Gene Expression Profiles Classify Human Osteosarcoma Xenografts According to Sensitivity to Doxorubicin, Cisplatin, and Ifosfamide

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Abstract

Purpose: In osteosarcoma, aggressive preoperative and postoperative multidrug chemotherapy given to all patients has improved patient survival rate to the present level of ~60%. However, no tumor marker is available that reliably can identify those patients who will or will not respond to chemotherapy.

Experimental Design: In an attempt to find leads to such markers, we have obtained microarray gene expression profiles from a panel of 10 different human osteosarcoma xenografts and related the results to their sensitivity to ifosfamide, doxorubicin, and cisplatin.

Results: The expression data identified genes with highly significant differential expression between poor and good responder xenografts to the three different drugs: 85 genes for doxorubicin, 74 genes for cisplatin, and 118 genes for ifosfamide. Technical validation with quantitative reverse transcription-PCR showed good correlation with the microarray expression data. Gene Ontology–guided analysis suggested that properties of the poorly responsive xenografts were resistance to undergo programmed cell death and, particularly for ifosfamide, a drive toward dedifferentiation and increased tumor aggressiveness. Leads toward metabolic alterations and involvement of mitochondrial pathways for apoptosis and stress response were more prominent for doxorubicin and cisplatin. Finally, small interfering RNA–mediated gene silencing of IER3 and S100A2 sensitized the human osteosarcoma cell line OHS to treatment with 4-hydroperoxyifosfamide.

Conclusions: The expression profiles contained several novel biomarker candidates that may help predict the responsiveness of osteosarcoma to doxorubicin, cisplatin, and ifosfamide. The potential of selected candidates will be further validated on clinical specimens from osteosarcoma patients. (Clin Cancer Res 2009;15(23):OF1–9)

Chemotherapy has clearly improved the outcome for patients with osteosarcoma. For patients with resectable primary tumors and no overt metastatic disease at diagnosis, long-term survival rates of ~70% can be achieved compared with the ~20% cure rate previously obtained by surgery alone (1, 2). One of the most powerful prognostic indicators is the degree of necrosis in the primary tumor induced by preoperative chemotherapy (3, 4). Current treatment regimens usually involve neoadjuvant and adjuvant chemotherapy with high-dose methotrexate, doxorubicin, cisplatin, and, more recently, ifosfamide. However, a plateau for what may be achieved with current drugs with known activity in osteosarcoma has been reached (5–7). Moreover, for osteosarcoma patients treated with the current highly aggressive chemotherapy regimens, acute and long-term toxicity is a significant problem, and it is likely that some of the patients with micrometastatic disease could be cured with less aggressive regimens (8). Hence, biomarkers that could identify chemoresistant tumors already at the time of primary biopsy would potentially benefit substantially to the management of osteosarcoma. Microarray approaches have extensively been used in many tumor types in attempts to identify biomarkers for further clinical validation. In osteosarcoma, relatively few such studies have been reported, presumably reflecting that osteosarcoma is a relatively rare disease for which it is difficult to obtain sufficient tumor tissue from untreated patients. Recently, however, interesting results have been obtained on pretreatment biopsied samples and on tumor tissue obtained during surgery after preoperative chemotherapy (9–11). However, these studies cannot access genes related to individual drugs using patient’s specimens. We have previously established a panel

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Received 11/2/08; revised 7/23/09; accepted 8/25/09; published OnlineFirst 11/17/09.

Grant support: The Norwegian Cancer Society and Cancer Genomics Laboratory startup fund of Mitchell Cancer Institute (J. Ju).

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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of osteosarcoma xenografts for the purpose of preclinical testing of novel drug candidates to treat osteosarcoma (12). Here, we report the results of microarray expression profiling of these xenografts to screen for potential markers for each of the three drugs used in current clinical treatment protocols, resulting in three different subsets of genes that with high significance distinguished between poor and good responders to doxorubicin, cisplatin, and ifosfamide. The advantage of our approach permitted comparison of the effects of three commonly used drugs individually to the expression profile for each of them, which is impossible using clinical specimens.

Materials and Methods

Animals, xenografts, and assessment of chemotherapy response. All procedures involving animals were done according to protocols approved by the National Animal Research Authority and conducted according to the European Convention for the Protection of Vertebrates Used for Scientific Purposes. Female BALB/c nu/nu mice bred at the nude rodent facility at the Norwegian Radium Hospital were used.

The animals were maintained, and experiments and assessment of chemotherapy response were conducted as described previously (12). In brief, the animals were 4 to 6 weeks old at the day of tumor implantation. Anesthesia was obtained with intraperitoneal injection of 0.1 mg/kg fentanyl, 5 mg/kg fluanisone, and 2.5 mg/kg midazolam. Tumor fragments (2 × 2 × 2 mm) were implanted subcutaneously in both flanks of nude mice. The animals were randomized for treatment according to tumor size when the average tumor diameters were ~6 mm. Each experiment contained four subgroups of mice that were treated intravenously with saline (control), 8 mg/kg doxorubicin (Nycomed Pharma), 5 mg/kg cisplatin (Platinol; Bristol-Myers Squibb), or 240 mg/kg ifosfamide (Holoxan; Asta Medica). In addition, tumors were harvested for the purpose of microarray gene expression analysis from a fifth subgroup of animals on day 0. The antitumor activity of each drug was quantified calculating the specific growth delay (SGD) after one and two relative tumor volume doubling times (SGD200 or SGD400) and the maximal growth inhibition (T/C%).

Ten human osteosarcoma xenografts were used, eight of which have been described previously (12), and in addition, two newly established xenografts were included. These originated from a 32-year-old male with an extremity located fibroblastic osteosarcoma of malignancy grade 4 (GKAMX) and from a 13-year-old female with an extremity located osteoblastic osteosarcoma of malignancy grade 4 (CTPX).

Sample preparation, array hybridization, and gene expression analysis. Frozen xenografts were grinded by a stainless-steel mortar and pestle under liquid nitrogen. The tissue powder was then transferred to a conical tube and total RNA was isolated using the Trizol reagent (Invitrogen) followed by DNase treatment for 30 min at 37°C (RNase-free DNase; Promega). CodeLink UniSet Human 20K Oligo Bioarray (GE Healthcare), containing ~20,000 gene probes, was used to generate gene expression profiles. All reagents were provided in the CodeLink expression assay kit (GE Healthcare), except where noted. cRNA synthesis was done as per manufacturer’s instructions. Using 2 μg total RNA, first-strand cDNA was generated by reverse transcriptase and a T7 primer. Subsequently, second-strand cDNA was produced using DNA polymerase I and RNase H. The resulting double-stranded cDNA was purified on a QiAquick column (Qiagen) and then fragmented using an in vitro transcription reaction using T7 RNA polymerase and biotin-11-UTP (Perkin-Elmer) at 37°C for 14 h. cRNA was purified on a RNasey column (Qiagen) and quantified by UV spectrophotometry, and 10 μg biotin-labeled cRNA was then fragmented by heating at 94°C for 20 min in the presence of magnesium buffer. The fragmented cRNA was hybridized overnight at 37°C in hybridization buffer to a UniSet Human 20K Bioarray in a shaking incubator at 300 rpm. After hybridization, the arrays were washed in 0.75× TNT buffer [1× TNT buffer: 0.1 mol/L Tris-HCl (pH 7.6), 0.15 mol/L NaCl, 0.005 mol/L sodium pyrophosphate, and 0.0025 mol/L sodium deoxycholate] with 0.1% SDS and then stained with streptavidin−biotin−alkaline phosphatase

Table 1. Response of human osteosarcoma xenografts to doxorubicin, cisplatin, and ifosfamide

<table>
<thead>
<tr>
<th>Xenograft</th>
<th>TD</th>
<th>SGD200</th>
<th>SGD400</th>
<th>T/C%</th>
<th>Efficacy</th>
<th>Response groups in expression analysis</th>
</tr>
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<tbody>
<tr>
<td>OHS</td>
<td>4</td>
<td>0.4</td>
<td>0.4</td>
<td>62</td>
<td>+++</td>
<td>Poor</td>
</tr>
<tr>
<td>CTPX</td>
<td>7</td>
<td>2.9</td>
<td>2.9</td>
<td>10</td>
<td>+++++</td>
<td>Good</td>
</tr>
<tr>
<td>SBX</td>
<td>6.5</td>
<td>0.8</td>
<td>1.7</td>
<td>28</td>
<td>++</td>
<td>Good</td>
</tr>
<tr>
<td>ALSKX</td>
<td>11</td>
<td>0.7</td>
<td>0.7</td>
<td>52</td>
<td>++</td>
<td>Good</td>
</tr>
<tr>
<td>AOX</td>
<td>10</td>
<td>1</td>
<td>0.5</td>
<td>58</td>
<td>++</td>
<td>Poor</td>
</tr>
<tr>
<td>TTX</td>
<td>6</td>
<td>4.8</td>
<td>1.7</td>
<td>17</td>
<td>+++</td>
<td>Good</td>
</tr>
<tr>
<td>GKAMX</td>
<td>4</td>
<td>0.9</td>
<td>1.5</td>
<td>25</td>
<td>++</td>
<td>Good</td>
</tr>
<tr>
<td>TFX</td>
<td>13</td>
<td>0.1</td>
<td>0.1</td>
<td>83</td>
<td>++</td>
<td>Poor</td>
</tr>
<tr>
<td>TSX pr1</td>
<td>4.5</td>
<td>0.3</td>
<td>0.2</td>
<td>71</td>
<td>++</td>
<td>Poor</td>
</tr>
<tr>
<td>KPDX</td>
<td>6</td>
<td>0.9</td>
<td>0.8</td>
<td>47</td>
<td>++</td>
<td>Poor</td>
</tr>
</tbody>
</table>

Abbreviations: TD, tumor doubling time; T/C%, maximal growth inhibition; SGD, specific growth delay.
NaCl, and 0.05% Tween 20] at 46°C for 1 h followed by incubation with Cy5-streptavidin (GE Healthcare) at room temperature for 30 min in the dark. Arrays were then washed in 1 × TNT four times for 5 min each. The slides were then dried by centrifugation and kept in the dark until scanning. Images were captured on an Axon GenePix 4200A scanner. The resulting image was quantified and the intensity of each spot was divided by the median spot intensity to provide a scaled and comparable number across multiple arrays. After dot grid and QC, CodeLink software generates export files for analysis by GeneSpring software 7.2 (Agilent).

Assessment of total RNA and cRNA concentration, purity, and quality. The concentrations of both total RNA and cRNA were determined by measurement of absorbance at 260 nm (A260) using Na-nodrop spectrophotometer. Only samples with A260:A280 ratio >1.8 were regarded as satisfactory pure. The quality of total RNA and cRNA was also evaluated by gel electrophoresis.

Quantitative reverse transcription-PCR. For real-time quantitative reverse transcription-PCR (qRT-PCR), the SYBR Green master mix from Applied Biosystems was used. qRT-PCR was done on an ABI 7500HT instrument under the following conditions: 25°C for 10 min and 37°C for 2 h for reverse transcription. Amplification was performed for 5 min initial denaturation at 95°C for 40 cycles of 15 s denaturation and 1 min annealing extension at 37°C. The gene expression C_v value from each sample was calculated by normalizing with internal housekeeping gene GAPDH and relative quantization values were plotted. qRT-PCR primers were purchased from Integrated DNA Technologies and Eurogentec (Belgium; S100A2). For the individual reactions, primers sequences listed in Supplementary Table S1 were used.

Cell culture and small interfering RNA gene silencing. The human osteosarcoma cell line OHS was routinely maintained in RPMI 1640 (BioWhittaker) supplemented with 10% fetal bovine serum (PAA Laboratories) and 2 mmol/L glutamine (Life Technologies). The following small interfering RNA (siRNA) sequences were purchased from Ambion: S100A2 (ID:17214) 5'-CAGGAAAAACACCAUACUCG-3' and IER3 (ID:6661) 5'-AGCAGGACGAGAGAAAGCCtt-3'. Silencer Negative Control #1 siRNA was used as a scrambled control. The effects of gene silencing with siRNA on sensitivity to 4-hydroperoxysfamide (D-18851; Universität Bielefeld) were assessed by the clonogenic assay. Briefly, OHS cells were seeded in 6-well plates (Costar 3516; Corning) and incubated overnight to achieve a confluency of 30% to 50%, before transfections with siRNA, using the Lipofectamine 2000 (Invitrogen) reagent according to the manufacturer's protocol, using 1 μg/mL Lipofectamine 2000 and 30 mmol/L siRNA. After transfections, the cells were incubated for 24 h and trypsinized and counted. Known numbers were then replated in triplicate wells and incubated for 24 h, allowing the cells to attach before treatment with indicated concentrations of 4-hydroperoxysfamide and finally incubated for 7 days to allow macroscopic colony development. Colonies were counted and the platting efficiency and surviving fraction for given treatments were calculated based on the survival of cells treated with 4-hydroperoxysfamide compared with vehicle-treated controls. 4-Hydroperoxysfamide was dissolved in water immediately before use to obtain stock concentrations that were added to the growth medium at a 1:30 ratio.

Western blot. Preparation of whole-cell lysates was done by addition of lysis buffer [150 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 7.5), 0.1% NP-40, 10 μg/mL of leupeptin hemisulfate, aprotinin, and pepstatin A, 20 mmol/L μ-glycerophosphate, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L sodium orthovanadate, and 100 mmol/L sodium fluoride] to dry cell pellets, left on ice for 15 min, sonicated, and frozen at -80°C. Upon use, the samples were centrifuged to remove cell debris. Total protein lysates from each sample (100 μg) was separated on 8% to 12% SDS-PAGE and transferred onto Immobilon-P membranes (Millipore) according to the manufacturer's manual. After blockage of nonspecific binding sites with 10% nonfat dry milk in TBS with 0.25% Tween, blots were incubated for 1 h at room temperature with a mouse monoclonal anti-S100A2 (clone SH-L1; Sigma), a rabbit polyclonal anti-IER3 (Abcam), or a mouse monoclonal anti-α-tubulin antibody (0.3 μg/mL; Oncogene Research Products). After washing, the blots were incubated for 1 h at room temperature with a rabbit anti-mouse or a goat anti-rabbit secondary antibody (DAKO) diluted 1:4,000. Signals were visualized using the enhanced chemiluminescence substrate (Amersham Pharmacia Biotech).

Statistical analysis. GeneSpring version 7.2 was used for normalization, filtering, statistical and Gene Ontology (GO) analysis of the microarray data, and hierarchical clustering of the selected genes. The expression data for each of the hybridizations were first normalized to their global median. Then, the expression of each gene was normalized to its median. Before the statistical analysis, genes flagged present in less than half of the conditions and that had an average expression signal below 200 in at least one of the groups were filtered out of the data sets. To identify genes with a high differential expression among the two groups analyzed, a one-way ANOVA test with a Benjamini and Hochberg multiple test correction allowing for a given false discovery rate of was done. Hierarchical clustering was done using the standard correlation option of the GeneSpring clustering tool. Finally, the integrated GeneSpring GO browser was used for category enrichment analysis.

Results

Response of human osteosarcoma xenografts to doxorubicin, cisplatin, and ifosfamide. Table 1 shows the classification of human osteosarcoma xenografts according to their response to doxorubicin, cisplatin, and ifosfamide. For analysis of the microarray expression data, we grouped the xenografts as good
Heat maps for genes that classify human osteosarcoma xenografts according to chemosensitivity. Levels of expression represent mean values from triplicate array hybridization and are presented as below (green) or above (red) the median expression of each gene (black). Blue, genes that were flagged absent in the representing arrays. These lists of genes were selected by a one-way ANOVA analysis with a Benjamini-Hochberg multiple test correction allowing false discovery rates of 5% for doxorubicin (A; 85 genes), 2% for cisplatin (B; 74 genes), and 0.5% for ifosfamide (C; 118 genes). GenBank accessions and full gene names are listed in Supplementary Table S2A to C.

Fig. 1. Heat maps for genes that classify human osteosarcoma xenografts according to chemosensitivity. Levels of expression represent mean values from triplicate array hybridization and are presented as below (green) or above (red) the median expression of each gene (black). Blue, genes that were flagged absent in the representing arrays. These lists of genes were selected by a one-way ANOVA analysis with a Benjamini-Hochberg multiple test correction allowing false discovery rates of 5% for doxorubicin (A; 85 genes), 2% for cisplatin (B; 74 genes), and 0.5% for ifosfamide (C; 118 genes). GenBank accessions and full gene names are listed in Supplementary Table S2A to C.
(+++++/+++ or poor -/-) responders. Four of the xenografts responded well doxorubicin, three to cisplatin, and five to ifosfamide. The sensitivity profiles for doxorubicin and cisplatin were similar with only one cisplatin-resistant xenograft responding well to doxorubicin. The sensitivity profile for ifosfamide was somewhat different from the two other drugs, with overall five responding xenografts, but two of the xenografts that responded poorly were sensitive to doxorubicin and cisplatin.

Differentially expressed genes. Expression profiles established from the 10 human osteosarcoma xenografts were based on mean values from triplicate hybridizations. When filtering was done as described in Materials and Methods, ∼3,600 genes were found eligible for statistical analysis. With a false discovery rate of 5%, 85 genes were identified as differentially expressed between good and poor responders to doxorubicin. Of these, 48 were expressed higher and 37 lower in the poorly responsive xenografts compared with those that responded well (Fig. 1A). Seventy-four genes were identified as differentially expressed between cisplatin poor and good responders (false discovery rate = 2%), 38 of which were expressed higher and 36 were lower in the poorly responsive group (Fig. 1B). For ifosfamide, 118 genes were identified as differentially expressed between poor and good responders (false discovery rate = 0.5%), and 67 and 51 of these were higher and lower expressed in the poorly responsive xenografts, respectively (Fig. 1C). Lists for all three expression profiles including full gene names and accession numbers are shown in Supplementary Table S2A to C. For all three expression profiles, clustering of significant genes resulted in two distinct groups in which poor or good responder xenografts clustered together. Among the genes that showed significant differential expression between xenografts that responded well or poorly to cisplatin, 21 were overlapping with the comparable gene list for doxorubicin. In contrast, only 2 genes were common in the expression profiles for cisplatin and ifosfamide and none when comparing the profiles for doxorubicin and ifosfamide.

qRT-PCR validation of selected genes. Ten of the genes identified as differentially expressed in poor or good responding osteosarcoma xenografts to doxorubicin, cisplatin, or ifosfamide were selected for validation of the microarray data by qRT-PCR: FABP3 and TGFBI (doxorubicin); DDP7, NCALD, and SSX1 (doxorubicin and cisplatin); and IER3, GADD45A, UBE2L6, EPB41L3, and ALPL (ifosfamide). The expression levels for these genes assessed by qRT-PCR confirmed the microarray data, illustrated as fold differences between poor and good responders for selected genes for each drug (Table 2). Moreover, there were significant correlations between results obtained by the two methods for all 10 genes (r = 0.71-0.98; P ≤ 0.02).

GO categories enriched of differentially expressed genes. GO categories that were enriched with a significance level of P < 0.05 for genes with either significantly higher or lower expression in poorly compared with good responding xenografts are shown in Supplementary Table S3. Categories containing altogether five or more genes are included. For xenografts responding poorly to doxorubicin, selective enrichment of genes with significantly higher expression in poor responders was found in ontology categories related to energy metabolism and mitochondrion and the ontology category response to abiotic stimulus. In contrast, extracellular region was the only the category that, with convincing selectivity, was significantly enriched by genes with low expression in doxorubicin poor responders. This category constituted genes that also received the annotations extracellular matrix (n = 4) and/or skeletal development (n = 3). For cisplatin, categories selectively enriched by genes upregulated in poorly responding xenografts to cisplatin were RNA metabolism, hydrolase activity, cytoplasm, and endoplasmic reticulum. As for doxorubicin genes with mitochondrial annotation were upregulated in poor responder xenografts, although the enrichment scores did not reach significance (n = 4; P = 0.11). For ifosfamide, genes with high expression in poor responders were most strongly overrepresented in the category of negative regulation of physiologic process, apoptosis, calcium ion binding, and microtubule cytoskeleton. The GO categories most significantly enriched by genes with lower expression in poorly responding xenografts were skeletal and organ development. Many of the same genes were also found in two other significant categories: regulation of transcription and extracellular region.

Functional validation of selected genes. To investigate whether any of the identified genes could be functionally related to chemotherapy resistance, siRNA-mediated gene silencing of two genes that were significantly higher expressed in xenografts resistant to ifosfamide, S100A2 and IER3, were done in the human osteosarcoma OHS cell line. Effects on sensitivity to 4-hydroperoxycisplatin, which is the active metabolite of ifosfamide, were then assessed by the clonogenic assay. siRNA reduced transcript levels of S100A2 to ~5% of the levels in control untransfected OHS cells as assessed by qRT-PCR 24 h after transfections (Fig. 2A). At the protein level, the expression of S100A2 was reduced 48 h after transfection and almost abolished after 96 h (Fig. 2A). For IER3, transcript levels were only reduced ~50% by the transfected siRNA and a transient reduction in protein level was seen after 48 h (Fig. 2B). For IER3, the

Table 2. Fold difference in expression levels of selected genes differentially expressed in poor and good responding xenografts to doxorubicin, cisplatin, or ifosfamide

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold difference (poor responders/good responders)</th>
<th>Microarray</th>
<th>qRT-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxorubicin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FABP3</td>
<td>2.8</td>
<td>3.7</td>
<td></td>
</tr>
<tr>
<td>NCALD</td>
<td>-3</td>
<td>-5.7</td>
<td></td>
</tr>
<tr>
<td>TGFBI</td>
<td>-13.8</td>
<td>-13.3</td>
<td></td>
</tr>
<tr>
<td>SSX1</td>
<td>-16.6</td>
<td>-3336</td>
<td></td>
</tr>
<tr>
<td>Cisplatin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DDP7</td>
<td>3.7</td>
<td>7.1</td>
<td></td>
</tr>
<tr>
<td>NCALD</td>
<td>-5.5</td>
<td>-8.9</td>
<td></td>
</tr>
<tr>
<td>SSX1</td>
<td>-4.7</td>
<td>-14.2</td>
<td></td>
</tr>
<tr>
<td>Ifosfamide</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IER3</td>
<td>11.7</td>
<td>19.3</td>
<td></td>
</tr>
<tr>
<td>UBE2L6</td>
<td>10.5</td>
<td>9.3</td>
<td></td>
</tr>
<tr>
<td>GADD45A</td>
<td>4.3</td>
<td>11.9</td>
<td></td>
</tr>
<tr>
<td>ALPL</td>
<td>-40.8</td>
<td>-36</td>
<td></td>
</tr>
<tr>
<td>EPB41L3</td>
<td>-52.4</td>
<td>-176</td>
<td></td>
</tr>
</tbody>
</table>

NOTE: Comparison of microarray and qRT-PCR results.
prominent bands detected by Western immunoblot analysis correspond to a nonglycosylated form of 17 kDa and a glycosylated form of 26 to 29 kDa, previously published by others (13, 14). Gene silencing with siRNA to S100A2 or a scrambled negative control significantly reduced colony formation of OHS cells compared with untransfected cells (bars, SD, duplicates); right, Western blot showing protein levels of S100A2 after siRNA-mediated transcript knockdown. Recombinant His-tagged S100A2 was used as a positive control. B, left, transcriptional levels of IER3 after siRNA-mediated transcript knockdown. Two dominant bands are observed, a nonglycosylated form of ∼17 kDa and a glycosylated form of ∼26 kDa. C, silencing of IER3 but not S100A2 or a scrambled negative control reduces colony formation of OHS cells (bars, SE, 3 experiments). D, silencing of IER3 and S100A2 sensitizes OHS cells to 4-hydroperoxyifosfamide (bars, SE, 3 experiments).

**Discussion**

In osteosarcoma, the introduction and optimization of aggressive preoperative and postoperative chemotherapy has decisively improved patient survival. However, a significant number of patients have tumors resistant to the drugs routinely used and hence receive a tough and toxic treatment without any therapeutic benefit. There is an obvious need for markers that predict tumor response and resistance, and gene expression profiling might provide promising leads that can be validated for their clinical potential. One key obstacle for performing such studies on clinical specimens is the difficulty in obtaining pretreatment osteosarcoma samples in an amount and quality...
needed for such studies, and because the patients receive multidrug chemotherapy, genes involved in resistance to the individual drugs cannot readily be identified. Our approach of using osteosarcoma xenografts with different sensitivities to clinically used drugs circumvents these problems. Outliers were selected with low false discovery rates; expression assessed with qRT-PCR correlated tightly with the array data.

We are aware of the fact that even a large panel of 10 xenografts may not cover the range of biological characteristics of clinical osteosarcoma. Moreover, from a statistical point of view, it is a relatively low number of tumors, and the reproducibility of results in xenografts with low or intermediate sensitivity is therefore of concern. Thus, the selection of genes that correlate with response to chemotherapy could change markedly if only one xenograft moved from one response category to the other. Future studies of this type should therefore be conducted in xenografts with either very high or very low sensitivity to the drug studied as was the case here for ifosfamide. However, we still feel that our xenograft panel represents a valuable tool for identifying new prognostic markers, and the present results support this conclusion. First, some of the identified genes have been shown previously to correlate with prognosis or chemotherapy response in osteosarcoma. Secondly, GO-guided analysis of the gene lists obtained identified genes and groups of genes that can be associated with known functions related to mechanisms of drug sensitivity and resistance.

Third, gene silencing of two of the identified genes with significantly enhanced expression in ifosfamide poor responder xenografts, IER3 and S100A2, sensitized human osteosarcoma OHS cells to treatment with 4-hydroperoxyifosfamide. Previously, osteosarcoma gene expression profiles that characterize poor or good responder osteosarcomas using clinical specimens from patients have been reported. Some of the genes identified in these studies were also found in our lists, such as MAGED1 (9), IER3, PAI1, and AXN2A (11), and HSP70 and MCM2 (10). Moreover, HSP27 and HSP70, which was associated with poor response to doxorubicin and cisplatin in our xenograft panel, have been shown previously to correlate with poor response to neoadjuvant chemotherapy when assessed by immunohistochemistry (15–17). In the previous studies, the combined effect of several drugs used in preoperative regimens was assessed by the degree of tumor necrosis at the time of surgery. The advantage of our approach permitted comparison of the effects of three commonly used drugs individually to the expression profile for each of them, which is impossible using clinical specimens. The advantage was clearly shown here in that it gives a better insight in the molecular mechanisms underlying resistance to the individual drugs, thereby providing the possibility to identify leads to genes and pathways that may be targeted therapeutically in addition to detect markers of putative prognostic value.

GO enrichment analysis was done to identify possible groups of genes associated to the chemoresistant phenotype. For doxorubicin, the most striking finding was that mitochondrial location and ontology categories related to energy metabolism were selectively enriched by genes with higher expression levels in xenografts responding poorly. This finding may link the lack of doxorubicin response to mitochondrial malfunctions or tumor hypoxia, phenomena with a well-established association to drug resistance (18–21). Moreover, the GO category response to abiotic stimulus contains genes that may interplay to inhibit mitochondrial/intrinsic apoptotic pathways, such as the heat shock proteins HSP27 and HSP70 (22), Bcl-2–associated athanogene 2 (23, 24), and SRI (Sorcin). Sorcin predicts poor outcome and response to chemotherapy in acute myeloid leukemia and may therefore be of particular interest (25, 26). Conversely, the category extracellular region was significantly and selectively enriched by genes downregulated. These genes included COL1A2, INHBA, and MGP, which are involved in skeletal development, and TGFBI, which is a cell adhesion molecule that can inhibit differentiation and promote apoptosis and chemosensitivity (27–29).

Genes with high expression in cisplatin poor responders were enriched by genes annotated to the endoplasmatic reticulum. One of these, RTN3, is an endoplasmic reticulum stress response gene that has been reported to facilitate the antiapoptotic activity of Bcl-2 (30). Further, two proapoptotic genes, PHLDA1 and PHLDA1, were strongly downregulated. PHLDA1 causes cellular accumulation of ceramide, which is an important second messenger of apoptosis (31), whereas downregulation of PHLDA1 contributes to apoptotic resistance in malignant melanoma (32). Interestingly genes that overlapped with the profile for doxorubicin include HSP70 as well as DPP7, which has been described as an important regulator of apoptotic threshold in resting lymphocytes (33).

For ifosfamide, genes involved in apoptosis regulation were only identified in the group of genes that had higher expression in xenografts that responded poorly (AXN4, Aven, IER3, GADD45A, and SH3KBP1). In addition, at least two other highly deregulated genes, FHL2 and MLF1, can promote survival through transcriptional regulation (34–37). This suggests that antiapoptotic mechanisms might be involved in mediating resistance to ifosfamide. Taken together, genes involved in development and transcriptional regulation and annotated to the category extracellular region indicate that poorly responding xenografts are characterized by dedifferentiation, tumor aggressiveness, and invasiveness. Genes with lower expression are involved in transforming growth factor–β signaling and osteoblastic and bone differentiation, including BMP4, MADH16, and ID3 (transforming growth factor–β pathway), MSX2 (38), MEF2C (39), COL1A2 (40), and ALPI. Conversely, genes with higher expression include the two transcription regulators CITED1 and TCF8 (41, 42) and three proteolytic factors PLAU (uPA), SERPINE1 (PAI-1), and CTS (cathepsin L), associated with tumor invasion and metastasis.

To further validate our findings, we performed siRNA-mediated gene silencing on two selected genes, differentially expressed between ifosfamide poor and good responder xenografts, IER3 and S100A2. Knockdown of each of these genes gave a modest but significant degree of chemosensitization. This is likely to reflect the notion that a network of genes, rather than a single gene, drives the chemoresistant phenotype. IER3, initially characterized as a radiation-inducible early response gene (43), exerts positive or negative regulation of apoptosis depending on the cell type (14, 44–49). We have shown that IER3 correlated inversely with response to ifosfamide in osteosarcoma xenografts and that a partial and transient silencing of IER3 sensitized osteosarcoma cells to ifosfamide and significantly impaired the ability of OHS cells to form colonies in vitro (11). Seemingly in contrast to this, the clinical study by Mintz et al. (11) showed that IER3 was overexpressed in biopsies from tumors with good histologic response. Because the preoperative...
chemotherapy in that study consisted of methotrexate, doxorubicin, and cisplatin, the findings are not necessarily contradictory to ours, as the effects of different drugs were used.

In summary, we have obtained gene expression profiles from a panel of osteosarcoma xenografts that in parallel were tested for their sensitivity to doxorubicin, cisplatin, and ifosfamide. Although the limitations of this approach are appreciated, it has produced detailed data that are practically impossible to obtain on osteosarcoma biopsies from chemotherapy-naïve patients. The xenografts showed a similar response to doxorubicin and cisplatin, whereas their chemosensitivity profile to ifosfamide was somewhat different. Despite these differences, the common features of the poorly responsive phenotype seem to be a drive toward dedifferentiation, tumor aggressiveness, and resistance to undergo programmed cell death. Moreover, we have shown that silencing of the S100A2 and IER3 genes sensitizes osteosarcoma cells to ifosfamide. The gene expression profiles here presented contain several novel biomarker candidates that may be used to predict the responsiveness of osteosarcoma to doxorubicin, cisplatin, and ifosfamide. The validity and potential of selected candidates will be studied in clinical specimens obtained from osteosarcoma patients.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Acknowledgments
We thank Gunhild M. Maelandsmo for helpful advice and Tove Øyjord, Thomas Halvorsen, and Marita Martinsen for excellent technical assistance.

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23. Thomas Halvorsen, and Marita Martinsen for excellent technical assistance.

Clin Cancer Res 2009;15(23) December 1, 2009 OF8 www.aacrjournals.org
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Clin Cancer Res  Published OnlineFirst November 17, 2009.

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