Identification of Potential Chemoresistance Genes in Osteosarcoma

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Abstract. *Background: Osteosarcoma (OS) is an aggressive bone malignancy that primarily affects children and adolescents. Patients with metastatic disease at diagnosis have only a 20% survival rate. The poor survival rate of these patients is largely due to their lack of responsiveness to chemotherapy. However, the mechanisms underlying osteosarcoma chemoresistance remain unknown. Materials and Methods: The effect of cisplatin, doxorubicin and etoposide was examined on OS cell lines. Affymetric Genechip analysis was used to examine differential gene expression. Results: A correlation between increasing metastatic potential and increasing chemoresistance was observed in the MG-63 cell line and sub-line model. Microarray analysis of these cell lines revealed the differential expression of several genes potentially involved in chemoresistance including ABCG2, ADD3, NMT2, WNT5a and PTN. Conclusion: The identification of genes contributing to chemoresistance and determining the role these genes play is critical in characterizing patient responsiveness and overcoming chemoresistance in osteosarcoma patients.*

Osteosarcoma (OS) is the most common type of primary malignant bone tumor in children and adolescents. Biologically, OS is characterized by the presence of malignant osteoid-producing spindle cells and is an extremely aggressive disease that is associated with a high degree of metastasis, most frequently to the lung (1). In fact, 15-20% of patients present with radiographically detectable metastatic lesions at the time of diagnosis and 80% of patients with localized tumors have microscopic foci at the time of initial presentation (2). Consequently, patients have historically been faced with a poor prognosis.

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Prior to the 1970's, the only treatment option available for OS involved amputation and/or radiation therapy with two-year survival rates averaging only 15-20% (1). Fortunately, a number of clinical studies demonstrated the benefits of multi-agent neo-adjuvant chemotherapy (3-5) and using this regimen five-year survival rates now average 70% for patients who present with non-metastatic disease. Despite these advances in treatment and survival, the addition of chemotherapy to surgical resection has been unable to improve the outcome of patients who present with metastatic disease (1). The poor survival rate of patients with metastatic disease is largely due to lack of responsiveness to chemotherapy and raises the question of whether these patients have intrinsic gene expression differences, or whether the cells that possess the ability for metastasis also have increased chemoresistance (7). Similarly, a number of patients with non-metastatic disease also initially respond poorly (<90% tumor necrosis) to the OS chemotherapeutic regimen. This group of patients is known to be at a higher risk of disease relapse and to have an overall poorer outcome even following complete primary tumor resection (6). These results suggest that these patients may also have intrinsic gene expression differences among their tumor cells, which may account for their lack of chemoresponsiveness. Yet, the exact mechanisms underlying poor responsiveness of OS patients to chemotherapy remain unknown.

Within the last few years, several different approaches have been used to investigate the mechanisms involved in OS chemoresistance. One method that appears to be gaining increasing popularity is gene expression profiling via microarray analysis. For example, Mintz *et al.* (7) performed expression profiling of 30 OS patients, 15 good responders to a 10 week treatment with doxorubicin, cisplatin and high dose methotrexate and 15 poor responders. Over one hundred genes were found to be differentially expressed between these two groups of patients with most genes found to be involved in either extracellular matrix microenvironment remodeling, or osteoclast differentiation.

Man *et al.* (8) performed gene expression profiling of 34 OS samples and identified 45 genes that could be used to predict and distinguish between good and poor chemotherapeutic responders. Additionally, Ochi *et al.* (9) compared the expression profiles of 6 responders and 7 poor responders and identified 60 genes which were potentially correlated to patient chemotherapeutic response. Thus, gene expression profiling appears to be a very valuable tool for the identification of genes involved in OS chemoresistance. However, if the identified genes in all three studies are compared it is evident that no common genes were found between these studies. Consequently, we were interested in performing microarray analysis of cell lines with differential chemoresponsiveness in order to determine whether we could identify any of the differentially expressed genes that were reported in the previous studies. Identification of common gene expression differences in tumor tissues and in established cell lines *in vitro* may aid in the characterization of mechanisms involved in OS chemoresistance.

Materials and Methods

Cell lines and reagents. The human OS cell lines SAOS-2 (HTB-85), U2OS (HTB-96), HOS, MNNG and 143B cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). LM5 cells were kindly provided by E.S. Kleinerman (M.D. Anderson Cancer Center, Houston, TX, USA). Hu09, Hu09 L13 and Hu09 H3 cells were provided by Dr. M. Tani (National Cancer Center Hospital, Tokyo, Japan), MG-63 cells were provided by Dr. G. Sarkar (Mayo Clinic, Rochester, MN, USA) and MG-63 M6 and MG-63 M8 cells were provided by Dr. W.T. Zhu (Tongji Hospital, Huazhong University of Science and Technology, Wuhan, China). All cell lines, except Hu09, Hu09 L13 and Hu09 H3 were cultured in Dulbecco's modified Eagle's medium (4.5 g/l glucose)/Ham F12 (1:1) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (FCS). Hu09, Hu09 L13 and Hu09 H3 cells were cultured in RPMI supplemented with 10% FCS. All cells were cultured at 37˚C in a humidified atmosphere of 5% CO_2. Etoposide, doxorubicin and cisplatin were purchased from Sigma (Buchs, Switzerland).

Cytotoxicity assay. Three thousand cells per well were plated in 96-well plates and allowed to adhere overnight. Different concentrations of doxorubicin (range: 0.001-30 μg/ml), cisplatin (range: 0.001-30 μg/ml), etoposide (range: 0.003-100 μg/ml) were added the following day. Cytotoxicity was measured 72h later with WST-1 reagent (Roche, Mannheim, Germany), as described elsewhere (10). Percentage growth inhibition was calculated by dividing the absorbance of drugtreated cells by that of untreated (control) cells and multiplying by 100. Statistical differences were determined with Student's *t*-test.

Microarray analysis. cRNA preparation: Total RNA was isolated using TRIzol (Invitrogen). The RNA quality was determined with a NanoDrop ND 1000 (NanoDrop Technologies, Delaware, USA) and a Bioanalyzer 2100 (Agilent, Waldbronn, Germany). Only samples with a 260 nm/280 nm ratio of between 1.8-2.1 and a 28S/18S ratio of within 1.5-2 were processed. RNA samples (2 μg) were reverse-transcribed into double-stranded cDNA and cRNA was *in vitro* transcribed in the presence of biotin-labeled nucleotides using an IVT Labeling Kit (P/N 900449; Affymetrix Inc., Santa Clara, CA, USA), purified and quantified using BioRobot Gene Exp – cRNA Target Prep (Qiagen AG, Switzerland). The labeled cRNA quality was determined using the Bioanalyzer 2100.

Array hybridization. Biotin-labeled cRNA samples (15 μg) were fragmented randomly to 35-200 bp at 94˚C in Fragmentation Buffer (P/N 900371; Affymetrix Inc.) and then mixed in 300 μl of Hybridization Mix (P/N 900720; Affymetrix Inc.) containing control Oligonucleotide B2 (P/N 900454; Affymetrix Inc.), prior to hybridization to GeneChip® Human Genome U133 Plus 2.0 arrays for 16 h at 45˚C. Arrays were then washed using an Affymetrix Fluidics Station 450 FS450_0001 protocol. An Affymetrix GeneChip Scanner 3000 (Affymetrix Inc.) was used to measure the fluorescent intensity emitted by the labeled target.

Statistical analysis. Array data were normalized to the 50th percentile of the respective chip (GeneSpring GX 7.3 Expression Analysis, Agilent Technologies, USA). Transcripts which were detected in the MG-63 cell line or the metastatic derivatives M6 and M8 were tested for significant changes with the Significance Analysis of Microarrays (SAM, available at http://www-stat.stanford.edu/~tibs/SAM) (11) running as an add-on in Microsoft Excel. The output criteria selected for SAM included ≥2-fold change at a threshold expected to produce fewer than 0.9 falsely detected genes or a false discovery rate of <0.5%.

RT-PCR. Total RNA was extracted from cultured cells using TRIzol reagent (Invitrogen) following the manufacturer's protocol. cDNA synthesis was performed using random primers and MultiScribe Reverse Transcriptase (Applied Biosystems, Foster City, CA, USA). RT-PCR for GAPDH transcripts was then performed and RNA was normalized according to GAPDH transcript content. PCR was carried out in the presence of Paq5000 reaction buffer (Stratagene, LaJolla, CA, USA), 0.8 mM dNTPs, 0.8 mM forward gene-specific primer, 0.8 mM reverse gene-specific primer and 2.5 U Paq5000 (Stratagene). Amplification of cDNA was performed for 35 cycles with a profile of 94˚C for 30 s, the primer specific annealing temperature for 40 s and 72˚C for 20 s. Resulting PCR products were electrophoresed in a 1.5% agarose gel containing ethidium bromide and photographed using a Versadoc System (Bio-Rad, Hercules, CA, USA). Primer sequences and primerspecific annealing temperatures used were: ADD3 forward 5'-GCAGCA CTAGAAGAAATCAGCAGGTCTAA-3', reverse 5'-ACTGTGA CATCACTTGGGTGAAAGGG-3', 65˚C. ABCG2 forward 5'-CAGGTGGAGGCAAATCTTCGT-3', reverse 5'-ACACACCAC GGATAAACTGA-3', 59˚C. CALCRL forward 5'-CCTAACTCAA GGACTTGGACCCAT-3', reverse 5'-AGGTTAGTAGCGTCA CATCAGGCAT-3', 66˚C. FZD8 forward 5'-TGCAGCGAAG GGACACTTGATG-3', reverse 5'-CTGGGTCTGGGAGGCTT CAAT-3', 65˚C. NMT2 forward 5'-AAATTCCGGTGTCCCTCAA TAAGGT-3', reverse 5'-GACGCTGAAGCAGGAGGATCAT-3', 65˚C. POSTN 5'-GGAGTAAGCAAGGGAGAAACGGT-3', reverse 5'-AATGTCCAGTCTCCAGGTTGTGT-3', 65˚C. PTN

forward 5'-TGACCTGAACACAGCCCTGAAGACC-3', reverse 5'-CCAGCATCTTCTCCTGTTTCTTGCCT-3', 68˚C. RELN forward 5'-ATGTGCTCAGGACGAGGGATTTGTGAT-3', reverse 5'CTGCACCATACACTTCAGGCCAAAGG-3', 68˚C. TRIM22 forward 5'-TCACAAACCACGGAGCACTCATCTACA-3, reverse 5'-GGGCTACTATGCAGAAGTGGGTAAAGGAAT-3', 67˚C. Wnt5a forward 5'-CCTCCTGAGACTGGCACTGTGT-3', reverse 5'-TTGTGGACTGATGCTGCTAACGAT-3', 65˚C. GAPDH forward 5'-TGAACGGGAAGCTCACTGGCATGG-3', reverse 5'-TGGGTGTCGCTGTTGAAGTCAGAGGAGA-3', 68˚C.

Immunoblotting. Cells ($1x10^6$) were lysed on ice with lysis buffer consisting of 50 mM Tris (pH 7.5), 150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid and 0.1% SDS. Lysates were cleared of insoluble material by centrifugation at 14000 rpm, 4˚C for 10 minutes. Protein content was measured with a standard Bradford assay and 80 μg of total protein were resolved by SDS-PAGE. Resolved proteins were then transferred to Immobilon-P membranes for immunoblotting. NMT-2 was detected using an NMT-2 antibody (BD Biosciences, Franklin Lakes, NJ, USA). WNT5a was detected using a WNT5a anti-body (Cell Signaling, Danvers, MA, USA). PTN was detected using a PTN antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Actin was detected with an actin antibody (Chemicon, Dietikon, Switzerland). Horseradish peroxidase conjugated (HRP) secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, Biotechnology) and resulting bands were detected using a Versadoc system (Bio-Rad, Munich, Germany).

Results

Increasing in vivo metastatic potential correlates with increasing chemoresistance in the parental MG-63 cell line and subline model. In initial experiments, the effect of cisplatin and doxorubicin, which are two currently used neo-adjuvant OS chemotherapeutic agents, and etoposide, which is solely an adjuvant OS chemotherapeutic agent generally administered to non-responders, was examined on OS cell lines. The cell lines included the 4 parental cell lines (SaOS, HOS, Hu09, MG-63) and their sublines (LM5, MNNG and 143B, L13 and H3, M6 and M8, respectively), which have differing *in vivo* metastatic potential. Each drug inhibited the growth of all cell lines tested (n=3) and the resulting IC_{50} values for each cell line are shown in Figure 1. Comparison among parental cell lines and their sublines revealed that the MG-63 sublines M6 and M8 demonstrated increased chemoresistance to all 3 drugs tested when compared to the responsiveness of the parental MG-63 cell line.

Microarray analysis reveals significant differential expression of 252 genes between MG-63 and the M8 subline. Due to the fact that the M6 and M8 sublines demonstrated increased chemoresistance to all drugs tested when compared to the parental MG-63 cell line, we next wanted to determine if there were any intrinsic differences in gene expression among these cell lines that might account

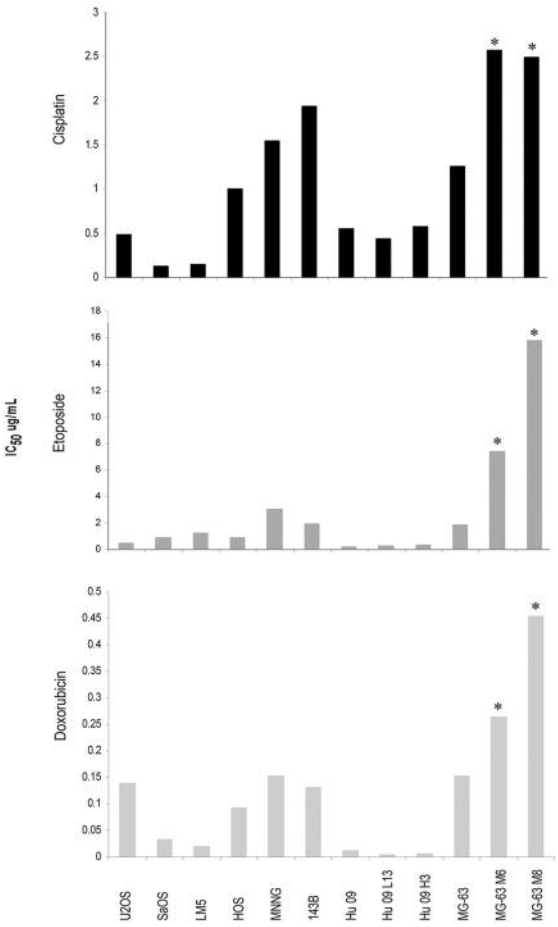

Figure 1. *Sensitivity of OS cell lines to various chemotherapeutic agents. IC_{50} values of all OS cell lines tested following 72 h treatment with cisplatin, doxorubicin, or etoposide. Values represent mean (n>3). Significant differences in IC_{50} values are indicated by*.*

for the observed differences in drug responsiveness. Consequently, total RNA was isolated in triplicate from each cell line and expression profiling of the MG-63, M6 and M8 cells was performed using the Affymetrix GeneChip® Human Genome U133 Plus 2.0 array, which allows for analysis of over 47000 transcripts. Subsequent GeneSpring analysis revealed no significant expression differences between the M6 and M8 sublines and thus, further analysis focused on the identification of intrinsic differences between the parental MG-63 cell line and the M8 subline. Analysis of these two cell lines identified significant expression differences of 252 genes, with 149 genes being up-regulated and 103 genes being down-regulated. The false discovery rate for this analysis was 0.5% or 1.3 genes. The selection of genes included only genes which had an average fold change of 2.0 or greater (Figure 2).

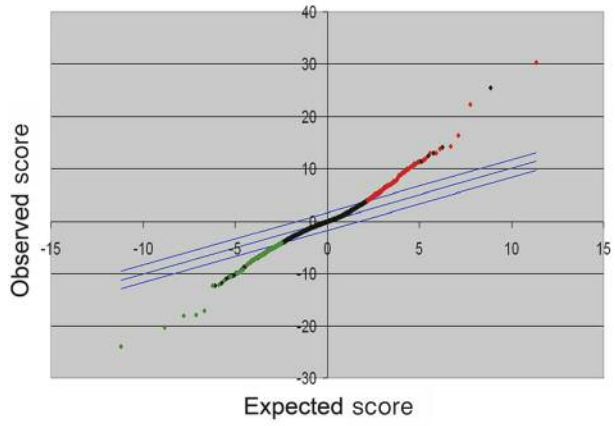

Figure 2. *Statistical analysis for microarray (SAM) plot. Dot plot of genes expressed in the MG-63 cell line and M8 cell line. Up-regulated genes are shown in red, down-regulated genes are shown in green and unchanged genes are shown in black.*

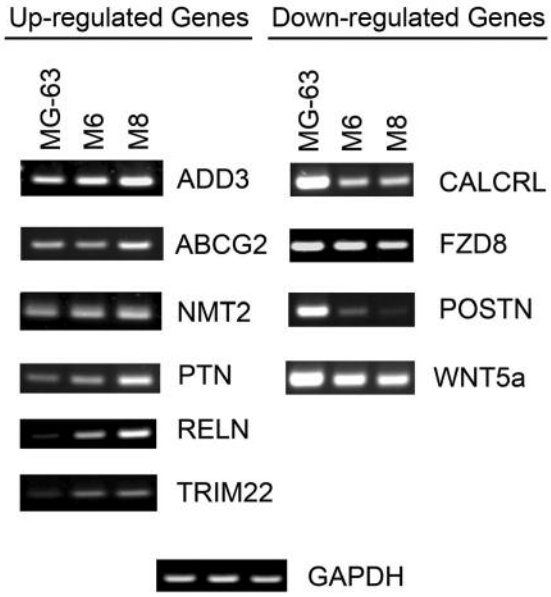

Figure 3. *RT-PCR validation of differentially regulated genes identified via microarray analysis. RT-PCR was performed for the indicated up-regulated and down-regulated genes in order to validate the microarray findings.*

Gene selection. In order to focus our gene selection further, a comparison of our 252 differentially identified genes to published reports examining chemoresistance genes potentially involved in OS were made. Interestingly, 4 of the genes identified as being differentially regulated in the MG-63 cell line system (*TRIM22, ADD3, PTN, WNT5A*) were also identified by Mintz *et al.* (2005) (7) as being differentially regulated between good and poor OS chemotherapeutic responders. In our analysis, the fold difference in expression of *TRIM22, ADD3, PTN* and *WNT5A* of Mg-63 m8 cells compared to the parental MG-63 cells was determined to be 4.4, 2.2, 3.3 and -3.1, respectively, while Mintz *et al.* found the fold difference to be -2.4, -2.2, 3.2 and -2.4, respectively. We then expanded our gene comparison and examined several reports involving gene expression profiling of over 30 cancer cell lines in order to identify chemoresistance genes. Using this strategy, we also identified *ABCG2* and *NMT2*. In order to validate the microarray expression results, RT-PCR was performed for each of the selected genes (Figure 3). Finally, to further confirm our microarray analysis, we also selected *RELN* as a highly up-regulated gene and *POSTN* and *CALCR* as highly down-regulated genes for RT-PCR analysis. Fold changes from the RT-PCR reactions confirmed the microarray expression fold changes (Table I).

RT-PCR and Western blot validate differential expression of selected genes. Western blot analysis was also performed for 2 of the selected up-regulated genes *(NMT2, PTN*) and 1 of the selected down-regulated genes (WNT5a) in order to validate the microarray findings at the protein level (Figure 4).

Discussion

In this study, we tested multiple OS cell line systems with three current OS chemotherapeutic agents and found that the MG-63 sublines M6 and M8 had increased chemoresistance when compared to the parental MG-63 cell line. Microarray analysis of total RNA from these cell lines identified 252 differentially regulated genes. Comparison of these results to previously published reports revealed 4 genes (PTN, ADD3, TRIM22 and WNT5a), which were also identified by Mintz *et al.* (7) following expression profiling of OS tumor samples from 15 good chemotherapeutic responders and from 15 poor chemotherapeutic responders. Although in the discussion Mintz *et al.* (7) focused more on differentially expressed genes involved in bone resorption and osteoclastogenesis, it is important to note that we also identified these genes in our analysis of the MG-63 cell line model. While a direct association between chemoresistance and aberrant expression of these genes has yet to be firmly established, it is significant that we and Mintz *et al.* (7) have identified common differentially expressed genes using two separate systems and therefore, the genes and their potential role in chemoresistance are discussed below.

PTN or pleiotrophin (PTN) is a cytokine that is highly expressed during embryogenesis but is restricted to the brain in adults (12). Up-regulated PTN expression has

Table I. *Selected genes and fold difference in expression in MG-63 M8 cells compared to the parental MG-63 cells.*

Accession number	Gene symbol	Microarray fold change	RT-PCR fold change±SD
AY140646	POSTN	–21.4	–18.5±3.8
NM_005795	CALCRL	–15.1	–4.2±0.5
AW340311	FZD8	–3.8	–2.5±1.4
NM_003392	WNT5A	–3.1	–3.0±0.8
AL134489	NMT2	2.2	1.4±0.1
AF098951	ABCG2	2.5	3.6±1.1
AI818488	ADD3	2.2	1.8±0.2
AL565812	PTN	3.3	2.9±0.5
AA083478	TRIM22	4.4	4.8±1.8
NM_005045	RELN	11.4	5.2±1.8

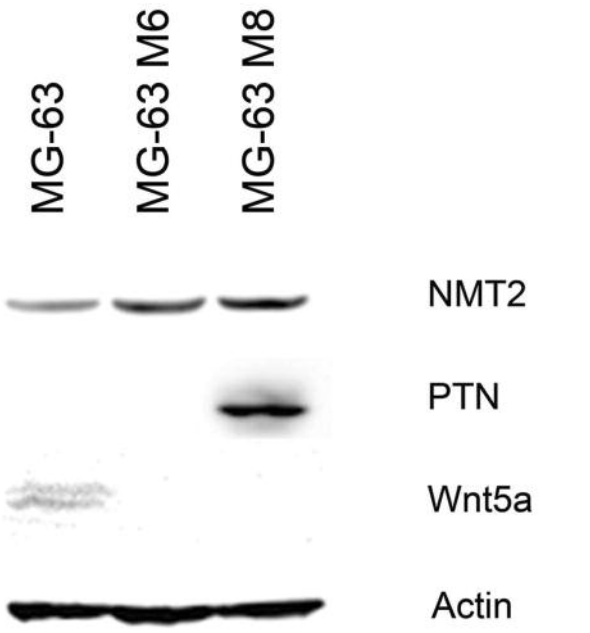

Figure 4. *Western blot validation of differentially regulated genes identified via microarray analysis. Cell lysates were subjected to immunoblot analysis for expression of PTN, NMT2, and Wnt5a.*

previously been shown to be associated with a variety of tumors including glioblastoma (13), multiple myeloma (14) and pancreatic cancer (15). Implantation of PTN-transformed fibroblasts into nude mice results in highly vascularized and aggressive tumors (16). Consequently, PTN is believed to contribute to the tumor vascularization and proliferation of tumor cells. In our study, we found that PTN levels were elevated in the M8 cell line (3.3-fold increase), which possesses greater *in vivo* metastatic potential and greater chemoresistance in comparison with the MG-63 parental cell line. Likewise, Mintz *et al.* (7) found that PTN expression was increased 3.2 fold in poor OS chemoresponders. Although a direct involvement of PTN in chemoresistance remains to be established, further examination of the role of PTN is obviously warranted.

ADD3 or adducin 3 is a protein associated with the cytoskeleton at the cell membrane and promotes the assembly of the spectrin-actin network at sites of cell-cell contact in epithelial tissues (17). Consequently, ADD3 seems to play an important role in cell membrane organization. In our analysis, expression of ADD3 was found to be higher in the more chemoresistant cell line M8 when compared to the parental cell line MG-63. By contrast, Mintz *et al.* (7) found expression levels of ADD3 were lower in poor chemotherapeutic responders. However, examination of chemotherapeutic resistance through gene expression profiling of 30 cancer cell lines revealed that the expression of ADD3 was associated with resistance to vinblastine, topotecan, paclitaxel, doxorubicin and 5-fluorouracil (18) and thus, may play a role in OS chemoresistance.

TRIM22 is a member of the tripartite motif (TRIM) protein family, which is a large family of proteins that are involved in several cellular processes, including proliferation, differentiation, oncogenesis and apoptosis (19). TRIM22 has been shown to be an interferon (IFN)-inducible p53 target gene and is thought to be involved in proliferation and differentiation (20). A direct role for *TRIM22* involvement in chemoresistance has yet to be reported, though the role of *TRIM22* in OS should be examined in future studies.

Wnt5a is a member of the Wnt family of secreted cysteine-rich proteins, which are proteins that play an important role in development (21). Wnt members bind to the Frizzled family of transmembrane receptors and activate several signaling pathways (22). Aberrant activation of Wnt signaling pathways has been shown to be involved in several types of cancer including gastric carcinoma, liver cancer and colon cancer (23). However, the role of Wnt5a remains to be a matter of debate due to the fact that Wnt5a has been shown to induce migration and invasion of breast carcinoma cells (24)and gastric cancer cells (25) while it has been shown to act as a tumor suppressor in thyroid carcinoma (26) and hematopoietic tumors (27). Thus, these data suggest that the role of Wnt5a varies according to tumor type. In the Mintz *et al.* (7) study and in ours, Wnt5a expression was found to be down-regulated in poor OS responders and the chemoresistant cell line M8, respectively. Likewise Blanc *et al.* (28) found that lower Wnt5a levels were associated with high-risk neuroblastoma while Jonsson *et al.* (29) reported they were associated with early relapse of invasive ductal breast carcinomas. However,

a definitive role for Wnt5a involvement in chemoresistance has yet to be established. Thus, taken together, loss of Wnt5a in OS may play a greater role in development of metastatic potential rather than chemoresistance, though this fact remains to be established.

In addition to the genes described above, we also identified increased expression in the M8 cell line of the ATP-binding cassette G2 gene or *ABCG2*, which is also known as the breast-cancer-related protein (BCRP). ABCG2 belongs to the ABC superfamily of transporter proteins and is known to efflux multiple chemotherapeutic agents including topotecan, irinotecan, mitoxantrone and doxorubicin (30-32). Overexpression of ABCG2 has previously not been reported for osteosarcoma. Consequently, our finding of increased ABCG2 expression in the M8 cell line may contribute to the increased resistance of the M8 cell line but may not play a significant role in overall OS chemoresistance. However, given the significant role of ABC transporters in the development of chemoresistance in other tumors (33), further investigation for a role of these proteins in OS is still warranted.

The identification of genes contributing to chemoresistance is a crucial first step in overcoming the hurdle of OS patient chemoresistance and improving overall OS patient survival. As described, we and Mintz *et al.* have identified 4 common differentially expressed genes that may be involved in OS chemoresistance. Although a direct correlation between these genes and chemoresistance has yet to be established, further investigation is warranted in order to determine their role. Identification of genes involved in OS chemoresistance could potentially be used to predict patient responsiveness to chemotherapy and aid in the development of novel OS therapies.

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