as xenografts in severe combined immunodeficient mice were obtained from Memorial Sloan-Kettering Cancer Center and used for gene expression profiling. The tumors were prepared using the following protocol: Poor histologically responsive primary tumors were isolated and grown in culture to confluence; thereafter, cells were harvested and injected into severe combined immunodeficient mice. The resulting xenograft tumors were isolated and stored at −80°C until ready for use in the gene expression profiling studies. Quantitative reverse transcription-PCR (RT-PCR) analysis was conducted on six independent osteosarcoma biopsy specimens (three Huvos grade I/II and three Huvos grade III/IV osteosarcoma tumors) obtained at Memorial Sloan-Kettering Cancer Center and stored at −80°C until ready for use. The Children’s National Medical Center Institutional Review Board approved the conduct of the laboratory analyses.

Gene Expression Profiling. Total RNA was isolated from tissues using TRIzol reagent (Invitrogen, Carlsbad, CA) as described previously (8). Five to twenty-five micrograms total RNA were obtained from each tumor tissue sample. Five micrograms extracted RNA from each sample were synthesized into double-stranded cDNA using the SuperScript choice system (Life Technologies, Inc., Carlsbad, CA) using an oligo(dT) primer containing a T7 RNA polymerase promoter (Genset, San Diego, CA). The double-stranded cDNA was purified by phenol-chloroform extraction and in vitro transcribed using the ENZO Bioarray RNA transcript labeling kit (Affymetrix, Santa Clara, CA). The biotin-labeled cRNA was purified using the RNeasy kit (Qiagen, Valencia, CA) and fragmented to 50–60 nucleotide fragments with alkaline treatment [200 mmol/L Tris-acetate (pH 8.2), 500 mmol/L MgCl2, 10 mmol/L deoxynucleotide triphosphate, 0.1 mol/L dithiothreitol, RNaseOUT (40 units/μL), and SuperScript II RT (200 units/μL)]. The double-stranded fragmented cRNA was purified using the RNeasy kit (Qiagen, Valencia, CA) and fragmented to 50–60 nucleotide fragments with alkaline treatment [200 mmol/L Tris-acetate (pH 8.2), 500 mmol/L MgCl2, 10 mmol/L deoxynucleotide triphosphate, 0.1 mol/L dithiothreitol, RNaseOUT (40 units/μL), and SuperScript II RT (200 units/μL)]. The samples were incubated at 25°C for 10 minutes, followed by a 50-minute incubation at 42°C. The reaction was terminated at 70°C for 20 minutes followed by a 20-minute incubation at 37°C with E. coli RNase H (2 units/μL).

Reverse Transcription and TaqMan Quantitative PCR Analysis. All reagents and primers used for reverse transcription and PCR amplification were obtained from Invitrogen. Total RNA was reverse-transcribed using a RT-PCR reaction mix consisting of oligo (dT) 0.5 μg/μL, random hexamers (50 ng/μL), 10× cDNA expression buffer, 25 mmol/L MgCl2, 10 mmol/L deoxynucleotide triphosphate, 0.1 mol/L dithiotreitol, RNaseOUT (40 units/μL), and SuperScript II RT (200 units/μL). The samples were incubated at 25°C for 10 minutes, followed by a 50-minute incubation at 42°C. The reaction was terminated at 70°C for 20 minutes followed by a 20-minute incubation at 37°C with E. coli RNase H (2 units/μL).

TaqMan PCR primers were designed using Invitrogen LUX (Light Upon eXtension) fluorogenic designer software and labeled with 6-carboxyfluorescein phosphoramidite (FAM). 18S rRNA predesigned primers were purchased and used as an internal control. Primer sequences for annexin 2 were (forward) 5′-CCTGAGGCCTCGAAGAATTG[FAM]G-3′ and (reverse) 5′-GGAAGATGTTAGGCTGGTT-3′, for biglecan, and (forward) 5′-CAGCGCTTCTTCCAACACCCG[FAM]G-3′ and (reverse) 5′-TCAGTGACGCGAGGGAAATG-3′, for desmoplakin, (forward) 5′-CACCAGCATGACCTGTCCCTCCAG[FAM]G-3′ and (reverse) 5′-GCC-TCGAACTACTATTCCTCCCAA-3′, for osteoprotegrin (OPG), (forward) 5′-CATTGAGCCACTTGAGGCTCTCAG[FAM]G-3′ and (reverse) 5′-TGGAGAGGATGTTAGGCTGGTT-3′, for plasminogen activator inhibitor type 1, (forward) 5′-CAGCGGAGGAAATGTCGATGC[FAM]G-3′ and (reverse) 5′-CACGCGGAGGAAATGTCGATGC[FAM]G-3′ and (reverse).
Results

Expression Profiling of Osteosarcoma. Expression profiling was conducted on 30 independent osteosarcoma biopsy samples. Fifteen of these samples were obtained from patients with an inferior response to chemotherapy (Huvos I/II), whereas the remaining 15 samples were obtained from patients with a favorable response to chemotherapy (Huvos III/IV). Grading of these biopsy samples was assigned after induction therapy by observing the percentage of necrosis visible after preoperative chemotherapy.

Expression profiling was conducted using Affymetrix U95Av2 chips comprised of 12,625 probe sets. The 12,625 probe sets were normalized via a linear scaling on MAS 5.0 and cropped for samples displaying at least one present call across the 30 osteosarcoma tumor set, resulting in a gene list of 9,030 genes. The 9,030 genes were analyzed using the Significance Analysis of Microarrays add-in to Microsoft Excel to ascertain the authenticity of genes deemed significant. One hundred and twelve significant genes were selected using a \( \alpha \) of 0.04248. The false discovery rate obtained for these 112 genes was 17.8%, indicating a high probability that these genes were selected due to statistical significance and not due to chance alone.

The 9,030 genes exhibiting at least one present call were further cropped for genes differentially expressed between Huvos I/II and III/IV tumors through the use of a permutational t test and differential fold change cutoff. Nine hundred and ten genes were differentially expressed \( (P < 0.05) \). To further select for genes with the largest average fold changes, we cropped the 910 differentially expressed genes by first selecting genes with an average fold change >2.0, and then selecting genes with an average expression of at least 500 in at least one class (Fig. 1). Of the 104 differentiated genes that met these criteria, 63 (60.5%) were up-regulated in poorly responsive tumors and 41 (39.5%) were down-regulated in poorly responsive tumors (Fig. 1). The 104 significant genes obtained from permutational t testing and fold-change restrictions displayed absolute concordance with the 112 significant genes selected by Significance Analysis of Microarrays software.

The 910 significant genes were examined to determine the overarching pathways involved in poor histologic response. Confirmation of the identified pathways was conducted by validating select genes obtained from the 104 highly differentially expressed genes.

Classification of Independent Tumor Samples. Xenograft samples were derived from aggressively growing, poorly responsive primary tumor cells grown in severe combined immunodeficient mice. Xenograft samples derived from primary osteosarcoma tumor cells were expression-profiled using Affymetrix U95Av2 Gene Chips. Independent validation of the 104 highly differentially expressed and statistically significant genes was conducted on this separate sample cohort using hierarchical clustering (Fig. 2). The xenograft specimens clearly clustered with the poorly responsive tumors using this unsupervised method.

RT-PCR Validation of Microarray Expression Results. RT-PCR validation was conducted on six independent osteosarcoma tumor samples (Table 2). Genes were selected for mRNA confirmation based on biologically and statistically significant genes through MAS 5.0 normalization methods. Quantitative RT-PCR was conducted on desmoplakin, OPG, plasminogen activator inhibitor type 1, biglycan, annexin A2, SPARC-like 1, and PLA2G2A. Fold changes obtained from quantitative RT-PCR confirm the microarray expression fold changes (Table 2).

Discussion

Microarray technology has provided the means for studying the molecular basis of human disease by examining thousands of genes simultaneously. Microarray technology holds particular promise in the study of the innate chemotherapy resistance in osteosarcoma patients, where presently there is an inability to salvage poorly responsive patients once induction therapy has been given. It is imperative to determine the biological factors that drive chemotherapy resistance in osteosarcoma patients with adverse histologic response to chemotherapy to predict survival outcome and to identify potential novel therapeutic targets at diagnosis. This is the first study to subclassify nonresponders, to identify new therapeutic targets for nonresponders, and to suggest chemotherapeutic opportunities for further testing.

We expression-profiled 15 independent Huvos I/II and 15 Huvos III/IV osteosarcoma biopsy samples and identified 910 statistically significant genes and 104 highly differentially expressed significant genes. Permutational analysis of this data set suggests a low false discovery rate, giving credence and biological significance to the poor-responsive gene expression signature. Gene expression validation was conducted through the unsupervised classification of independent therapy-resistant tumor xenografts and quantitative RT-PCR in an independent set of osteosarcomas. We propose that a poor-prognosis osteosarcoma expression signature involves a drive toward osteoclastogenesis, ECM remodeling, and tumor progression. We further speculate that functionally diverse genes cooperatively promote an altered ECM environment to promote tumor survival (Fig. 3).

Confimation of Differential Expression between Classes on Independent Tumor Samples. We have generated a genetic fingerprint of chemotherapy-resistant tumors (Fig. 1). A selection of these genes involved in osteoclastogenesis and ECM remodeling was validated via quantitative RT-PCR on an independent tumor sample set (Huvos I/II \( n = 3 \); Huvos III/IV \( n = 3 \)). Quantitative RT-PCR validation was conducted on desmoplakin, OPG, plasminogen activator inhibitor type 1, biglycan, annexin 2, PLA2G2A, and...
Figure 1. Chemotherapy resistance genes in osteosarcoma. One hundred and four highly differing genes were identified through statistical significance and fold change criteria in 30 individual osteosarcoma microarray samples. These genes differentiating poor and good histologic response to chemotherapy are represented in this cluster gram. Levels of expression are represented on a scale from the lowest expression (dark green) to the highest expression (bright red). Fold differences are represented in the last column. A negative fold difference indicates a decreasing fold change between Huvos I/II versus Huvos III/IV tumors, whereas a positive fold difference indicates an increasing fold change.
The dramatic fold characterized by a prevailing pattern of genes involved in osteoclastogenesis and ECM modulating genes in poor-prognosis samples.

To further confirm that the significant genes identified in the microarray data were indeed biologically significant, we investigated whether this gene list (Fig. 1) was able to segregate osteosarcoma tumors characterized by aggressive growth behavior and poor therapeutic response. Xenograft samples grown from primary aggressive and chemotherapy-resistant osteosarcoma tumors were expression-profiled on Affymetrix U95Av2 GeneChips. Using an unsupervised strategy, xenograft samples clearly clustered with Huvos I/II samples (Fig. 2), concluding that genes identified from osteosarcoma tumors in this preliminary study can be definitively classified Huvos I/II osteosarcoma tumors. We recognize that the validation cohort was limited but the excellent classification ability of this signature using unsupervised analysis coupled with the RT-PCR validation data illustrates a robust signature.

**Poor-Prognosis Tumors Are Associated with Osteoclastogenesis and Bone Resorption.** Statistically significant genes are characterized by a prevailing pattern of genes involved in osteoclast differentiation and bone resorption. The dramatic fold change decrease of $-5.44$ of OPG is a striking implication of the dysregulation of osteoclastogenesis and ECM modulating genes in poor-prognosis samples.

Using an unsupervised strategy, xenograft samples clearly clustered with Huvos I/II samples (Fig. 2), concluding that genes identified from osteosarcoma tumors in this preliminary study can be definitively classified Huvos I/II osteosarcoma tumors. We recognize that the validation cohort was limited but the excellent classification ability of this signature using unsupervised analysis coupled with the RT-PCR validation data illustrates a robust signature.

<table>
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<th>GenBank identifier</th>
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<th>Average fold change: Huvos I/II versus III/IV</th>
<th>Quantitative RT-PCR</th>
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<td>AL031058</td>
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<td>$-4.1 \pm 1.22$</td>
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</table>

Table 2. Quantitative RT-PCR confirmation of transcriptional changes of six genes differentially expressed in an independent set of chemotherapy-responsive and nonresponsive osteosarcoma tumors

The tumor-mediated bone remodeling cycle arises from the interaction between the tumor and bone microenvironment. In metastatic breast cancer, tumor cells mediate osteoclast activity through the inhibition of OPG and expression of parathyroid-related peptide. The inhibition of OPG allows for the differentiation of osteoclast multinucleated cells through the interaction of receptor activator of nuclear factor $\kappa$B and its ligand. Osteoclastic activity liberates cytokines and growth factors embedded in the bone matrix. These growth factors, such as TGF-$\beta$, stimulate the tumor cell and facilitate tumor gene expression through SMAD and mitogen-activated protein kinase pathways, resulting in the cyclic mediation of bone resorption.

Multiple genes involved in the osteoclast differentiation signaling pathway were identified among the 910 statistically significant genes. An increased expression of TGF-$\beta$ (P = 0.03; fold increase, 1.6), MAPK (P = 0.02; fold increase, 1.4), and SMAD (P = 0.005; fold increase, 1.6) genes were observed in Huvos I/II tumor samples (Fig. 3; ref. 14). The mitogen-activated protein kinase pathway has been shown to be a major component of SMAD signaling by TGF-$\beta$ osteolytic bone metastases in breast cancer (14).

Additionally, expression of annexin 2 (P = 0.049) and PLA2G2A (P = 0.025), genes indirectly involved in bone resorption, were up-regulated in Huvos I/II samples. Annexin 2 is a $\text{Ca}^{2+}$-dependent phospholipid and membrane-binding protein that enhances osteoclast formation and bone resorption (15). Additionally, PLA2G2A has been implicated in osteoclastic bone resorption (16). Increased levels of PLA2G2A have additionally been associated with various tumors, such as prostate cancer, pancreatic carcinoma, small bowel adenocarcinomas, and colorectal carcinoma (17). More importantly, PLA2G2A has proven to play a crucial role in the production of prostaglandins, potent regulators of bone formation, and resorption. Prostaglandin E$_2$ has been shown to promote osteolysis in response to cytokines and growth factors, such as TGF-$\beta$ (18). Prostaglandin is intricately tied to OPG and receptor activator of nuclear factor $\kappa$B ligand expression and has shown to suppress OPG mRNA expression to stimulate bone resorption (19, 20). Additionally, osteolytic metastases of breast cancer have been shown to be caused by a prostaglandin E$_2$-dependent mechanism (19).
Given these data implicating osteoclasts in tumor growth and therapy resistance, drugs inhibiting osteoclast formation and function may represent a possible novel adjunct therapy for osteosarcoma. Bisphosphonate compounds inhibit osteoclast differentiation and bone resorption by targeting gene transcription and enzyme metabolism in mature osteoclasts, leading to osteoclast apoptosis and loss of bone-resorptive activity (21).

Bisphosphonates have been shown to inhibit osteosarcoma cell lines and tumor growth. Aminobisphosphonate pamidronate has been implicated as a potent inhibitor of osteosarcoma cell line growth. The administration of pamidronate was shown to reduce cell viability and proliferation in seven osteosarcoma cell lines, namely HOS, MG-63, OST, SaOS-2, SJSA-1, U2-OS, and ZK-58. Observed reduction of cell viability occurred in a dose-dependent manner (22). Additional studies conducted with the administration of the aminobisphosphonate pamidronate to the UMR 106-01 clonal rat osteosarcoma cell line showed a decrease in osteosarcoma cell growth, an induction of apoptosis, and an altered expression of osteoclast-regulating factors (23). The bisphosphonate, zoledronic acid, has additionally shown to induce apoptosis in the human osteosarcoma cell lines HOS, BTK-143, MG-63, SJSA-1, G-292, and SAOs2 (24).

Bisphosphonates are approved by Food and Drug Administration to treat bone loss associated by osteoporosis, Paget’s disease, and osteolytic metastases of breast cancer. The potential for bisphosphonates merits further evaluation as an effective tool to decrease osteosarcoma cell growth, both for its proven ability to decrease osteosarcoma cell growth in vitro and its minimal side effects (22).

**Poor-Prognosis Tumors Are Associated with ECM Remodeling.** In addition to osteoclast-stimulating genes, ECM modulating proteins were differentially expressed between Huvos I/II and III/IV tumors. Huvos I/II tumors display a marked increase of genes involved in cell migration, proliferation, and remodeling. For instance, thrombospondin 4 (P = 0.04) is an extracellular binding protein involved in cell proliferation and migration (25). Platelet endothelial cell adhesion molecule (P = 0.027) and perlecain (P = 0.0072) are both implicated in angiogenesis pathways as well as tumor growth promotion (26, 27). Biglycan (P = 0.025) is involved in ECM remodeling through connective tissue metabolism (28), SPARC-like 1 (P = 0.001) is additionally involved in remodeling cellular architecture through the rounding of adherent cells (29).

Poorly responsive tumors display a decrease in genes involved with cytoskeleton stability and homeostasis. For instance, keratin 19 (P = 0.015) and desmoplakin (P = 0.008) function in maintaining cytoskeletal architecture and cell stability (30, 31). Poorly responsive tumors additionally display a decrease in plasminogen activator inhibitor type 1 (P = 0.04), a protein that functions in the inhibition of cell migration and the invasive phenotype of cancer cell lines (32). A decrease in these genes strongly implies an increase in motility in poorly responsive osteosarcoma tumors.

**Poor-Prognosis Tumors Are Associated with Tumor Progression and Apoptosis Resistance.** Analysis of the 104 highly differentially expressed genes revealed functionally diverse genes involved in tumor apoptosis resistance and tumor progression in poorly responsive osteosarcoma tumor samples. Poorly responsive tumors show an up-regulation of two metallothionein compounds, namely metallothioneine 1G (P = 0.045) and metallothioneine 1L (P = 0.007). Metallothioneine, a heavy metal–binding protein class characterized by high cysteine content, is associated with chemotherapeutic resistance. Metallothioneins have been found to play a physiologic role in metabolizing and eliminating cytotoxic heavy metals found in chemotherapy agents commonly used to treat osteosarcoma, such as cisplatin (33).

Additionally, BAX and ICAM2 genes were differentially expressed in Huvos I/II tumors. A decrease in the proapoptotic BAX gene (P = 0.002) is observed in Huvos I/II tumor samples. ICAM2 (P = 0.029) gene was increased in Huvos I/II tumors. ICAM2 has been shown to mediate a block in apoptosis through the phosphatidyl inositol-3-kinase pathway (34).

A number of oncogenic factors were increased in Huvos I/II tumors. RAB4B (P = 0.016), a member of the RAS oncogene family, was highly increased in poor-prognosis samples (fold increase of 5.7). Additionally, pleiotrophin (P = 0.024) expression was increased.

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**Figure 3.** Multigenic mediation of poor histologic response to chemotherapy. This schematic model depicts a hypothesis underlying chemotherapy resistance in osteosarcoma as mediated by several genes differentially expressed between Huvos I/II and III/IV tumors. Poor-prognosis tumors might progress and develop a malignant phenotype through the increased expression of oncogenic factors RAB4B, myc, and pleiotrophin and a decreased expression of proapoptotic BAX. The elevated expression of migration and invasion genes could cooperatively function to alter ECM microenvironment. The decrease in OPG and increase in bone resorption genes could mediate a drive toward osteoclastogenesis. Thereafter, the destruction of bone matrix might release stored growth factors, such as TGF-β, which establish a positive feedback toward tumor progression and ECM remodeling. This model represents a starting point for further hypothesis testing to abrogate the tumor in resistant cases.
in poor-prognosis samples. Pleiotrophin has been implicated in the malignant phenotype of breast and pancreatic cancer (35–37). Additionally, the v-myc oncogene (P = 0.034) and the myc-associated zinc factor (P = 0.006) were increased in Huvos I/II samples by fold increases of 2.43 and 1.82, respectively. Ribosomal protein S2 (P = 0.04), s23 (P = 0.008), L12 (P = 0.014), s27 (P = 0.008), L31 (P = 0.011), L28 (P = 0.008), and s14 (P = 0.007) were up-regulated in poor-prognosis osteosarcoma samples. These ribosomal proteins further implicate the myc oncogene, as these proteins are regulated by myc expression.

Summary

This study has defined a set of 104 genes that classify poor histologic response to chemotherapy in osteosarcoma tumors at the time of diagnosis and validate these findings in a small independent cohort of primary and xenograft tumors. While speculative, we hypothesize that chemotherapy-resistant osteosarcoma tumors mediate tumor growth and survival through altered proteolytic mechanisms that function in a multiform fashion to mediate osteoclast activation, tumor survival, and modified ECM environment. Although further investigation is required to validate this proposed model, the defined differentially expressed genes raise the possibility that the use of bisphosphonate analogues should be considered a testable hypothesis as potential therapeutic interventions to suppress the bone remodeling and tumor osteolysis involved in osteosarcoma chemotherapy resistance.

References

11. Kakonen SM, Selander KS, Chirgwin JM, et al. Transforming growth factor-β stimulates parathy-
roid-hormone-related protein and osteolytic metas-
tases via Smad and mitogen-activated protein 
kine signaling pathways. J Biol Chem 2002;277: 
24571–8.
269:2966–971.
glandin E2-mediated bone resorption associated with inflam-
ma
cov
15. Krieger NS, Bushinsky DA, Frick KK. Cellular 
mechanisms of bone resorption induced by metabolic 
ac
d
17. Okada Y, Pilbeam C, Rai
d s L, Tanaka Y. Role of 
cyc
ci
toxygenase-2 in bone resorption. J Urool 2003;265:
185–95.
18. Brown JE, Neville-Webbe H, Coleman BE. The role of 
bi
siphosphonates in breast and prostate cancers. Endocr 
19. Sonnemann J, Eckert SF, Truchtenbrod B, Boos J, 
20. Winkelmann W, van Valen F. The bisphosphonate 
ana
gues should be considered a testable 
hypothesis as potential therapeutic interventions to 
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References

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