CANCER GENOMICS & PROTEOMICS *3*: 373-382 (2006)

Expression Analysis for the Identification of Genes Involved in Acquired Resistance to Cisplatin in Osteosarcoma Cells

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Abstract. *Background: Clinical observations indicate that tumour cells can acquire tolerance when an anticancer drug is administered repeatedly. In the present study, the gene expression in cisplatin-resistant cells was analysed to identify early changes in gene expression in the course of cisplatin exposure. Materials and Methods: After establishing a cisplatin-resistant human osteosarcoma subline (OST/R) and two additional sublines by more brief repeated exposure, cDNA expression microarrays were used to study genes linked with prolonged exposure to cisplatin of human cancer cells. Results: OST/R cells showed increased expression of 17 genes and reduced expression of 14. Genes associated with DNA repair, apoptosis, cell cycle progression, and proliferation were associated with the acquired resistance. Genes showing early changes were also identified. Conclusion: Identification of genes showing altered expression in the early stages of development of resistance to cisplatin may help to improve the therapeutic effectiveness of this drug.*

The prognosis in osteosarcoma has been significantly improved by the development of chemotherapy; in particular, cisplatin is now widely used (1-6). However, drug resistance has become an important issue as growing numbers of these tumours are showing resistance to chemotherapeutic regimens (7, 8). Resistance may be classified as intrinsic or acquired. In intrinsic or natural resistance, the drug is ineffective upon the first administration, which is considered to result from the tumour's genetic composition (9). On the other hand, acquired resistance occurs only after repeated administration of anticancer drugs. In this study gene expression in cisplatin-resistant cancer cells was analysed and changes in gene expression during drug exposure were systematically examined using cDNA microarray technology (10). Additionally, a real-time polymerase chain reaction (PCR) assay was used to quantitate gene expression for comparison between the two analytical methods (11-13).

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Key Words: Osteosarcoma, cisplatin, drug-resistance, DNA microarray.

Materials and Methods

Cell growth inhibition assay in human osteosarcoma cells. OST and MG63 osteosarcoma cells, used as the parental cell lines, were maintained in RPMI-1640 supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin, at 37˚C in humidified air with 5% CO_2. Cell survival was measured by WST-1 assays (14, 15). Cells were seeded at a density of 10^4 cells/well in 96-well microtitre plates and preincubated for one day to permit adhesion to the plates. The cells were exposed for 1 h to various concentrations of cisplatin then cultured for 2 days and WST-1 reagents were added to the cultures for a 90-min incubation. Then absorbance was measured at 450 nm against a reference wavelength of 630 nm with an Easy Reader (EAR 340 AT; SLT-Lab Instruments, Austria). Absorbance of the no-drug control was defined as 100%, while absorbances of the dilution series were expressed as percentages of the control. The 50% inhibition concentration (IC_{50}) was defined by a 50% reduction in the cell survival curve.

Establishment of a human osteosarcoma subline resistant to cisplatin (OST/R). The OST/R cell line was established by subcloning and maintaining OST cells in 0.7 mg/ml cisplatin for more than 3 months (16, 17). The cell survival rate was measured by WST-1 assays. Acquired drug resistance was determined as the change in the IC_{50}. When relative resistance was calculated by dividing the IC_{50} for OST/R cells by the IC_{50} for OST cells, OST/R cells were 7.2 times more resistant to cisplatin than the parental OST cells (Figure 1).

Establishment of two cell lines (OST/3, OST/5) by short-term exposure to cisplatin. Two additional cell lines were established to analyze early changes in gene expression related to cisplatin exposure.

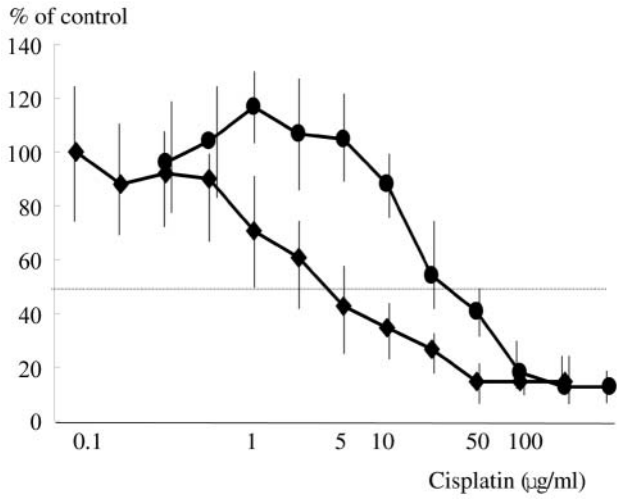

Figure 1. *Dose-response curves for cisplatin in OST and OST/R cells. OST (◆) and OST-R cells (●) were exposed to various concentrations of cisplatin for 1 h. Relative survival is expressed as a percentage of survival in control cultures without cisplatin treatment. The IC_{50} in the OST cell line was 3.5 μg/ml and was 25 μg/ml in the OST/R cells, showing that OST/R cells were 7.2 times more resistant to cisplatin than the parental OST cell line.*

These cell lines were generated by administration of either three doses (OST/3) or five doses (OST/5) for 1 h once weekly.

Labeling, hybridization, and scanning of the cDNA microarray. After total RNA was isolated from cultured cells and treated with DNase, cDNA probes were synthesized using the Atlas Pure Total RNA Labeling System (BD Biosciences Clontech; Tokyo, Japan). Probes then were hybridized to BD Atlas cDNA Expression Arrays (Cat. #7851-1). The array used in this study consisted of 1176 cDNA fragments associated with human cancer spotted on positively charged nylon membranes. After hybridization, the array was exposed to radiographic film at –70˚C with the aid of an intensifying screen (Fuji Film, Tokyo, Japan). Spots on the film were analyzed densitometrically with Array Gauge software (version 1.2; Fuji Film).

Real-time PCR. A real-time PCR assay was used to confirm macroarray results. TaqMan MGB probes (Assays-on-Demand Gene Expression Products, Applied Biosystems, Foster City, CA USA) were used for analysis with the ABI PRISM 7000 Sequence Detection System (Applied Biosystems). PCR was carried out for genes selected based on the macroarray results. The following primers were used for real-time PCR analysis; NTHL1 (assay ID, Hs00267385_m1) and CDK6 (assay ID, Hs00608037_m1).

Statistical analysis. Significant differences were identified by Student's *t*-test, requiring a *p*-value less than 0.05.

Results

Genes up-regulated in the OST/R cell line according to the macroarray (Figure 2 and Table I). The expression of 17 genes was increased in OST/R cells. The genes showing higher expression in the resistant cell line included those involved in DNA damage signaling and repair, (*e.g.*, nth endonuclease III-like 1 or NTHL1), or the cell cycle (*e.g.,* cyclin-dependent kinase 6 or CDK6).

Genes down-regulated in the OST/R cell line according to the macroarray (Figure 2 and Table II). The expression of 14 genes was reduced in OST/R cells. The genes with decreased expression in OST/R cells encoded products including cytoskeletal proteins, such as keratin 10 (*KRT10*) and 14 (*KRT14*), or apoptosis-associated proteins, such as CASP2 and RIPK1 domain containing adaptor with death domain (*CRADD*).

Changes in gene expression with short-term cisplatin exposure (Table III). The analysis of the changes in gene expression after cisplatin exposure identified genes whose expression increased or decreased after a few brief exposures to cisplatin (three or five). For example, expression of the cell cycle regulator *CDK6* did not change after three treatments, but increased after five. No change in expression of the DNA damage repair gene *NTHL1* was seen after either three or five cisplatin treatments, in contrast to the increase in cells rendered by long-term drug exposure (OST/R).

Gene expression analyses with real-time PCR (Figure 3). To confirm the occurrence and extent of changes in the expression of *NTHL1* and *CDK6*, real-time PCR was carried out. The results were consistent with the gene expression data obtained by macroarray analysis.

Study of MG63 cells. When resistance was examined in another cisplatin-sensitive human osteosarcoma cell line (MG63) after long-term exposure to cisplatin, the IC_{50} ratio in MG63 was 1.8, consistent with development of resistance by the cells.

Discussion

Research concerning drug resistance, particularly resistance to cisplatin, will, hopefully, lead to improved outcomes (18, 19). Our present clinical experience, however, suggests that the effectiveness of cisplatin-based chemotherapy lessens with repeated administration. Tumour cells are believed to acquire tolerance to cisplatin gradually, so, the effectiveness of chemotherapy might be maintained if acquisition of tolerance could be prevented by blocking associated changes in gene expression. Among the many varied genes participating in acquired resistance, we suspect that the acquisition of tolerance could be most efficiently prevented by targeting genes showing altered expression in the early stages of cisplatin exposure.

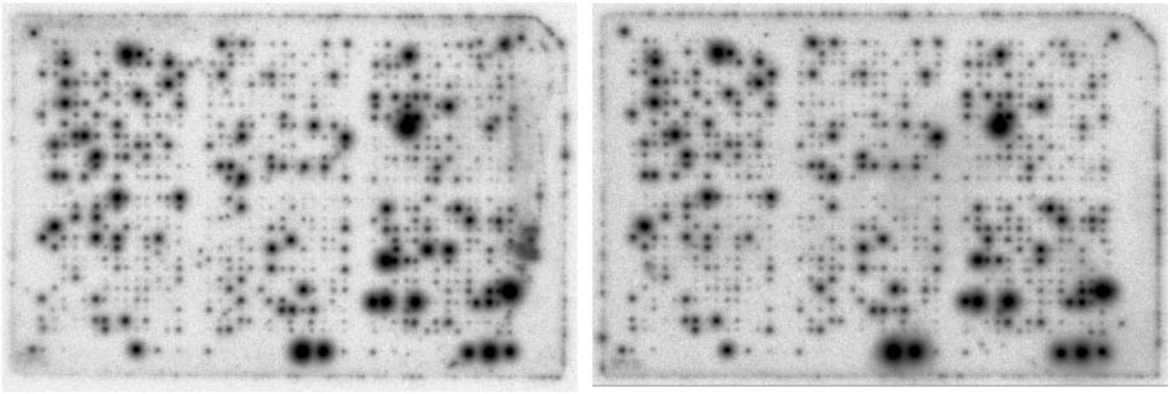

Figure 2. *Gene expression in OST cells and OST/R cells. ^{32}P-labeled cDNA probes were generated from each poly A RNA sample and hybridized to a BD Atlas Human Array (#7851-1). Membranes were exposed to radiographic film with the aid of an intensifying screen at –70˚C for 1 day. The cDNAs appear as single spots.*

Table I. *Up-regulated genes in the OST/R cell line.*

Functional classification	Gene name	Symbol
Oncogenes and tumor suppressors	Interferon induced transmembrane protein 1 (9-27)	IFITM1
	V-myc myelocytomatosis viral oncogene homolog (avian)	MYC
	Fms-related tyrosine kinase 1	FLT1
	Prefoldin 5	PFDN5
	Non-metastatic cells 2, protein (NM23B) expressed in	NME2
	SHB (Src homology 2 domain containing) adaptor protein B	SHB
Cell cycle	Cyclin-dependent kinase 6	CDK6
Intracellular transducers/effectors/modulators	S100 calcium binding protein A4	S100A4
	Guanine nucleotide binding protein 10	GNG10
	Rho GDP dissociation inhibitor (GDI) alpha	ARHGDIA
DNA synthesis, recombination and repair	Nth endonuclease III-like 1 (*E. coli*)	NTHL1
Cell adhesion receptors/proteins	Cadherin 5, type 2, VE-cadherin (vascular epithelium)	CDH5
Protein turnover	Cathepsin D (lysosomal aspartyl protease)	CTSD
	Serine (or cysteine) proteinase inhibitor, clade E	SERPINE2
Metabolism	Fatty acid synthase	FASN
Translation	Ribosomal protein L32	RPL32
Cytoskeleton/motility proteins	Tubulin, gamma 1	TUBG1

Table II. *Down-regulated genes in the OST/R cell line.*

Functional classification	Gene name	Symbol
Intracellular transducers/effectors/modulators	Dual specificity phosphatase 8	DUSP8
	Ras homolog gene family, member B	RHOB
Apoptosis associated proteins	Heat shock protein 75	HSP75
	CASP2 and RIPK1 domain containing adaptor with death domain	CRADD
Cell adhesion receptors/proteins	Integrin, beta 4	ITGB4
	Integrin, alpha 7	ITGA7
Protein turnover	Serine protease inhibitor, Kunitz type, 2	SPINT2
Cytoskeleton/motility proteins	Keratin 7	KRT7
	Keratin 10	KRT10
	Keratin 8	KRT8
	Keratin 14	KRT14
	Keratin 18	KRT18
	Keratin 2A (epidermal ichthyosis bullosa of Siemens)	KRT2A
Extracellular matrix proteins	Laminin, beta 2 (laminin S)	LAMB2

Table III. *Changes in gene expression associated with short term cisplatin exposure.*

Gene name	OST/3	OST/5	OST/R
Interferon induced transmembrane protein 1 (9-27)			
V-myc myelocytomatosis viral oncogene homolog (avian)			
Fms-related tyrosine kinase 1			
Prefoldin 5			
Non-metastatic cells 2, protein (NM23B) expressed in			
SHB (Src homology 2 domain containing) adaptor protein B			
Cyclin-dependent kinase 6			
S100 calcium binding protein A4			
Dual specificity phosphatase 8			
Ras homolog gene family, member B			
Guanine nucleotide binding protein 10			
Rho GDP dissociation inhibitor (GDI) alpha			
Heat shock protein 75			
CASP2 and RIPK1 domain containing adaptor with death domain			
Nth endonuclease III-like 1 (*E. coli*)			
Cadherin 5, type 2, VE-cadherin (vascular epithelium)			
Integrin, beta 4			
Integrin, alpha 7			
Cathepsin D (lysosomal aspartyl protease)			
Serine protease inhibitor, Kunitz type, 2			
Serine (or cysteine) proteinase inhibitor, clade E, member 2			
Keratin 7			
Keratin 10 (epidermolytic hyperkeratosis; keratosis palmaris et plantaris)			
Keratin 8			
Keratin 14 (epidermolysis bullosa simplex, Dowling-Meara, Koebner)			
Keratin 18			
Tubulin, gamma 1			
Keratin 2A (epidermal ichthyosis bullosa of Siemens)			
Fatty acid synthase			
Ribosomal protein L32			
Laminin, beta 2 (laminin S)			

 , increase gene expression.

 , decreased gene expression.

Cisplatin blocks DNA synthesis by forming bridges between the two DNA strands. The mechanisms of cisplatin resistance include interference with this bridging, the acceleration of DNA repair, and the reduction of drug availability (18, 20-22). Recently, these mechanisms of acquired drug resistance have been linked to changes at the genetic level, with many genes being implicated. For instance, multidrug-resistance-associated protein (*MRP*), p53, p73α, glutathione S-transferase II, O6-methylguanine-DNA methyltransferase (*MGMT*), Ets-1, bcl-2, Lung resistance-related protein (*LRP*), *ERCC1* and *BRCA1* have been implicated (19, 23-35). Perego *et al.* have demonstrated that stable transfection of US-OS cells with a cDNA encoding bcl-xL, a participant in apoptosis, conferred low-level cisplatin resistance (27). Wilson *et al.* have examined the contribution of a transcription factor, Ets-1, to cisplatin resistance in C13 cells, finding that Ets-1 expression was associated with cisplatin resistance (28). Resistance-associated genes have a variety of effects, including increased inactivation of cisplatin by thiol-containing molecules, increased repair of DNA damage, and down-regulation of apoptotic pathways. Consequently, no single mechanism can solely account for development of resistance to anticancer drugs, and the concurrent analyses of expression of many genes is needed, such as screening using cDNA microarrays. Simultaneous analysis of several thousand gene expression profiles is now possible using cDNA microarray technology, and many changes in gene expression in cisplatin-resistant cells have been observed (36-42). Results of these studies have differed to some extent because different genes were selected for analysis. However, the general consensus was that changes in DNA repair, apoptosis, and drug inactivation were associated with resistance.

Many investigations of cisplatin resistance have used osteosarcoma cells because these cells generally are sensitive

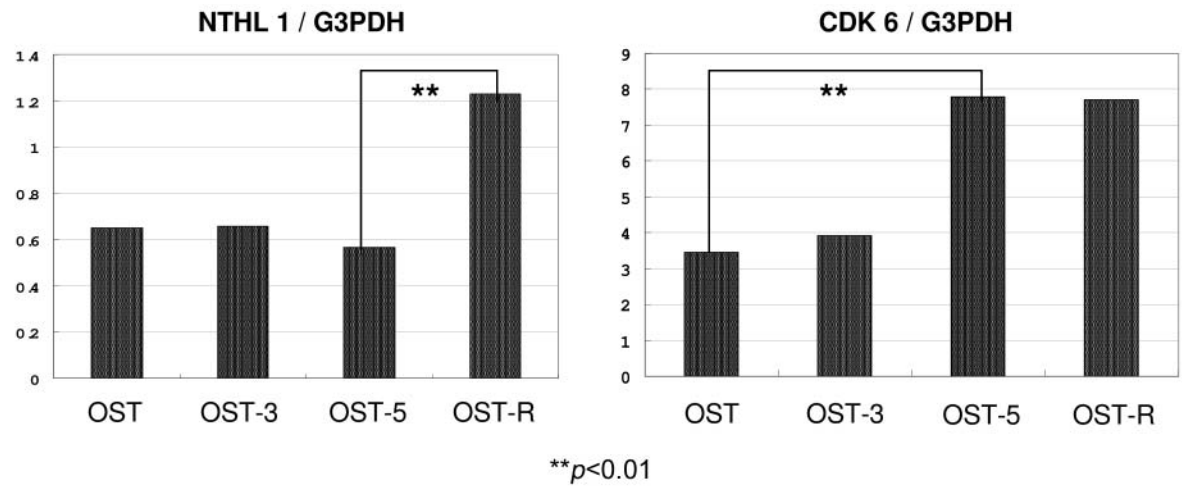

Figure 3. *Quantitative PCR analysis of mRNA expression of genes selected based on the microarray results. Total RNA was extracted from each cell line and analyzed by real-time PCR.*

to cisplatin-based chemotherapy (19, 43). Recently microarrays have been used investigationally to predict chemotherapeutic responses in osteosarcoma patients (44, 45). However, as Man *et al.* have pointed out, predictor gene lists have differed between studies because of differences in chemotherapeutic agents and in constituents of the arrays (45). One area yet to be subjected to microarray analysis has been development of drug resistance in osteosarcoma cells upon exposure to cisplatin. Accordingly, we used a cDNA macroarray analysis to identify genes associated with cisplatin resistance in osteosarcoma cell lines. Advantages of a cDNA macroarray as apposed to a microarray include high sensitivity and reproducibility of the searches. Further, analysis is possible with a very small amount of mRNA because a ^{32}P-labeled radioactive probe can be used. Finally, costly instrumentation is not needed for analysis. A disadvantage of macroarrays is a lower number of genes per array than with a microarray.

A large variety of genes appeared to be likely to participate in acquired resistance in our OST/R cells, including 17 up-regulated genes. Interferon-induced transmembrane protein 1 (*IFITM1*) encodes a cell-surface protein that may modulate cell adhesion and influence cell differentiation (46). Cell adhesion to the extracellular matrix has been shown to inhibit apoptosis. Whiteside *et al.* and Roberts et al. have suggested further study of the biological significance of *IIP 2-27* in cisplatin cytotoxicity and subsequent development of cisplatin resistance (37, 47). Cathepsin D (*CTSD*), a lysosomal proteinase, has been reported to promote cancer cell proliferation, invasion and local dissemination (48, 49). Cadherin 5, type 2 (*CDH5*), is a calcium-dependent adhesive protein that mediates cell-cell interactions as a vascular epithelial cadherin. Deficiency or truncation of the Cdh5 protein has been reported to induce endothelial cell apoptosis and limit endothelial cell survival. On the other hand, suppression of apoptosis is associated with cisplatin resistance (50-52).

Altered cell-cycle regulation is associated with cisplatin resistance. Cyclin-dependent kinase 6 (*CDK6*), which regulates major cell-cycle transitions in eukaryotic cells, is associated with cyclins D1, D2 and D3 in lysates of human cells, and is activated by co-expression with D-type cyclins. *CDK6* and the homologous peptide CDK4 link growth factor stimulation with the onset of cell-cycle progression (53). Nth endonuclease III-like 1 (*NTHL1*) is a DNA repair gene (54). The expressed and purified protein product has DNA glycosylase activity on DNA substrates containing oxidized pyrimidine residues, as well as apurinic/apyrimidinic lyase activity (55). DNA repair genes frequently have been reported to participate in cisplatin resistance (18, 20, 21, 36, 47, 52).

A variety of other genes also showed increases. S100 calcium-binding protein A4 (*S100A4*) cosegregates with CAGA, CAGB and calcyclin (56). Roberts *et al.* have reported that S100 calcium-binding proteins A2 and A10 have been associated with resistance to platinum drugs (37). Guanine nucleotide-binding protein 10 (*GNG10*) regulates activities of various enzymes and ion channels (57). Toshimitsu *et al.* have found guanine nucleotide-binding protein beta polypeptide 2-like 1 to be overexpressed in a cisplatin-resistant cell line (40). Rho GDP-dissociation inhibitor alpha (*ARHGDIA*) belongs to the RAS gene superfamily, which encode small guanine nucleotide exchange (GTP/GDP) factors and may be kept in a GDP-bound inactive state by interaction with GDP-dissociation inhibitors (58). Sakamoto *et al.* have reported decreased

expression of Rho GTPase-activating protein 1 in a group of chemotherapy-resistant tumours (36). Tubulin gamma 1 (*TUBG1*) is essential for nuclear division and microtubule assembly (59). Sakamoto *et al.* have reported increased expression of tubulin beta and tubulin alpha in chemotherapy-resistant cancers (36).

Fourteen down-regulated genes were identified in the OST/R cells. Among these, dual-specificity phosphatase 8 (*DUSP8*), a member of one class of protein-tyrosine phosphatase, dephosphorylates phosphotyrosine, phosphoserine, and phosphothreonine as well as negatively regulating a member of the mitogen-activated protein (MAP) kinase family associated with cellular proliferation and differentiation (60). The ras homologue gene family member B (*RHOB*), which stimulates actin stress fiber production and formation of focal adhesions, has various other functions including an influence on programmed cell death and cell transformation (61, 62). The protein encoded by the *CRADD* gene has been shown to induce cell apoptosis (63). Integrin beta 4 (*ITGB4*) is a transmembrane glycoprotein receptor that mediates cell-to-matrix and cell-to-cell adhesion, as well as transducing signals that regulate gene expression and cell growth and has various other functions (64). A reduction in integrin beta 4 protein has been related to changes observed during cell adhesion and cancer progression (65). Further, peritoneal spread of cancer cells has been reported to be inhibited by induction of integrin beta 4 and consequent integrin beta 4-induced apoptosis.

Keratin expression has been altered in association with cisplatin resistance; we found gene expression for keratins 2A, 7, 8, 10, 14, and 18 to be decreased in OST/R cells. Sakamoto *et al.* have reported that keratin 7 was underexpressed in clinical samples of tumours showing resistance, while Whiteside *et al.* have reported reduced expression of cytokeratin 19 in cisplatin resistance (18, 36). Keratin 10 (*KRT10*) encodes a member of the type I cytokeratin family, which in turn belongs to the superfamily of intermediate filament proteins. Expression of K10 prevents cell proliferation through inhibition of Akt kinase activity, resulting in impaired NF-kappa B function (66). Keratin 14 (*KRT14*) encodes a member of the keratin family, the most diverse group of intermediate filaments. Susceptibility of keratinocytes to caspase-8-mediated apoptosis has been found to be increased after K14-TRADD interactions (67). Katabami *et al.* have reported an association of cisplatin resistance with a marked reduction of K14 in human squamous cell lung carcinoma lines (68). Keratin 18 (*KRT18*), together with its filament partner keratin 8 (*KRT8*), may be the most ubiquitous member of the intermediate filament gene family. Parekh *et al.* have found that a cisplatin-resistant human ovarian adenocarcinoma cell line contained far less cytokeratin 18 than the corresponding cisplatin-sensitive cell line (69). Keratins 8 and 18 remain associated in heteropolymeric aggregates during apoptosis (70). On the other hand, Iwaya *et al.* have linked decreased amounts of cytokeratin 8/18 in the cytoplasm of breast cancer cells with a poor prognosis, while Woelfle *et al.* have reported that down-regulated expression of cytokeratin 18 promotes progression of human breast cancer (71, 72). These results suggest that these keratins in sufficient quantities can promote apoptosis. Kunitz type serine protease inhibitor (*SPINT2*) inhibits activity of hepatocyte growth factor activator (73). Overexpression of this gene has been linked with suppression of invasion and peritoneal carcinomatosis (74). *SPINT2* was found to be decreased in resistant cells in our study, although Huerta *et al.* (42) and Nakatani *et al.* (38) have reported overexpression.

The genes implicated in the mechanism of cisplatin resistance in osteosarcoma cells thus belong to one of four groups characterized by effects favouring proliferation, suppression of apoptosis, advancement through cell-cycle check points, or DNA repair. Up-regulated genes affecting cell proliferation were *IFTTM1, MYC, FLT1, PFDN5, SHB, CTSD, SERPINE2, S100A4, GNG10, ARHGDIA, FASH* and *TUBG1*. Cadherin 5 was the only up-regulated apoptotic gene. The up-regulated cell cycle gene was cyclin-dependent kinase 6. Up-regulated DNA repair genes included Nth endonuclease III-like 1. Down-regulated genes involved in suppression of cell proliferation were DUSP8 and SPINT2. Down-regulated genes concerned with suppression of apoptosis were *RHOB, HSP75, ITGB4* and *KRT7, 8, 10, 14* and *18*.

The genomic context of the genes obtained from these results was examined, and found that it was variable. However, we consider that the alterations in gene expression are associated with chromosomal alteration. The comparative genomic hybridization (CGH) method has been used similar to the microarray method for examining the genes participating in cisplatin resistance. Wasenius *et al.* have reported that chromosomal changes occur during the development of acquired resistance to cisplatin in human ovarian carcinoma cells (75). We consider that similar results would be obtained using the CGH method.

Changes in gene expression over time, in relation to the number of cisplatin exposures, and to how cells acquired tolerance were next examined. Few reports have examined time-dependent gene expression during resistance acquisition (43, 76), except for one report of a novel time-course cDNA microarray analysis (47). The studies in which squamous cell lung cancer cell lines were treated weekly with an IC_{50} dose of cisplatin, identified genes not previously associated with cisplatin resistance. However, some implicated genes had known associations with cancer progression, suggesting that cancer cells become more malignant as they develop drug resistance. We generated drug-resistant cell lines and established a novel model

system, in line with clinical methods, to identify genes associated with the development of cisplatin resistance. The results indicated that expression of some genes had already changed by the third exposure to cisplatin, while others showed altered expression after five exposures. The findings of this study suggest that a variety of genes participate in the early stage of acquired cisplatin resistance in OST cells.

Acknowledgements

We thank Dr. Gong Ming for helping in statistical analysis.

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Received July 25, 2006
Accepted September 27, 2006